ROLE OF FORMIN-2 IN ACTIN-MICROTUBULE COORDINATION DURING AXONAL PATHFINDING AND ITS CHARACTERIZATION IN AXONAL BRANCHING

A thesis

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Tanushree Kundu

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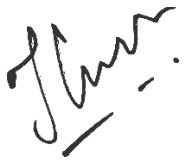
DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.



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Tanushree Kundu

20133245

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Role of Formin-2 in actin-microtubule coordination during axonal pathfinding and its characterization in axonal branching**", submitted by Tanushree Kundu was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: Dr. Aurnab Ghose

Supervisor

DEDICATION

"To My Grandmother & Late Grandfather"

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Neural circuits are formed by directed translocation of axonal growth cones to their synaptic targets and specific patterns of branching. The axon, with the growth cone at the tip, moves in a directed fashion by sensing the environmental cues through structures like the filopodia. As the neuron reaches its target tissue, it innervates the tissue to form multiple connections. This is achieved by the arborization of the terminal end of the axon or by collateral branching of the axon. These processes of outgrowth, guidance and branching are driven by active and coordinated remodelling of the underlying cytoskeletal components. One such cytoskeletal regulator is Formin-2 (Fmn2), an actin nucleator, which is highly expressed in the developing and adult central nervous system (Leader and Leder, 2000) and has been implicated in cognition (Law *et al.,* 2014; Agís-Balboa *et al.,* 2017). Recent studies from our lab have provided glimpses into the mechanism of Fmn2 function during the development of the nervous system. Fmn2 is involved in maintaining optimum outgrowth speed and directionality of migration. Moreover, deficiency of Fmn2 resulted in pathfinding defects of spinal commissural neurons (Sahasrabudhe *et al.,* 2016). Since axonal pathfinding requires coordination between actin and microtubule cytoskeletons, this study investigated the role of Fmn2 in mediating actin-microtubule crosstalk. We find that Fmn2 facilitates the exploration of microtubules into the peripheral domain of the growth cone.In the filopodia, Fmn2 stabilizes the microtubules, most likely, by physically coupling themto the F-actin bundles. This coupling appears to occur through the tail region of Fmn2, which binds to both actin and microtubule. In the absence of Fmn2, disruption of actin-microtubule crosstalk in filopodia results in deficits in sensing and/or turning that underlie the axon guidance defects. Along with the requirement of Fmn2 in the growth cone, Fmn2 was found to be involved in axonal collateral branching. Axonal branching is an important component of connectivity patterns in neural circuits. A collateral is initiated by the seeding of F-actin bundles from actin accumulation in the axonal shaft which result in the formation of a filopodium on the axonal shaft. This protrusion is stabilized by microtubule innervation and ultimately matures into a stable branch (Gallo,2015). Fmn2 was found to localize at the base of axonal filopodia and its deficiency reduced branching by half. On the other hand, Fmn2 lead to increased branching when over-expressed. Moreover, the density of these protrusions is dependent on the actin nucleation activity of Fmn2. Preliminary evidence suggests that Fmn2 may facilitate microtubule presence in axonal filopodia,

in a manner analogous to that uncovered in the growth cone filopodia, thereby ultimately aiding their maturation into branches. In conclusion, this study provides a mechanistic understanding of Fmn2 in processes critical to the development of neuronal circuits and its mediation of actin-MT cross-talk in the developing neuron.

SYNOPSIS

1.1. Introduction

During development, the nervous system forms the neural circuitry through which neurons communicate with each and different parts of the body. Genetic defects could lead to improper development of these trajectories which could result in aberrant trajectories (Geidd *et al.,* 2015). These improper connections are a major cause of neurodevelopmental disorders such as autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), schizophrenia and intellectual disability affect cognition, learning and memory, behaviour as well as language and motor skills (Cioni *et al.,* 2016; Mullin *et al.,* 2013). To gain a better understanding of these disorders, it is critical to study, dissect out the mechanism(s)of circuit formation at the molecular, cellular and behavioural levels.

During development, neurons undergo several stages of development (Stiles and Jernigan, 2010; The Mind's Machine, 2nd Edition, 2016):

1. Neurogenesis – neurons are born from progenitor cells mainly in the ventricular zone of the neural tube.
2. Neuronal migration and differentiation – neurons migrate to their destination by crawling along radial glial cells which act as guides and then different into different to form specific connections.
3. Synaptogenesis and synapse maturation – The neuron polarizes to form an axon which travels long distance to form connections with different parts of the body.

The molecular mechanisms underlying the developmental processes of axonal migration and branching have been investigated in this thesis. Both of these processes are dependent on the underlying structural elements – the cytoskeleton. The major cytoskeletal systems are actin and microtubules.

Actin monomer is a 42-kDa protein, that exists in monomeric or globular (G) or filamentous (F) actin forms. G-actin undergoes polymerization to form filamentous or F-actin. F-actin is a single stranded linear right-handed helix of actin polymers with a pitch of ~71.5nm (Pollard and and acts a slow ATPase. F-actin is polar in nature with two dynamically different barbed and pointed ends. The rate of elongation occurs from the barbed end (Blanchoin *et al.,* 2014). The polymerization reaction can be divided into several steps (Pollard and Cooper, 1986; Pollard *et al.,* 2000; Chesarone and Goode, 2009; Mattila and Lappalainen 2008) involving 1) activation, wherein the G-actin monomer undergoes a conformational change.2) nucleation -wherein 3to 4 actin sub-units form a nucleation seed. This is the rate limiting step of the reaction. (3) elongation- actin is rapidly polymerized from the actin seeds from the barbed end. In cells, the nucleation and elongation are regulated by a number of proteins. Prominent nucleators are Arp 2/3 complex activated by the Wiskott-Aldrich Syndrome protein (WASP)/WAVE family of proteins (Goley and Welch, 2006), formins (Faix and Grosse, 2006; Pring *et al.,* 2003; Otomo *et al.,* 2005) and spire (Bosch *et al.,* 2007). Elongation is regulated by Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of proteins and formins (Reinhard et al, 1992; Ferron *et al.,* 2007). Disassembly of filaments is regulated by actin depolymerizing factor (ADF)/cofilin (Bamburg *et al.,* 1980) and myosin (Kohler and Baush, 2012; Blanchoin *et al.,* 2014). Singles filaments are disassembled by altering their mechanical properties such as persistence length (McCullough *et al.,* 2011) or increasing the helical twist (McGough *et al.,* 1997). Actin filaments are arranged in two major architectural forms- 1) the branched actin network. Arp2/3 complex is the major factor involved in the nucleation of branched filaments. Arp2/3 complex sits on the side the actin filament and nucleates actin filaments at a fixed angle of 70° (Mullins *et al.,* 1998; Pollard *et al.,* 2000). The filaments are elongated by either Ena/VASP ((Koestler *et al.,* 2008) or formins (Yang *et al.,* 2007). 2) Parallel actin architecture. The actin bundles are arranged in linear unbranched filaments that are bundles together. Ena/VASP and formins are involved in this process (Goode and Eck, 2007).

Another cytoskeletal element involved in cell shape and migration are microtubules. Microtubules are polymers of α- and β-tubulin dimers (~55kD) that form hollow tube-like structures by joining 8-17 protofilaments (dimers bind in head-to-tail fashion) (Akhmanova and Steinmetz, 2008). Microtubules are another major cytoskeletal system involved in regulating cell shape and migration. These filaments are quite dynamic and undergo phases of polymerization and depolymerization. In cells, microtubules nucleate from ɣ-tubulin ring complexes (ɣ-TURC) (Muroyama and Lechler, 2017; Mitchison and Kirschner, 1984 I) and spontaneously polymerize from the plus end using tubulin-GTP. Microtubules switch from polymerization to depolymerization spontaneously based of availability tubulin-GTP, biochemically referred to as "catastrophe". When they switch back to polymerizing phase it is termed as "rescue". The phase transitions between polymerization and shrinkage occurs stochastically as the microtubule grows in-vitro and is termed as dynamic (Mitchison and Kirschner, 1984 II; Akhmanova and Steinmetz, 2008). Regulation of microtubule dynamics occurs through multiple microtubule binding proteins which either promotes its assembly or destabilize them or bundle them (Goodson and Jonasson, 2018). One such category is the End-binding (EB) proteins, which are +TIP tracking proteins, which dynamically track the plus end of a polymerizing microtubule (Schuyler and Pellman, 2001; Lansbergen and Akhmanova, 2006). These proteins fall off as the microtubule undergoes catastrophe (Sandblad *et al.,* 2006; Beiling *et al.,* 2007). Another population of microtubules exist, which are quite stable with half-life of more than one hour (Kries, 1987). They undergo a number of post-translational modifications, including, detyrosinationa and acetylation ((Janke, 2014).

The neuronal cytoskeleton consists of actin at the periphery of the axon and growth cone, while microtubules are more central in localization. The tip of the axon, the growth cone, is structurally classified into three regions- peripheral region (P) which is composed of branched lamellipodial actin network and unbranched bundled actin which form the finger-like filopodia. Microtubules occupy the central region (C) with a few dynamic one that explore the peripheral domain. And between the two is the transition zone (T), composed of actin arcs (Dent and Gertler, 2003). The advancement of growth cone can be described in three stages: Protrusion, engorgement and consolidation (Goldberg and Burmeister, 1986; Halloran and Kalil,1994; Lowery and Vactor, 2009). In the protrusion stage, the filopodia and lamellipodia undergo rapid elongation of the actin filaments which push the membrane forward. The balance is maintained by the pull from the

retrograde flow. As the substrate and cytoskeleton couple, the growth cone forms attachment by mechanically linking the focal contact proteins to the substrate which in turn bind to F- actin to generate traction which tilts the balance towards polymerization, attenuates retrograde flow. During the engorgement phase, these actin arcs depolymerize, thereby clearing the region for MTs to advance into the P-domain. The final step is consolidation, after the growth cone has advanced the MT are compressed by the re – established actomyosin arcs and stabilized into bundles by the MT – associated proteins. These iterative cycles of advancement, engorgement and consolidation result in axon elongation (Mitchison and Kirschner, 1988; Suter and Forscher, 2000). Growth cones also require actin- microtubule for turning (Nader *et al.,* 2008; Nader *et al.,* 2012; Buck and Zheng, 2002). This cross-talk can occur either through the actin-treadmilling and retrograde flow, which are tightly couple to microtubules and restricts their presence n the P-domain. Or, it could occur in the filopodia wherein the actin bundles guide the microtubules or MT can be captured at the mesh-like cell cortex (Coles and Bradke, 2015). The regulation of growth cone navigation can occur via three Rho-GTPases -RhoA, RAC1 and CDC42 (Govek *et al.,* 2005).

Another important developmental process involved in establishment of the circuitry is branching. Axons undergo branching to form connections with different parts of the body. The major form of axonal branching is collateral branching, wherein the branches arise from the axon shaft (Gibson and Ma, 2011). Branch formation involves several stages of cytoskeletal dynamics upon cue induction (Gallo and Letourneau, 1998; Danzer *et al.,* 2002). The sequential steps begin with formation of an actin patch, followed by patch elaboration. Then the patch gives rise to nascent protrusion which is then stabilized by entry of a dynamic microtubule and finally matures into a branch (Gallo, 2006; Orlova *et al.,* 2007; Ketschek and Gallo, 2010; Anderson *et al.,* 2011). Here too, actin-microtubule cross-talk is required for the branching process. One of prominent protein families implicate in this cross-talk are Septins. Septins are referred to as the fourth cytoskeleton and play a major role in branching (Cho *et al.,* 2011; Hu *et al.,* 2012; Ageta-Ishihara *et al.,* 2013). The major cellular structure involved in growth cone turning and axonal branching is- the filopodia. Filopodia are slender finger-like projections that are the primary chemosensory structures involved in growth cone guidance (Zheng *et al.,* 1996; Rajnicek *et al.,* 2006). The formation of filopodia, according to the convergent elongation model (CEM), starts with actin nucleation by nucleators such as Arp2/3 to gives rise to a mesh of actin. The branched actin filaments then

converge together and rapidly polymerize with the help of formins and Ena/VASP to push the membrane and protrude outwards (Svitkina *et al.,* 2003; Yang and Svitkina,2011; Mattila and Lappalainen 2008).

Formins are class of proteins identified by their formin homology1 and 2 (FH1 and FH2) domains (Castrillon and Wasserman, 1994; Higgs, 2005; Grunt *et al.,* 2008). They are involved in nucleation (Pruyne *et al.,* 2002; Sagot *et al.,* 2002) and elongation (Evangelista *et al.,* 2002; Kovar *et al.,* 2003) of actin filaments. The steps in mechanism of formin-mediated actin polymerization (Chesarone *et al.,* 2009; Campellone and Welch, 2010), begin with release of auto-inhibition. The formins are auto-inhibited and require activation by dimerization. Once, activated they nucleate with their FH2 domains. They strength of nucleation varies widely. Elongation from the barbed end occurs by binding to both terminal actin sub-units with the formin dimer – a closed conformation. This prevents further actin binding. Then one of the FH2 dimer steps towards the barbed end either before or after actin monomer addition. The FH2 dimers undergo iterative cycles of alternating between the two states to resemble a "stair- stepping" mechanism of actin elongation (Dong *et al.,* 2003; Brandt *et al.,* 2007; Paul and Pollard, 2009).

Fmn2 is a non-Diaphaneous related formin (DRF) and a member of the sub-family of FMNs which interacts with an another nucleator spire (Quinlan *et al.,* 2007; Montaville *et al.,* 2014). The tail domain of Fmn2 contributes to actin bundling (Viczarra *et al.,* 2011, 2014; Roth-Johnson *et al.,* 2014).

Fmn2 is highly expressed in the developing and adult CNS of mice and humans (Leader and Leder, 2000) and implicated in cognitive disorders such as mental retardation (Perrone *et al.,* 2012) and intellectual disability (Almuqbil *et al.,* 2013; Law *et al.,* 2014) and sensory processing dysfunction (SPD) (Marco *et al.,* 2018). The mechanism of function of Fmn2 in the developing nervous system was first elicited by Saharabudhe et al. in 2016. Fmn2 was found to important for axonal path finding *in-vivo*.

1.2. Objectives

1) Axonal guidance occurs through the growth cone machinery at the tip of the axon. The growth cone senses the environment through chemosensory structures, the filopodia. The

growth cone motility and directionality are governed by the underlying cytoskeleton rearrangements. And indeed, the study (Sahasrabudhe *et al.,* 2016) found that reduction of Fmn2 affected both growth cone speed and directionality in-vitro along with defects in axonal pathfinding *in-vivo*. Since growth cone navigation is dependent on coordination between actin and microtubules (Dent and Gertler, 2003; Lowery and Vactor, 2009; Buck and Zheng, 2002; Geraldo and Gordon-Weeks, 2009), the first objective was to investigate mechanisms of actin-microtubule crosstalk via Fmn2.

2) Collateral branching, wherein the branches appear from the shaft of the axon, is the major form of branching involved in establishing the circuitry (Cohen-cory *et al.,* 2010; Snider *et al.,* 2010). Branch formation begins with an actin patch that is formed mainly through Arp2/3 and matures and undergoes actin filament rearrangement to form a protrusion (Armijo-Weingart and Gallo, 2017; Ketschek and Gallo, 2010). The protrusions are then stabilized and mature into a branch with the entry of dynamic microtubules (Kornack and Giger, 2005; Dent *et al.,* 1999; Ketschek *et al.,* 2015). During the formation of a protrusion it is not yet clear how the patches convert to a protrusion as the patches are not protrusive. There is evidence that actin elongators Ena/VASP (Dwivedy *et al.,* 2007) are promote branching but it is not known whether formins contribute this process of branch formation. Owing to the enrichment of Fmn2 in the developing nervous system (Leader and Leder, 2000), the second objective was to assess the role of Fmn2 in axonal branching.

1.3. Major findings of the study

1.3.1. Role of Fmn2 In Actin-Microtubule Coordination During Axonal Pathfinding

During development, axonal guidance occurs through the growth cone machinery at the tip of the axon and deficiency of Fmn2 resulted in defects in axonal pathfinding *in-vivo* (Sahasrabudhe *et al.,* 2016). Since growth cone navigation is dependent on coordination between actin and

microtubules (Dent and Gertler, 2003; Lowery and Vactor, 2009), the mechanisms of actin-microtubule crosstalk via Fmn2 was investigated.

Fmn2 influences microtubule organization, exploration and stability in the growth cone in-vitro and in-vivo.

Depletion of Fmn2 in neuronal growth cones, altered the organization of microtubules to a more bundles form, indicating instability during movement. Exploration of microtubule into the filopodia was also reduced. The overall stability, as measured by postranslational modifications and EB3 dynamics, was also affected on depleting Fmn2 levels.

Loss of Fmn2 affects actin-MT alignment in the growth cone

In a motile growth cone, the exploratory microtubules invade the peripheral domain and are guided along actin bundles (Sabry *et al.,* 1991; Williamson *et al.,* 1996; Scheafer *et al.,* 2002; Zhou *et al.,* 2002). We found that Fmn2 decorates the actin bundles all along the filopodia and Fmn2-enriched filopodia commonly had MT innervation. Therefore, using super-resolution microscopy we quantified the alignment of actin bundles and MT and found that depletion of Fmn2 reduced the percentage of microtubules aligned to actin bundles. Moreover, even when they were aligned the lengths of microtubule aligned to the actin bundle were reduced, suggesting that Fmn2 facilitates the alignment of microtubule to actin and its subsequent presence in the filopodia. *In-vivo* imaging of EB3 dynamics in growth cones of sensory RB neurons in zebrafish embryos, also revealed that MT stability was affected with depletion of Fmn2.

Fmn2 cross-links actin and microtubule through the FSI domain in the filopodia

The alignment of actin and microtubule stabilized the microtubule in the filopodia by slowing down the polymerization and catastrophe speed, thereby, increasing the time spent by microtubule in the filopodia. The alignment of the two cytoskeletal systems was achieved by of physical coupling between actin and microtubule through the FSI domain of Fmn2.

Actin-MT cross-talk is required for growth cone turning

Functionally, the loss of alignment of actin and microtubule due to depletion of Fmn2, lead to destabilization of MT in filopodia. We found that microtubule stability was contributing factor to filopodial stability. Moreover, the loss of cross-talk via Fmn2 lead to defects in sensory guidance and growth cone turning.

Discussion

Microtubule organization is causal to growth cone motility. Microtubules become bundled just before undergoing a membrane collapse and forming a new axon (Tanaka ad Kirschner, 1991). So, the presence of a more bundled form in Fmn2 depleted growth cone indicates that this a consequence of poorly spread growth cones and increased retrograde flow (Sahasrabudhe et al, 2016).

Studies on Cappuccino (*drosophila* ortholog of Fmn2) have been shown to crosslink actin and microtubules in-vitro (Rosales-Nieves *et al.,* 2006). Binding of microtubule to Capu occurs primarily through non- specific charged interactions in the FSI domain, although FH2 domain has also been implicated in this interaction (Roth-Johnson *et al.,* 2014). These domains are conserved from *drosophila* to human. Moreover, biochemical studies from the lab show that FH2FSI region of *gallus* Fmn2 binds both actin and MT. Live-TIRF imaging of actin and microtubule filaments in the presence of Fmn2 showed co-localization of the two cytoskeletal filaments (Priyanka Dutta, unpublished data).

We hypothesize that when a growth cone senses a cue, higher recruitment of Fmn2 enables increase in actin polymerization and bundling in the chemotactic filopodia. Engagement with substrate which attenuate retrograde flow which allows greater number of microtubules to asymmetrically invade the peripheral region. The microtubule entry in filopodia is aided by Fmn2, where one-half of Fmn2 dimer attaches to the incoming microtubule and stabilizes it. The other half remain attached to the actin filament, which, promotes actin-MT coupling for growth cone turning.

1.3.2. Role of Fmn2 in axonal branching

Another important process involved into establishment of neuronal circuitry is axonal branching. Collateral branch formation involves multiple discreet steps that require rearrangement of both actin and microtubule cytoskeletons (Orlova *et al.,* 2007; Ketschek and Gallo, 2010; Ketschek *et al.,* 2015). The process begins with mesh of actin filaments called an actin patch formation followed by patch maturation and reorganization of the filaments to rise to a protrusion. The patches are not protrusive and require actin elongation (Dwivedy *et al.,* 2007) and bundling proteins but it is not clear that being the major actin elongators, what role formins play in this process of branch formation.

Fmn2 promotes protrusion density through its actin nucleation/elongation activity in the axon

Depletion of Fmn2 reduced the density of protrusions on the axons, whereas, overexpression led to increased number of protrusions. Single point mutation (I1226A) blocking actin nucleation/elongation of Fmn2 was unable to rescue the density of protrusions.

Fmn2 localizes to the base of axons and pre-determines protrusion formation

Live-imaging of gFmn2-GFP revealed that Fmn2 localized to curved, deformed membranes and formed cheveron-shaped before the formation of a protrusion. Moreover, these structures persisted even after the protrusions were initiated. Fmn2 also co-localized with actin structures- almost immobile actin patch and another rapidly moving actin trail. Although, actin patch is the predominant actin structure that gave rise to a branch, actin trails were also found to be capable of initiating branch formation.

Fmn2 promotes patch size and lifetime

Depletion of Fmn2 reduced the size and lifetime of actin patches, without affecting patch density. It also caused patches to be more mobile. Moreover, Fmn2 knockdown caused multiple cycles of patch formation and disappearance taking place in the same spot of an axon, thereby, leading to a "blinking" phenomenon, indicating an overall instability of these actin structures.

Fmn2 promotes branching of axons in-vitro and in-vivo

The number of axons that displayed branches after Fmn2 depletion was reduced by half. This phenomenon was rescued by morpholino resistant Fmn2 and nucleation/ elongation activity

mutant as well. Thus, Fmn2 may be involved in two independent functions 1) initiating protrusions and 2) stabilising existing protrusions, which are most likely mediated via distinct domains. Observing branching in motor neurons of zebrafish revealed that depletion of Fmn2 caused drastically reduced branching *in-vivo*, as well.

Discussion

Formins has been predicted to be involved in axonal branching, but there is not much evidence seen till now. We discovered that Fmn2 localizes to the base of every protrusion. This persistent expression at the base could serve to guide the incoming microtubules or acts a filter or provide structural support. In literature, only a couple of proteins (of the same family) have been reported to form chevron structures at the base of protrusions. These are septin -6 and -7 (Hu *et al.,* 2012; Nölke *et al.,* 2016) and BAR (Bin/amphiphysin/Rvs) proteins such as IRSp53(Chung *et al.,* 2015) and SR-GAPs (Coutinho-Budd *et al.,* 2012). Actin is present all along the cortex of the axon, but Fmn2 appears to be sensitive to curvature of the membrane. The mechanism of this recruitment of Fmn2 is yet unknown.

Fmn2 does not affect patch formation but promotes actin patch maintenance. Actin patches are under the control of antagonistic factors and there is a balance between actin assembly and disassembly (Smith *et al.,* 2001; Nakano *et al.,* 2001).Fmn2 appears to be regulating the patch dynamics by tipping the balance towards actin elongation and stabilizing the F-actin filaments after patch formation.

Every protrusion does not lead to a mature branch. This frequency of transition from a nascent protrusion to a mature branch was unexpectedly much higher in rescue experiments with nucleation/elongation dead mutant of Fmn2. This could be either due increase in availability of free-actin pool which direct more actin in existing protrusions (the ones that were formed despite reduction in overall protrusion density) and enable maturation or due to the still intact microtubule binding FSI domain ofFmn2.

We hypothesize that actin patch formed is co-inhabited by Fmn2, which leads to its maturation. Fmn2 also co-localizes to sites of membrane deformation. The deformation of the membrane and its bud-like maintenance due to curved actin bundles reduces the force required to push it further

to form a protrusion. Polymerization of parallel bundles of actin filament via Fmn2 provide the pushing force necessary for protrusion formation. The protrusion is then stabilized by guided entry of dynamic microtubules.

Chapter 2

INTRODUCTION

2.1. Brain development

The human brain, with about a 100 billion cells and more than a trillion connections, is certainly the one of the most complex object man has ever come across. Every sensation experienced and behaviour generated by us is governed by the nervous system. The nervous system itself develops from the ectoderm – outermost tissue of the embryo (Sanes *et al.,* 2005). The neural ectoderm then forms a neural plate along the dorsal side of the embryo and the neural plate wraps around itself to form a neural tube. In the vertebrate brain, the neural tube gives rise to three vesicles: prosencephalon (forebrain), mesencephalon (midbrain) and telencephalon (hindbrain). Then, the neural progenitor cells in the neural tissue start proliferating and differentiate into the specific neuronal cell types or glial cells. The neurons and glial cells which stay in the brain, spinal cord and retina form the central nervous system (CNS) and those which migrate to form the nerve and the ganglia become a part of the peripheral nervous system (PNS). After the completion of migration, the neurons send out axons and dendrites to other neurons and connect via synapses and gap junctions to establish the neural circuitry in the CNS. And those in the PNS innervate different parts of the body and form synaptic connections (Sanes *et al.,* 2005). Our current knowledge of the molecular mechanisms of these developmental processes is still quite limited. Even more rudimentary is understanding of how do abnormalities in connectivity either due to genetic or epigenetic changes lead to neurodevelopmental disorders.

Neurodevelopmental disorders (NDD)

Neurodevelopmental disorders such as autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), schizophrenia and intellectual disability affect cognition, learning and memory, behaviour as well as language and motor skills (Cioni *et al.,* 2016; Mullin *et al.,* 2013). Although some cases of intellectual disability are associated with specific genes, most of these disorders are a result of a combination of genetic, social, psychosomatic and physical interactions (America's Children and the Environment, Third Edition, 2015). Genetic origin of these NDD's could be due to defects in genes leading to mislocalized developmental trajectories in different brain regions resulting in aberrant circuit connectivity (Geidd *et al.,* 2015). Circuit defects could also be due to problems in synapse formation after reaching the target (Washbourne, 2015),

leading to imbalances in excitatory: inhibitory synapses which is particularly important for higher order cognitive processing (Sohal and Rubenstein, 2019). Therefore, dissecting and integrating the mechanism(s) of circuit formation at the molecular, cellular and behavioural levels is critical to gaining a better understanding and identification of novel targets and therapies for these disorders.

2.2. Neuron: development and structure

At the cellular level, neurons possess a distinctive architecture with a nucleus, a cell body and long cellular processes- the **axon** and **dendrites**. These two distinct morphological and functional extensions are required by the neuron in order to communicate with other neurons and tissues.

Establishment of neuronal circuit

During development, neurons undergo several stages of development (Figure **2. 1**) (Stiles and Jernigan, 2010; The Mind's Machine, 2nd Edition, 2016):

1. **Neurogenesis**

The neurons are born from neural progenitor cells in the ventricular zone of the neural tube and recently, from progenitors in the ventral telencephalon. At first, the neural progenitor cells undergo symmetric division to increase their numbers. Then gradually shift to asymmetric division where it divides to give rise to a neuron and another progenitor cell. Progenitor cell undergoes subsequent rounds of asymmetric division but these early undifferentiated neurons, known as neuroblasts, are post-mitotic cells and do not undergo further divisions; instead they migrate to different regions of the brain and spinal cord (central nervous system or CNS).

2. **Neuronal migration and differentiation**

Neurons of the CNS undergo migration by crawling along radial glial cells, which act as guides for translocation across several millimetre distances. Neurons born in the ventral telencephalon undergo tangential migration involving guidance molecules that direct the neuronal migration.

Post- migration, neurons differentiate into distinct classes of neurons that make connections with specific targets. Early neuronal progenitors are capable of forming any type of neuron, but as development progresses the ability to form distinct types of neuron becomes limited.

3.Synaptogenesis and synapse maturation

In order to make connections with other neurons and tissues, neurons undergo several morphological and polarity developments to form an axon and dendrites (Figure **2. 2**). In an immature neuron these cellular processes are called neurites (stage 2) and one of these neurites polarizes to become a long and dominating process called axon (stage 3). The process of polarization involves the neuronal cytoskeleton. Actin polymerization, as seen in CDC42 knockout mice that are unable to establish polarity, and microtubule stability are key processes that govern this process (Lalli, 2012). The other neurites mature to form short and branched processes called dendrites (stage 4). The axon elongates with a dynamic structure called a growth cone at its tip. The growth cone is guided by attractive or repulsive cues to reach its target where its forms a synapse. The axon also forms branches to form synapses with multiple target regions. The synapses undergo rearrangement and pruning based on cognitive input and cell apoptosis.

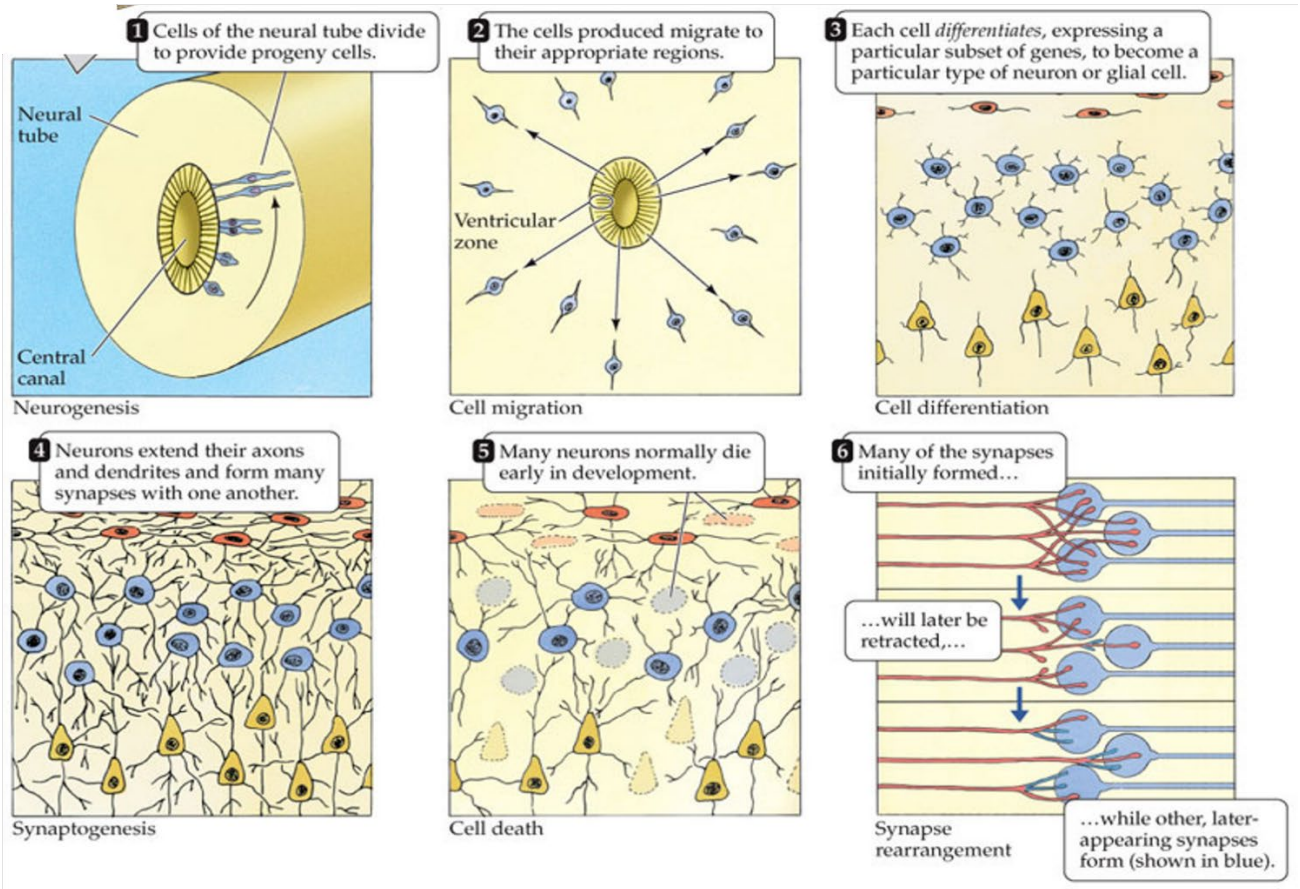

Figure 2. 1. Shows the different stages of establishment of neuronal circuit. Reproduced from The Mind's Machine, 2nd Edition, 2016.

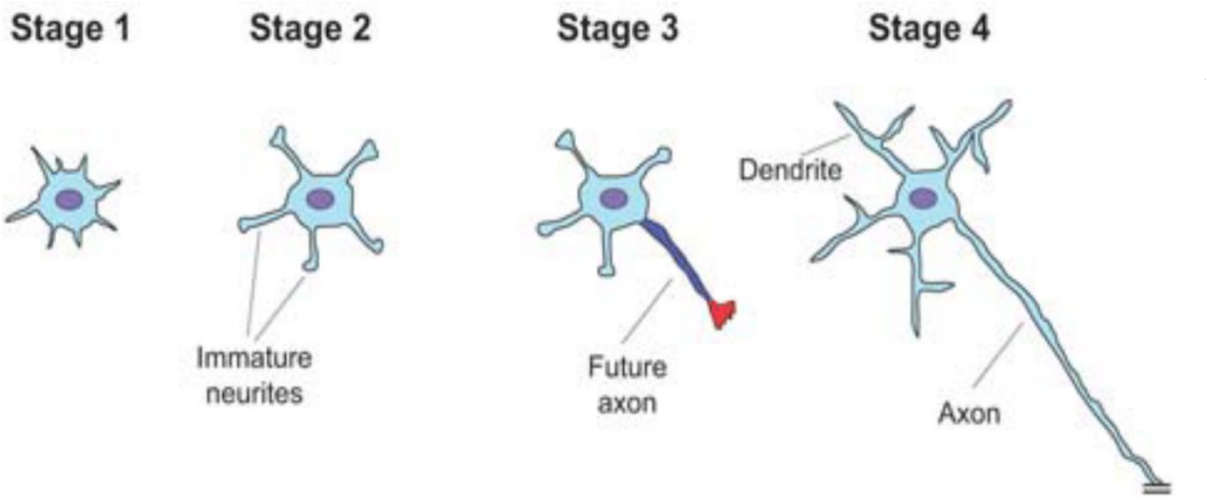

Figure 2. 2. Shows the different stages of development of polarity. Reproduced from Lalli, 2012.

The molecular mechanisms underlying the developmental processes of axonal migration and branching have been investigated in this thesis. Both of these processes are dependent on the underlying structural elements – the cytoskeleton.

2.3. The cytoskeleton

The cytoskeleton is a dynamic network of protein filaments that is present in all cells, including bacteria. It encompasses the entire cytoplasm of all eukaryotes and gives the cell its structure and shape. The cytoskeletal system is involved in cell migration, division, cell signalling and intracellular transport. The dynamic nature of the filaments enables them to undergo polymerization and depolymerization based on cellular function. The three major cytoskeletal systems are: **actin**, **microtubules** and intermediate filaments. This thesis will focus on the first two widely studied systems.

2.3.1. Actin

Actin monomer is a 42-kDa protein with a diameter of about 4-7nm (Dominguez and Holmes, 2011). The monomeric structure is a globule that contains two lobes separated by a cleft where ATP and Mg^{2+} is usually bound. Monomeric actin or G- (globular) actin is present at a concentration of ≤100μM in the cell (Pollard *et al.,* 2000). G-actin undergoes polymerization to form filamentous or F-actin.

F-actin

F- actin is a single stranded linear right-handed helix of actin polymers with a pitch of ~71.5nm. The helix is staggered by 2.75nm and cross every 35.8nm (Pollard and Cooper, 1986). F-actin is a slow ATPase which converts ATP to ADP at a rate of $0.3s^{-1}$. F-actin is polar in nature with two dynamically different barbed and pointed ends. The rate of elongation is 10 times faster from the barbed end (Blanchoin *et al.,* 2014).

Mechanism of actin polymerization

The polymerization reaction can be divided into 3 steps (Pollard and Cooper, 1986; Pollard *et al.,* 2000; Chesarone and Goode, 2009; Mattila and Lappalainen 2008) (Figure **2. 3**): 1) **activation** of the monomer- G-actin is activated by binding to salt Mg^{2+} and undergoes conformational change. Activated actin monomer forms nuclei 10 times faster than un-activated actin and also undergoes faster polymerization. 2) **nucleation** -the nucleus for polymerization is formed with a trimer or tetramer of actin molecules. Trimer is the lowest number actin molecules that are required to be bound for the elongation and is the rate-limiting step of the spontaneous polymerization reaction. (3) **elongation**- the critical concentration of actin required for polymerization from actin seed is 0.1 μM at the barbed end and 0.6 μM at the pointed end. This rapid elongation from the barbed end while preventing polymerization at pointed end is seen after the addition of Mg^{2+} and another actin binding protein called profilin in-vitro (Pollard and Cooper, 1986; Dominguez and Holmes, 2011; Blanchoin *et al.,* 2014).

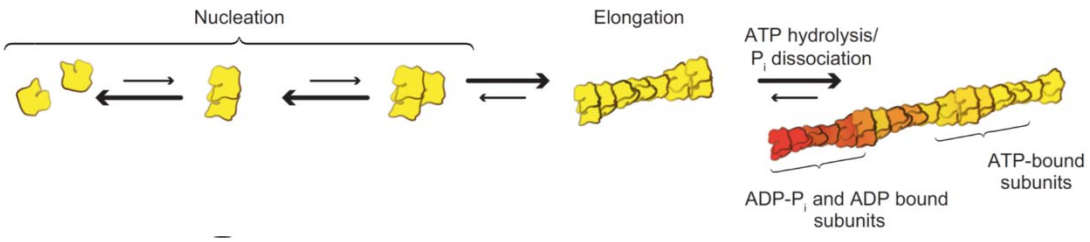

Figure 2. 3. Shows the nucleation and elongation stages of actin monomers (yellow). Adapted from Blanchoin *et al.,* 2014.

Regulation of actin polymerization

In cells, the prominent nucleators are a) **Arp 2/3** complex activated by the Wiskott-Aldrich Syndrome protein (**WASP)/WAVE** family of proteins (Goley and Welch, 2006) b) **formins**, a class of proteins identified by their FH2 domains involved in both nucleation and elongation (Faix and Grosse, 2006; Pring *et al.,* 2003; Otomo *et al.,* 2005) c) **spire**, a class of proteins that bind and nucleate actin through the 4 tandem WASp-homology 2 (WH2) domain repeats while remaining associated with the pointed end to allow free barbed end polymerization. Spire also interacts with formin, *cappuccino* (Quinlan *et al.,* 2005; Bosch *et al.,* 2007) d) **Cordon bleu** (Cobl), a protein nucleates monomeric actin through 3 WH2 domains (Ahuja *et al.,* 2007).

In cells, the rate of actin elongation from nucleated seeds is dramatically increased by actin elongators.

The two prominent actin elongators are a) **Ena/VASP** proteins and b) **formins**. Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins form a tetramer that accelerate barbed end elongation and also protect the barbed end from capping proteins (limit the rate and extent of elongation of barbed ends) (Reinhard et al, 1992; Ferron *et al.,* 2007). Unlike formins, Ena/VASP do not nucleate actin (Bear *et al.,* 2011; Barzik *et al.,* 2005). Similar to Ena/VASP proteins, formins accelerate barbed end elongation rates and also possess anti-capping

activity (Kovar, 2006; Kovar *et al.,* 2006; Goode and Eck, 2007). The mechanism of action of formins will be discussed in depth later in this chapter (Figure **2. 4**).

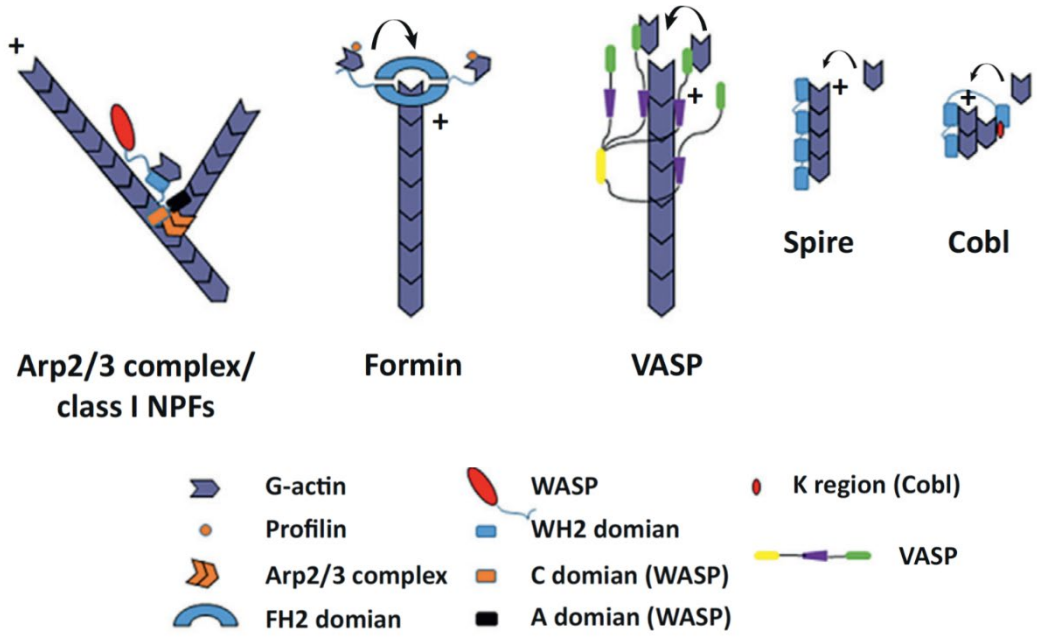

Figure 2. 4. Different classes of actin nucleators and elongators. Adapted ftom Siton-Mendelson and Bernheim-Groswasser, 2017.

Disassembly of actin filaments

Actin filaments undergo disassembly by two separate manner:1) via **ADF/cofilin**. Actin deploymerizing factor (ADF) was the first actin disassembly protein to be discovered (Bamburg *et al.,* 1980). Since then, several proteins have discovered and are grouped under the class of ADF/cofilin family with vertebrates expressing ADF, cofilin1 and cofilin2(Bernstein and Bamburg, 2010). ADF/cofilin disassembles actin filaments by destabilizing instead of depolymerizing (from the pointed end) (Blanchoin *et al.,* 2014; Winterhoff and Faix, 2015). It does so by cooperatively binding to actin filaments and alters their mechanical properties such as increasing the helical twist (McGough *et al.,* 1997) and decreasing the persistence length (the highest length the polymer reaches before buckling) (McCullough *et al.,* 2011). This weakens the lateral contacts between actin subunits in the filament (Paavilainen *et al.,* 2008) and severs the filaments between boundaries of bare and ADF/cofilin-decorated actin filament (Suarez *et al.,* 2011). 2) via **myosin**. Another mode of disassembling actin filament is through myosin contraction (Kohler and Baush, 2012; Blanchoin *et al.,* 2014). Myosin II is a motor protein that utilizes ATP

to pull or contract actin filaments (Tyska and Warshaw, 2002). The disassembly occurs in two steps; first the bundles dissociate, the filament fragments (Haviv *et al.,* 2008). Myosin, first, reorganizes branched filaments into anti-parallel bundles and separates them, while parallel bundles are not affected, thereby displaying an "orientation selection" based mechanism (Reymann *et al.,* 2012). Then, at a single filament level, myosin disassembles actin filament similarly to ADF/Cofilin, by affecting the filament's mechanical properties. Actin filaments fragment by buckling due to myosin contraction (Murell and Gardel, 2012).

Actin architecture

Actin filaments form different architectures that are involved in maintaining cell shape and migration. Actin, along with myosin motors, is mainly involved in generating forces which cause protrusion and contraction at the leading edge (Kasza and Zallen, 2011).

Branched actin architecture

At the leading edge of a cell, multiple actin filaments form a branched network that are involved in the generating the mechanical force required to move the cell forward. This structure called the lamellipodium is made of a dense network of branched actin (Figure **2. 5** A.). Arp2/3 complex is the major factor involved in the nucleation of branched filaments. Arp2/3 complex sits on the side the actin filament and nucleates actin filaments at a fixed angle of 70°. It caps the pointed end and promotes elongation at the barbed end. But Arp2/3 alone is a slow acting complex, it is activated by nucleating-promoting factors (NPFs) from Wiskott-Aldrich Syndrome protein (WASP)/WAVE family of proteins through their characteristic WH2 domains (Mullins *et al.,* 1998; Pollard *et al.,* 2000; Strickler *et al.,* 2010). Being an actin nucleator, Arp2/3 is not the only protein involved in forming the actin meshwork but also involves elongators such as Ena/WASP (Koestler *et al.,* 2008) and formins (Yang *et al.,* 2007).

Parallel actin architecture

Actin filaments also form parallel bundles, with the barbed end oriented in the same direction. These bundles are found in protrusive structures of the cell such as filopodia (Figure **2. 5** B). Structurally, a filopodium is composed of 10-30 continuous actin filaments that are unbranched,

parallel (with their barbed end pointed towards the cell membrane) and bundled (Svitkina *et al.,* 2003). The main two major class of proteins involved in the formation of parallel bundles are Ena/VASP and formins (Yang *et al.,* 2007; Goode and Eck, 2007). These two class of protein sits at the tips of filopodia and are involved in processive elongation of the filament (Mattila and Lappalainen 2008). These polymerizing, parallel actin filaments are bundled by crosslinking proteins such as fascin (DeRosier and Edds, 1980) as well as formins (Roth-Johnson *et al.,* 2014) and VASP (Schirenbeck *et al.,* 2006). Cross-linking with fascin is dynamic; it does not cross-link well on preformed filaments but is more efficient when filaments are growing and undergoes reversible interactions with actin (Kureishy *et al.,* 2002). The cross-links are rigid and aid in mechanical resistance (Vignjevic *et al.,* 2006; Aratyn *et al.,* 2007).

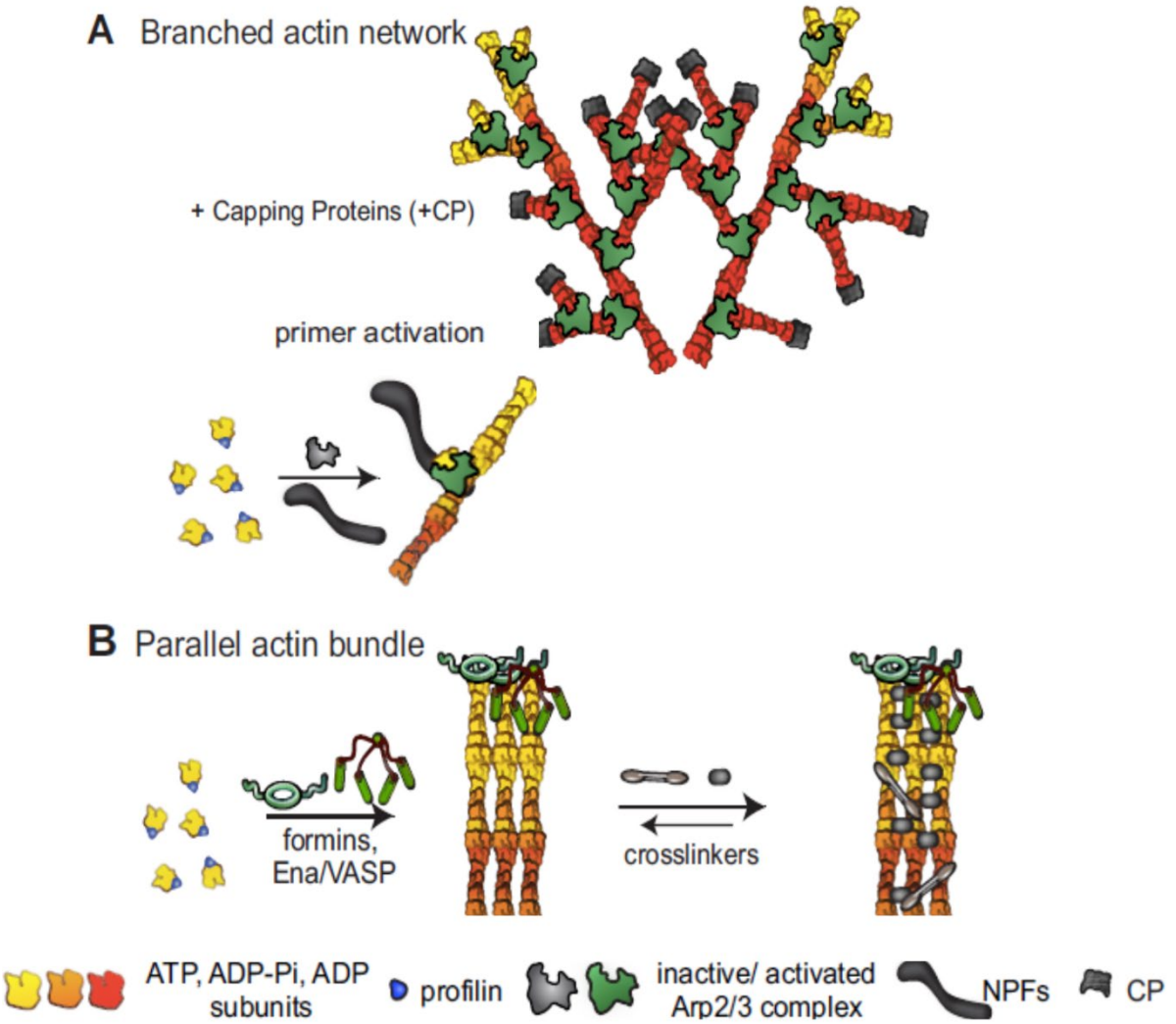

Figure 2. 5. Actin architecture. A. shows branched actin network formation B. Shows parallel actin bundle formation. Adapted from Blanchoin *et al.,* 2014.

2.3.2. Microtubules

Microtubules (MT) are tube-like polymers of α- and β-tubulin dimers (~55kD). A few hundred of these dimers bind in a head-to-tail fashion to form a protofilament using GTP as the energy source (Figure **2. 6** a). Around 8-17 of these protofilaments associate laterally to form a hollow tube of one microtubule with inner and outer width of 17nm and 25nm, respectively (Akhmanova and Steinmetz, 2008) (Figure **2. 6** b). Microtubules are another major cytoskeletal system involved

in regulating cell shape and migration. These filaments are quite dynamic and undergo phases of polymerization and depolymerization.

Microtubule dynamics

Microtubules occupy central regions of cells and these polar filaments nucleate from ɣ-tubulin ring complexes (ɣ-TURC) with the minus-end attached to the complex (Muroyama and Lechler, 2017; Mitchison and Kirschner, 1984 I) and the plus end polymerizes spontaneously (Vulevic and Correia 1997) and rapidly from GTP-loaded tubulin subunits. The polymerization reaction occurs through GTP hydrolysis and the polymer remains relatively straight and is GTP-capped. Switch from polymerization to depolymerization phase occurs when the conversion of GTP-tubulin to GDP-tubulin causes a profound bend in the subunits. This curvature most likely destabilizes the lateral interaction between the protofilaments and leads to "catastrophe". When the polymer again starts re-polymerizing that biochemical switch is called the "rescue". The phase transitions between polymerization and shrinkage occurs stochastically as the microtubule grows in-vitro and is termed as dynamic instability (Figure **2. 6** c) (Mitchison and Kirschner, 1984 II; Akhmanova and Steinmetz, 2008).

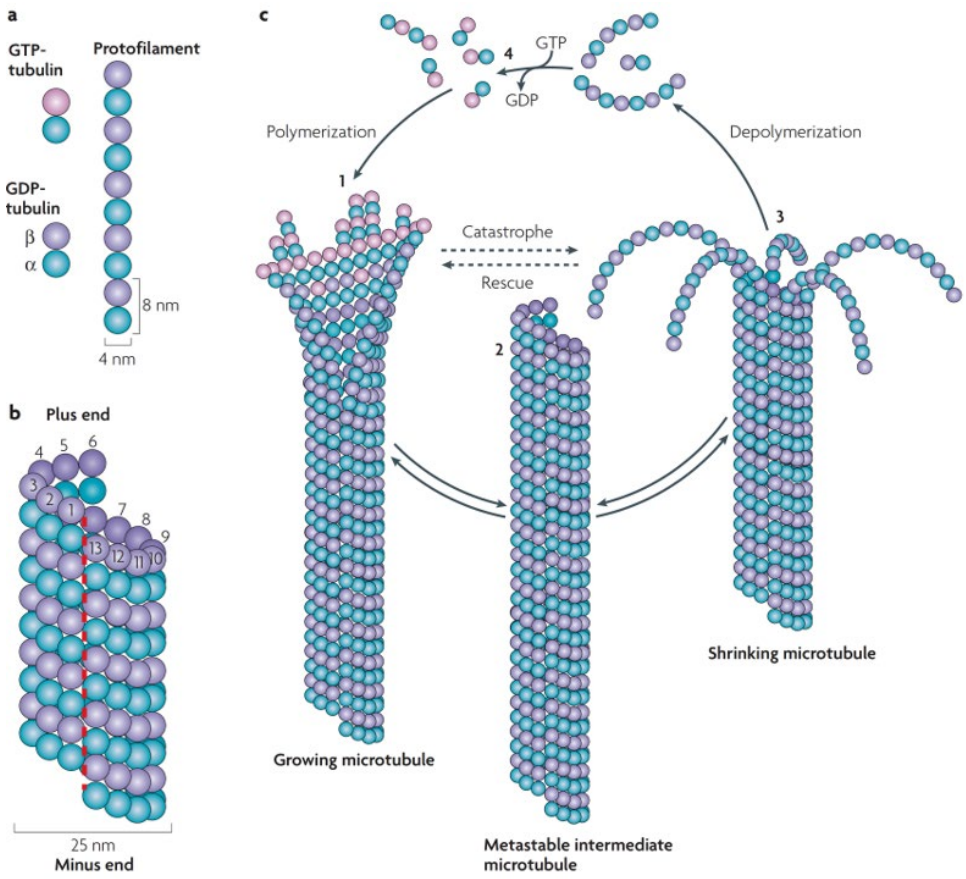

Figure 2. 6. Microtubules dynamics. a) tubulin heterodimer forming a single protofilament. b) microtubule hollow tube. c) shows microtubules undergoing phases of spontaneous polymerization and depolymerization, i.e., dynamic instability. Adapted from Akhmanova and Steinmetz, 2008.

Regulation of microtubule dynamics

Microtubules are under tight regulation *in-vivo* with proteins that both promote its assembly (Al-Bassam and Chang 2011; Komarova et al. 2009) and also proteins that destabilize microtubules (Cassimeris 2002; Gupta et al. 2013); proteins that bundle or cross-link (Walczak and Shaw 2010) or sever microtubule(Roll-Mecak and McNally 2010) or motor proteins that bind and use the microtubule as tracks for cargo transport (Sweeney and Holzbaur 2016) (Figure **2. 7**) (Goodson and Jonasson, 2018). Prominent among the microtubule regulators are a group of microtubule-associated proteins or MAPs. MAPs bind to tubulin at multiple sites and cross-link multiple subunits and stabilize them (Walczak and Shaw 2010; Dixit et al. 2008). They stabilize microtubules by decreasing the dissociation of tubulin subunits and facilitate microtubule assembly (Horio and Murata, 2014).

A specific category of MAPs is +TIP tracking proteins, which dynamically track the plus end of a polymerizing microtubule (Akhmanova and Steinmetz, 2008; 2015). **End-binding (EB)** proteins are the family 3 (EB1,2 and 3) proteins that accumulate at the growing plus end (Schuyler and Pellman, 2001; Lansbergen and Akhmanova, 2006). Highly conserved, the N-terminus of these proteins is necessary for MT binding (Hayashi and Ikura, 2003), whereas the C-domain is required for dimerization (Honnappa *et al.,* 2005). The proposed mechanism of tracking plus end of microtubule by EB proteins is through recognition of certain tubulin sites that are otherwise inaccessible due to contacts between protofilaments in the rest of the microtubule. At the plus end, however, the tubulin site is exposed for binding with the EB proteins. Another important feature of these EB proteins is that they fall of the microtubule lattice immediately as the microtubule undergoes catastrophe (Sandblad *et al.,* 2006; Beiling *et al.,* 2007; Tirnauer *et al.,* 2002).

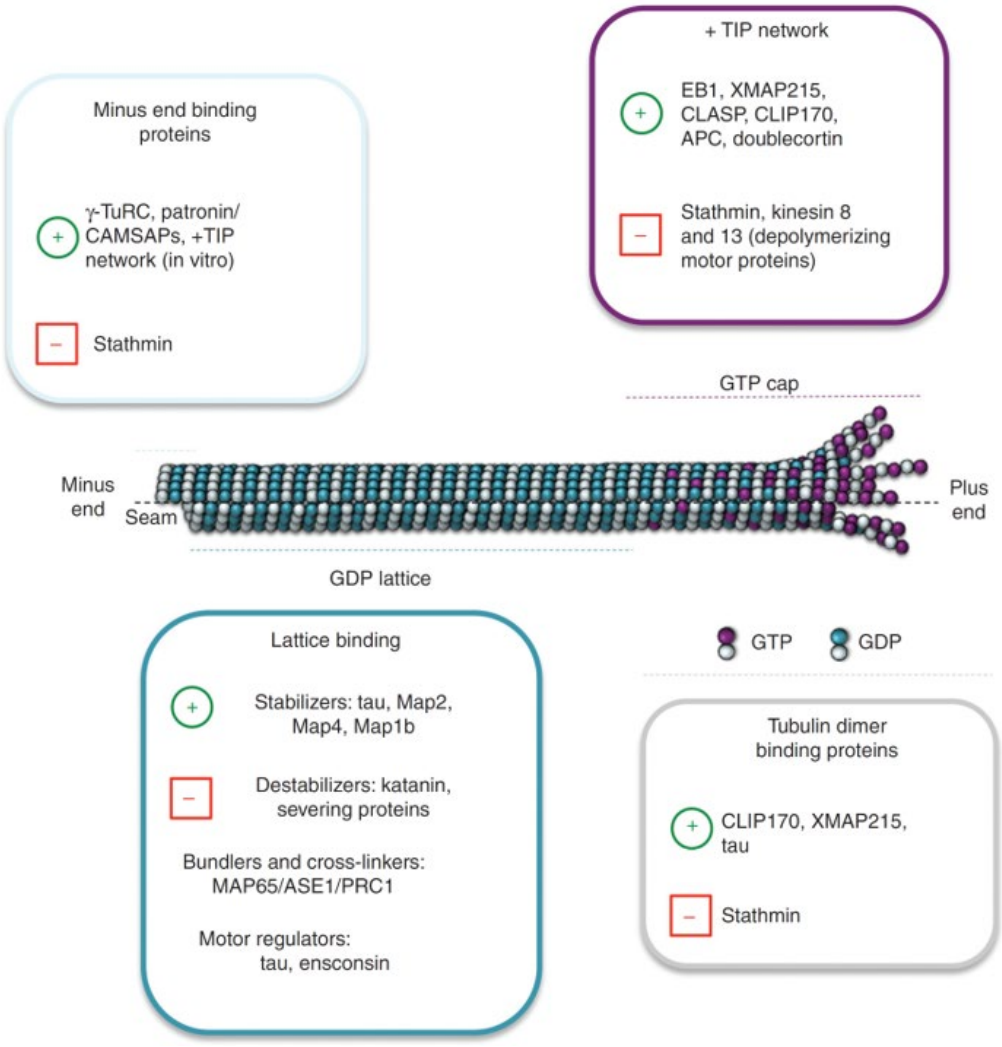

Figure 2. 7. Classes of different microtubule binding proteins regulating microtubule dynamics. Adapted from Goodson and Jonasson, 2018.

Dynamic microtubules have a rapid turnover with a half-life of about 10 minutes *in-vivo*. However, a proportion of microtubules are quite stable with a half-life of more than 1 hour (Schulze and Kirschner, 1987). These microtubules are more stable and less dynamic, non-growing (Kries, 1987) and undergo a number of posttranslational modifications (Janke, 2014) (Figure **2. 8**). One of the most widely known posttranslational modification is the detyrosination of α-tubulin at C-terminus exposing glutamate residue. Along with acetylation, detyrosination of microtubules is utilized to identify stabilized microtubules (Webster *et al.,* 1987).

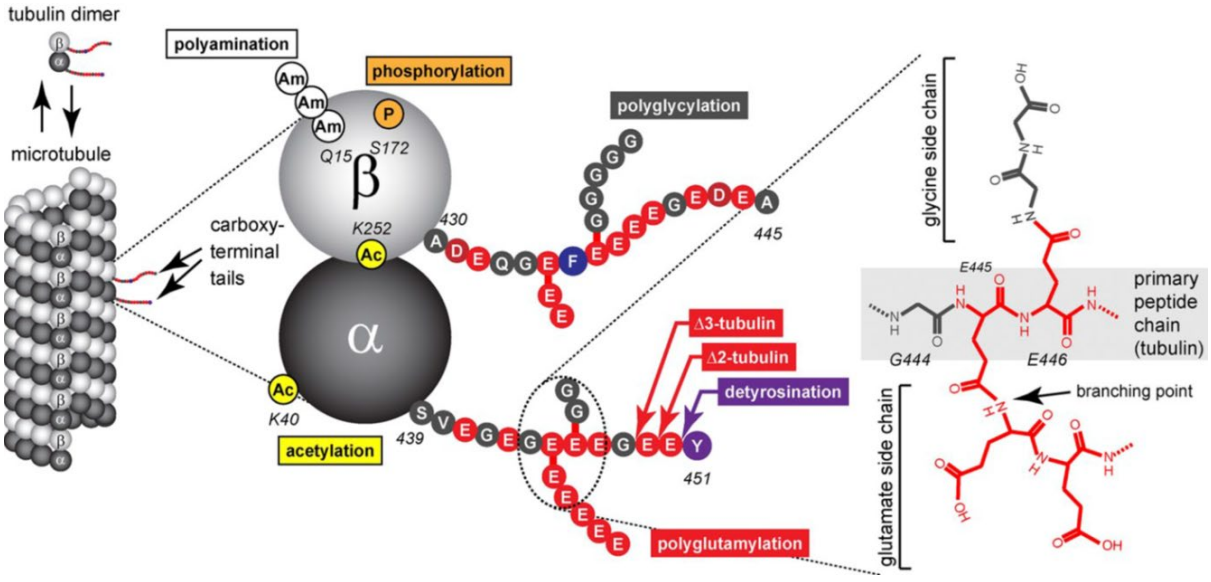

Figure 2. 8. Tubulin code. Different posttranslational modifications in tubulin regulating microtubule stability. Adapted from Janke, 2014.

2.4. Neuronal cytoskeleton

The neuronal architecture is composed of three major cytoskeletal elements: Actin, microtubules and neurofilaments. These filaments are very stable and mostly polymerized. Actin is mainly present at the periphery of the cell all along the axon and the growth cone. The core is made up microtubule bundles which run along the shaft of the axon and present mainly in the central region of the growth cone. In between microtubules and actin is present the intermediate filaments or, in this case, neurofilaments. Neurofilaments are mainly confined to the axon shaft (Bagnard, 2007; Lowery and Vactor, 2009).

2.4.1. Growth cone cytoskeleton

Based on the underlying cytoskeletal organization, the growth cone is structurally classified into three regions (Figure **2. 9**) (Bridgman and Dailey, 1989; Forscher and Smith, 1988; Smith,1988; Dent and Gertler, 2003). The **peripheral (P)** domain contains bundled actin filaments, that form the finger-like filopodia separated by mesh-like branched F-actin network which form a sheet-like lamellipodial veil. The **central (C)** domain encloses stable MTs that enter the growth cone in their bundled form from the axon shaft. The MTs remain largely restricted to the C-domain along with a large number of vesicles and other cellular organelles with a few dynamic, exploratory MTs that explore the P domain. Finally, at the interface between the P and C domains, lie the actomyosin contractile structures (termed actin arcs) that are perpendicular to F-actin bundles and form a hemi-circumferential ring, termed as the **transition (T)** zone (Schaefer *et al.,* 2002). As the growth cone advances, a new P-domain is formed ahead of the previous one and a new C-domain takes up the position of the original P-domain. This cycle continues as on as the growth cone advances.

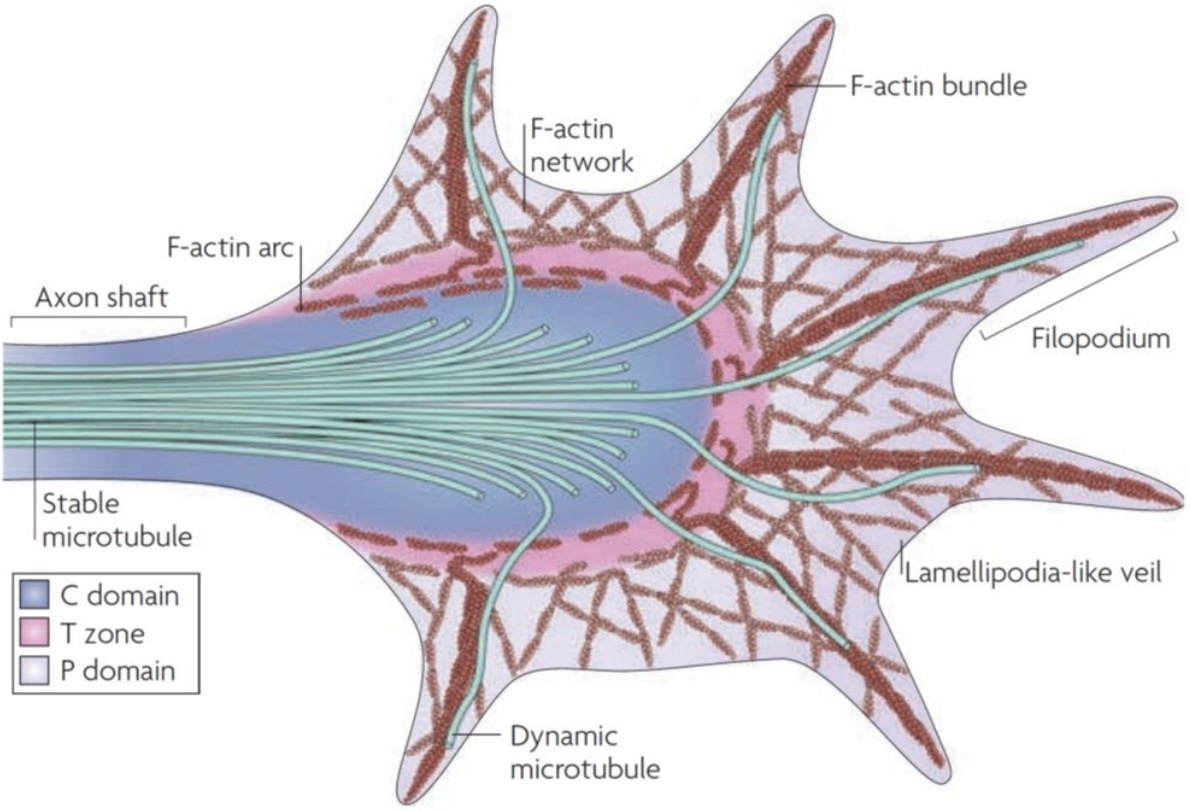

Figure 2. 9. Growth cone cytoskeleton. Shows the 3 regions – P-domain consisting of branched actin network and parallel bundles and exploratory microtubules; T- zone consisting of actin arcs and C- domain consisting of microtubules. Adapted from Lowery and Vactor, 2009.

2.5. Growth cone movement

The advancement of growth cone can be described in three stages: **Protrusion**, **engorgement** and **consolidation** (Goldberg and Burmeister, 1986; Halloran and Kalil,1994; Harris *et al.,* 1987; Dent and Gertler, 2003; Lowery and Vactor, 2009). In the **protrusion** stage the filopodia and lamellipodia undergo rapid extension owing to the actin polymerization at the leading edge. The actin polymers are carried back from the edge to the center of the growth cone (F –actin retrograde flow) where they are disassembled. This forward push from the F – actin assembly from the barbed end along with the disassembly from the pointed end (F-actin treadmilling) and backward pull from the retrograde flow are kept in balance during the idling phase of the growth cone motion. The retrograde flow is generated by a combination of the contractility of myosin II motor present at the transition zone and the "push" provided by the F-actin polymerization at the leading edge

(Medeiros *et al.,* 2006). According to the substrate-cytoskeletal coupling model (Mitchison and Kirschner, 1988; Suter and Forscher, 2000), when the growth cone attaches to a substrate at focal contact points, ECM (extra-cellular matrix) proteins get mechanically coupled to focal adhesion proteins like vinculin and talin (form molecular clutches) (Hu *et al.,* 2007), which in turn bind to F-actin and generate traction forces to reduce the F- actin retrograde flow locally (Lin and Forscher,1995). The membrane is pushed forward due to the protrusive forces caused by actin polymerization in the region ahead of the attachment. The T-zone is rich in actin arcs formed by end-to-end annealing of actomyosin bundles that form a mechano-chemical barrier for MTs attempting to invade the P-domain (Schaefer *et al.,* 2002). During the **engorgement** phase, these actin arcs depolymerize, thereby clearing the region for MTs to advance into the P-domain (Lowery and Vactor, 2009). The final step is **consolidation**, after the growth cone has advanced the MT are compressed by the re – established actomyosin arcs and stabilized into bundles by the MT – associated proteins. These iterative cycles of advancement, engorgement and consolidation result in axon elongation.

Growth cone turning: actin-microtubule crosstalk

Growth cones, during motion, interact with the surrounding environment to receive signals about the direction of their growth and the response requires rearrangement of the cytoskeletal elements (Koh, 2006; Robles and Gomez, 2006; Wolf *et al.,* 2008; Govek *et al.,* 2005; Ensslen-Craig and Brady-Kalnay, 2004). To steer the growth cone in a certain direction, the symmetry needs to be broken. While actin-adhesion systems have been implicated in generating traction force to move forward; microtubules, which are prominent mostly in the central domains, could break the symmetry by sending out exploratory microtubules on one side of the P-domain. These dynamic microtubules could act as guidance sensors giving directionality to the growth cone movement (Sabry *et al.,* 1991; Challacombe *et al.,* 1997; Lin and Forscher, 1993, 1995; Lee and Suter, 2008). It has been shown that stabilizing dynamic microtubules on one-side of the growth cone by local photoactivated-release of microtubule-stabilizing drug Taxol, caused the growth cone to turn on that side (Buck and Zheng, 2002). This suggested that in order to turn towards an attractive cue, microtubules were somehow captured and stabilized towards the cue receiving side of the growth

cone (Nader *et al.,* 2008; Nader *et al.,* 2012; Buck and Zheng, 2002). Local application of repulsive cues such as myosin light-chain kinase inhibitor (MLCK) led to focal (restricted to a small space) loss of actin bundles from the peripheral region of only one side of the growth cone, which caused growth cone collapse on that side and turn away from the cue. Moreover, it was found that microtubules had also undergone rearrangements and now were visible only in the areas where actin bundles were present and absent from the side that had lost the actin bundles (Zhou *et al.,* 2002). Thus, both actin and microtubule are required for growth cone turning.

In neurons multiple modes of actin-microtubule crosstalk have been observed (Figure **2. 10**) (Coles and Bradke, 2015). **First** (Figure **2. 10** A.), actin- treadmilling and retrograde flow is tightly coupled to MT translocation and restricts the entry of MT's into the P-domain. Retrograde flow can directly sweep away the exploratory MTs (Shaefer *et al.,* 2002; Lee and Suter, 2008). **Second** (Figure **2. 10** B.), actin bundles act as guides for extending microtubules in the P-domain (Challacombe *et al.,* 1997; Zhou *et al.,* 2002). While in the filopodia, actin-microtubule crosslinking could occur through multiple modes, such as, it could be a) +tip mediated. Spectraplakins like Bpag/dystonin and ACF7/MACF bind to EB proteins through growth arrest specific 2 (GAS2) domain and directly to actin through the calponin homology (CH) domain or form a crosslinking complex (Sanchez-Soriano *et al.,* 2009; Alves-Silva *et al.,* 2012). It could be also through b) direct interaction via a single protein. MAP2C crosslinks both actin and microtubule through the carboxy- terminal domain (Jaworski *et al.,* 2009). c) through motor proteins, such as dynein (Grabham *et al.,* 2007), which use microtubules as tracks for transport. **Third** (Figure **2. 10** C.), is through microtubule capture at the branched actin network present in cell cortex, through cell adhesion molecules. Cell-adhesion molecules (CAMs) like, apCAM (Lee and Suter, 2008) and NCAM (Perlson *et al.,* 2013) couple with the actin cytoskeleton resulting in retrograde flow attenuation, thereby facilitating exploration and capture of microtubules at the cell edge.

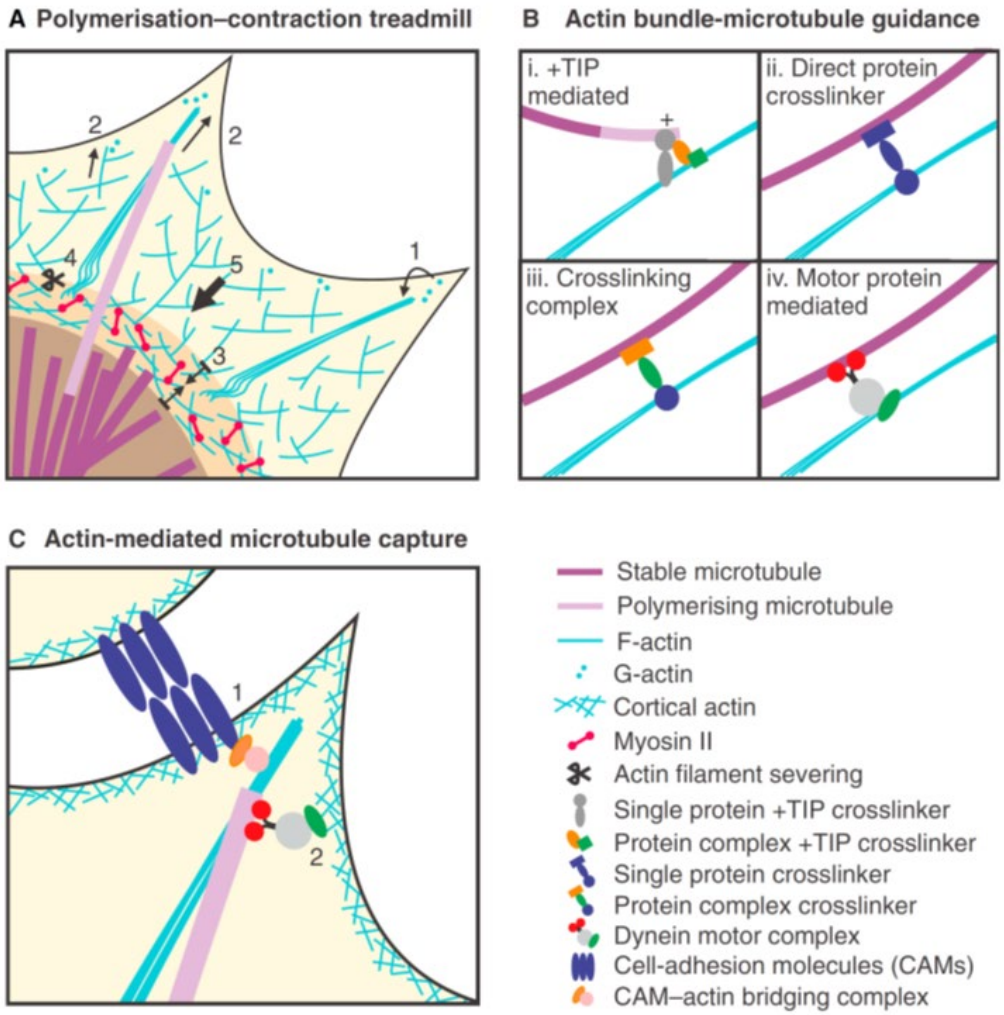

Figure 2. 10. Shows different modes of actin-microtubule crosslinking. A. MT coupled to actin retrograde flow. B. Direct cross-linking between actin bundles and MT. C) MT capture at branched actin cortex through adhesion molecules. Adapted from Coles and Brake, 2015.

Regulation and signalling in growth cone turning

Transduction of signalling from extracellular cues such as ephrins (Iwasato et al. 2007; Shamah *et al.,* 2001) netrins (Dickson, 2002; Chilton, 2006) slits (Piper *et al.,* 2006) and semaphorins (Oinuma *et al.,* 2004) into the cell and to the cytoskeletal regulators occurs through aclass of proteins termed as Rho-GTPases- RhoA, RAC1 and CDC42 (Govek *et al.,* 2005). Rho-GTPases are activated by GTPase activating proteins (GAPs) and inactivated by guanine nucleotide exchange factors (GEF) family of proteins (Watabe-Uchida *et al.,* 2006; Koh, 2006). There are

multiple GAPs and GEFs and ligand-receptors can bind to either a GEF or GAP to inhibit or activate the Rho-GTPases (Koh, 2006; Heasman and Ridley, 2008). But functionally this case is avoided by spatial compartmentalization of these GAPs and GEFs (Pertz *et al.,* 2008). Effector molecules downstream of Rho-GTPases steer growth cone navigation either by activating the actomyosin machinery (Gallo, 2006) and destabilization factors such as cofilin (Wen *et al.,* 2007) to cause disassembly of actin bundles (via **RhoA**) or by activating the actin nucleation (Machesky and Insall, 1998; Rohatgi *et al.,* 1999) and elongation factors (Krugman *et al.,* 2001; Nakagawa *et al.,* 2003) to enable actin polymerization (via **CDC42** and **RAC1**). (Figure **2. 11**).

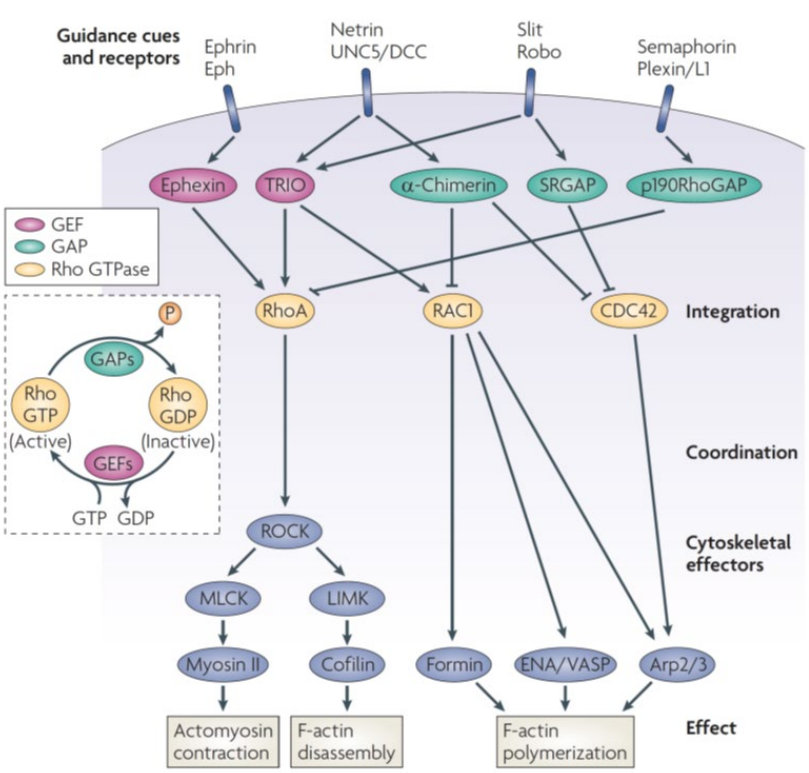

Figure 2. 11. Shows the extracellular cues and intracellular signalling involved in growth cone navigation. Adapted from Lowery and Vactor, 2009.

2.6. Axonal branching

Another important developmental process involved in establishment of the circuitry is branching. The growing neurons form multiple branches to reach multiple targets and arborize to form synaptic connections. But unlike, growth cone-mediated migration and guidance, axonal branching has received much less attention. Branching can occur from either the axon or the

growth cone, resulting in different branching morphologies (Figure **2. 12**) (Gibson and Ma, 2011). These morphologies have been designed to perform specific functions. First form is **arborization** which occurs at axon terminals to form multiple synapses once the neuron has its target tissue. The second and the third form occur to innervate multiple target tissues. This can occur via **growth cone bifurcation** where the growth cone bifurcates into two axons to innervate different tissues. Growth cone bifurcation is the simplest form of branching and is seen in central sensory neurons of the spinal cord. They split either obliquely or perpendicularly to grow rostrally and caudally in the spinal cord to reach different synaptic targets (Davis *et al.,* 1989; Schmidt *et al.,* 2007; Gallo, 2010). Growth bifurcation is not the primary form of branching to innervate multiple tissues (Harris *et al.,* 1987), and instead aids in establishing the basic organization of the nervous system. The third form of branching originates from the axon and is termed as **collateral branching**. The branches appear *de novo* from the axon shaft. Collateral branching is the major form through which neuronal arborization and circuitry is established (Cohen-cory *et al.,* 2010; Snider *et al.,* 2010).

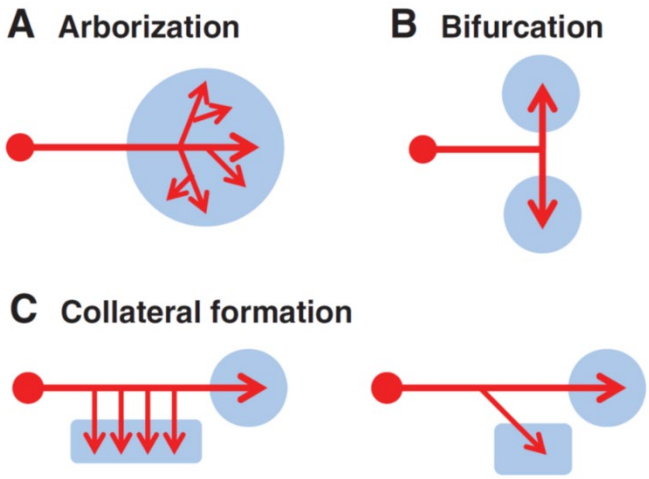

Figure 2. 12. Different morphologies of branching. A. Arborization morphology. B. Growth cone bifurcation. C. Collateral branching. Adpated from Gibson and Ma, 2011.

Molecular control of the branching process determines the location, number, size and complexity of branching. The discrete developmental steps involved in collateral branching can be specified as a) branch formation, b) branch maturation, c) guided migration and d) pruning.

Branch formation

2.6.1. Extracellular cues

Branch formation is initiated by extracellular cues. Location of a branch formation along the axon could be induced by a local induction or local inhibition and global promotion (Figure **2. 13**) (Gibson and Ma, 2011). Local induction of extracellular cues such as netrin1 (Dent *et al.,* 2004; LeBrand *et al.,* 2004), growth factors such as nerve growth factor (NGF) (Gallo and Letourneau, 1998), brain derived growth factor (BDNF) (Danzer *et al.,* 2002; Marler *et al.,* 2008) promote branch formation. On the other hand, factors such as semaphorin 3A (Dent at al., 2004) and slit1a (Campbell *et al.,* 2007) inhibit branch formation. Moreover, these promoting and inhibiting factors can act in a concerted fashion to achieve fine tuning required to sculpt the neuronal arborization. One such case is of branching in retinal ganglion neurons (RGC) in the optic tectum, wherein branch promoting factor BDNF is complemented with gradients of branch restricting factor ephrin A to regulate branching and terminal arborization of these neurons (Cohen-cory and Fraser, 1995; Marler *et al.,*2008). In response to extracellular cues, the axon regulates local cytoskeletal dynamics to initiate a branch.

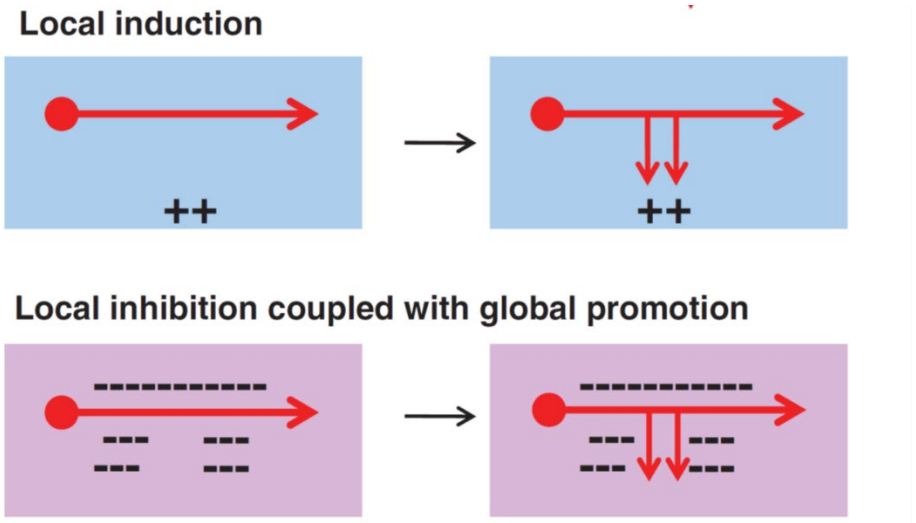

Figure 2. 13. Effect of extracellular cues on axonal branching. Adapted from Gibson and Ma, 2011.

Stages of branch formation

Early stages of axonal branching are regulated by dynamics of the actin cytoskeleton whereas, microtubule dynamics is crucial for branch stabilization and maturation (**Error! Reference source not found.**).

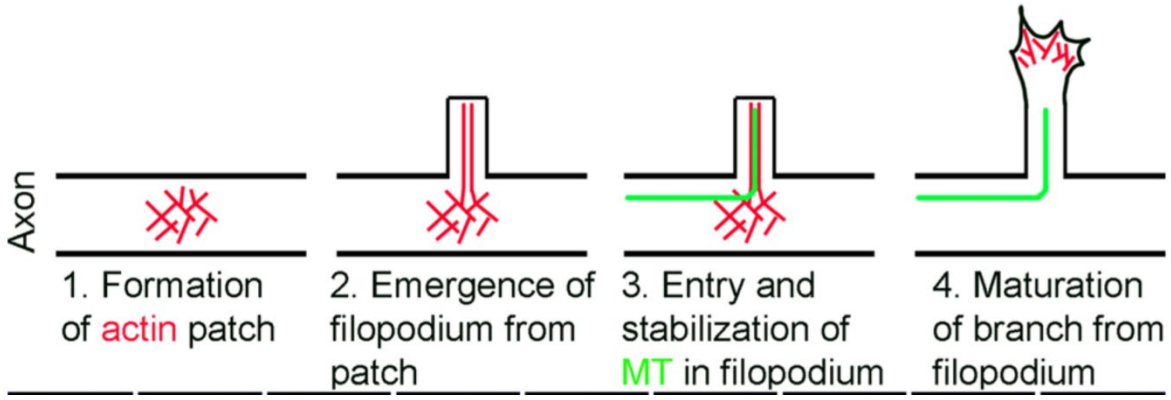

Figure 2. 14. Progressive stages of axonal branching. Adapted from Gallo, 2015.

2.6.2. Actin dynamics in branch formation

The first step in the emergence of an axon collateral downstream of extracellular cues is an actin **patch formation** (Figure **2. 14** 1) (Gallo, 2006; Orlova *et al.,* 2007; Ketschek and Gallo, 2010; Anderson *et al.,* 2011). Levels of actin is relatively lower in the axon shaft compared to the dynamic growth cone. The actin is sub-membranous and marks a thin cortex outlining the axon. Actin patch is focussed accumulation of branched actin filaments that is formed spontaneously and are transient (Ketschek and Gallo, 2010; Spillane *et al.,* 2012, 2013). The patch is formed mainly by the WAVE1 (Kim *et al.,* 2006a; Mingorance-Le Meur and O'Connor, 2009) activated Arp2/3 complex that nucleates branched actin filaments (Spillane *et al.,* 2011, 2012, 2013). Another nucleator, cordon-blue, has also been shown to promote axonal branching in hippocampal neuron and may act in concert to initiate a patch (Ahuja *et al.,* 2007). The next phase after patch initiation is **patch elaboration**. During this phase, the patch is stabilized and increases in lifetime. In chick sensory neurons, cortactin associates with Arp2/3 to stabilize the actin patch (Spillane *et al.,* 2012). In another study, inhibition of RhoA-GTPase and RhoA-kinase (ROCK), resulted in increased patch lifetime, without affecting patch initiation. This inhibition of RhoA and ROCK reduced myosin II activity which increased the probability of a patch giving rise to a filopodia (Loudon *et*

al., 2006). Although every patch pre-determines a filopodia, only about 20% of patches give rise to a filopodia (Armijo-Weingart and Gallo, 2017; Ketschek and Gallo, 2010). Actin patch themselves are not protrusive, but they act as sites for nucleation and rapid elongation through polymerization and bundling of parallel actin filaments which possess enough force to push the membrane to form a **protrusion** (Figure **2. 14** 2). Actin regulatory proteins, such as actin elongator Ena/VASP (Dwivedy *et al.,* 2007) family of proteins and actin bundling protein drebrin (Ketschek *et al.,* 2016) have been demonstrated to promote collateral branching. In general, the mechanisms of axonal filopodia are similar to growth cone filopodia formation and will be discussed in greater depth in the next section.

2.6.3. Microtubule dynamics in axonal branch formation

In the axon shaft, microtubules are arranged in long arrays of parallel bundles with their polarity away from the soma (Sharp *et al.,* 1997; Yu *et al.,* 1997). About half of the microtubule population are quite stable and less dynamic (Bass *et al.,* 1991, 2016). As with patches to filopodia, only a subset of filopodia mature into a branch. Maturation of a filopodia into a branch requires entry of microtubules into the protrusion (Kornack and Giger, 2005; Dent *et al.,* 1999; Ketschek *et al.,* 2015) as microtubules provide structural support as well a track for cargo and organelle transport. **Entry of microtubule** into the filopodia can occur either through +tip polymerization (Conde and Caceres, 2009; Kornack and Giger, 2005; Armijo-Weingart and Gallo, 2017) of dynamic microtubule or through active transport of fragmented microtubules (Figure **2. 14** 3). Microtubule severing proteins like spastin and katanin promote branching by severing the microtubule into small fragments which are then transported into the filopodium (Qiang *et al.,* 2006; Yu *et al.,* 2008; Yu *et al.,* 1994). But before entering a filopodia, bundled microtubules undergo local reorganization and de-bundling at sites of branch formation (Armijo-Weingart and Gallo, 2017). Microtubules undergo go de-bundling and splay apart prior to emergence of an axon collateral (Ketschek *et al.,* 2015; Gallo and Letourneau, 1998; Dent *et al.,* 1999; Kornack and Giger, 2005). Microtubule de-bundling is regulated indirectly by actin through the actomyosin contractile machinery. Inhibition of myosin II promoted microtubule de-bundling, which was strongly correlated with microtubule entry into axonal filopodia, thus suggesting that local splaying apart of microtubules may facilitate microtubule invasion into nascent protrusions (Ketschek *et al.,*

2015). DCX (Horesh *et al.,* 1999) and MAP1B (Takemura *et al.,* 1992) also stabilize microtubules apart from bundling. Microtubule stability is another key contributor to axonal branching as it affects both microtubule de-bundling and dynamic entry of microtubules into filopodia (Dent and Kalil, 2001). Loss of α-tubulin acetyltransferase1 (αTAT1), an enzyme that acetylates tubulin post-translationally, led to excessive branching in mice. Tubulin acetylation is correlated with microtubule stability and this work showed that de-stabilization of microtubules caused increased probability of microtubule de-bundling and entry into filopodia at branch sites and increased microtubule plus-end dynamics (Wei *et al.,* 2017).

2.6.4. Actin-microtubule crosstalk in branching

Apart from indirect interactions, direct actin-microtubule crosstalk has also been implicated in this process of axonal branching. Among crosslinkers, most prominent are **Septins**, a class of GTP binding proteins that can homo-hetero polymerize to form filaments (Nakahira *et al.,* 2010; Beise and trimble, 2011; Spilliotis and Nelson, 2005). Septins are referred to as the fourth cytoskeleton and play a major role in branching (Cho *et al.,* 2011; Hu *et al.,* 2012; Ageta-Ishihara *et al.,* 2013). Septins recognise sites of membrane curvature (Bridges *et al.,* 2016) and bend the actin cytoskeleton (Marvrakis et al. 2014). Septin 6 and 7 hetero-polymerize and coordinate with actin and microtubules to promote branching. Septin 6 localizes to actin patches and recruits cortactin to stabilize patches. Septin 7, on the other hand, binds and facilites entry of dynamic microtubules into axonal filopodia (Hu *et al.,*2012). Septin 7 achieves this by negatively regulating microtubule stability (Ageta-Ishihara *et al.,* 2013). **Drebrin** is an actin bundling protein that localizes to actin patches and increases the transition frequency of patches to filopodia. It also promotes targeting of microtubule tips into nascent protrusions (Ketschek *et al.,* 2016).

2.6.5. Signalling in axonal branching

Downstream signalling after local induction of extracellular cues like netrin1 and BDNF starts with increase in calcium transients (Tang and Kalil, 2005; Gomez and Zheng, 2006) that recruits molecules like RHO GTPases (Hall *et al.,* 2011) and protein kinase glycogen synthase kinase 3β (GSK3β) (Etienne-Manneville, 2010; Goold and Gordon-Weeks, 2004; Kim *et al.,* 2006). Multiple pathways of downstream signal transduction have been elucidated (Figure **2. 15**) and all

of them impinge on cytoskeletal re-organization and dynamics to regulate axonal branching (Kalil and Dent, 2014).

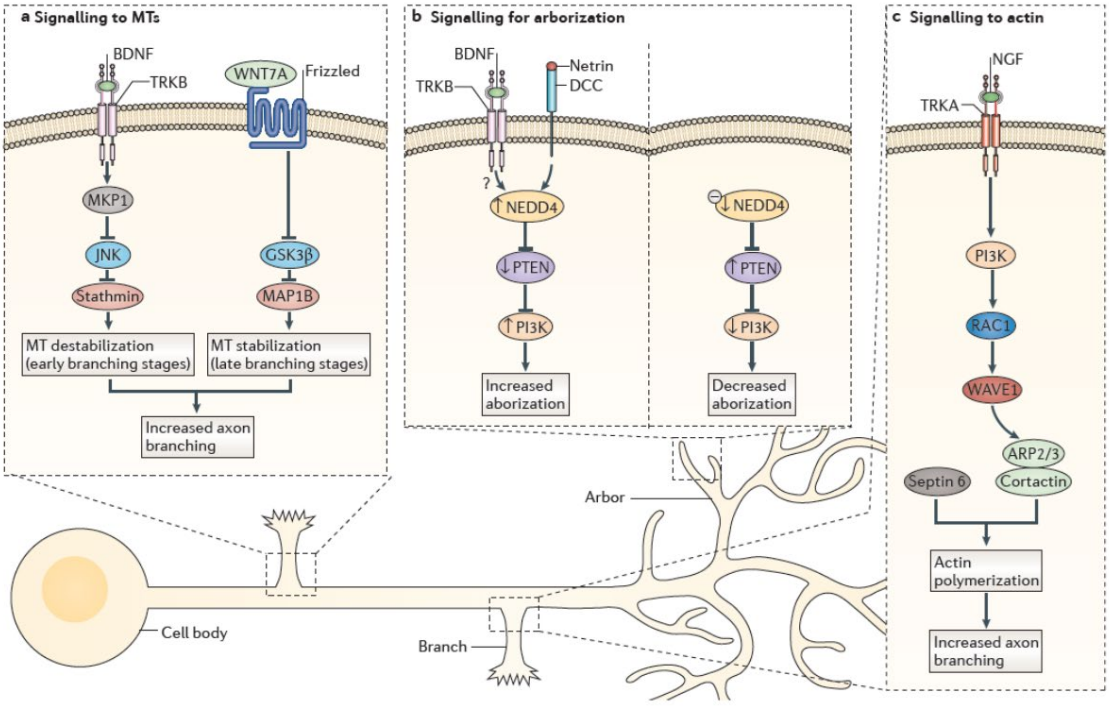

Figure 2. 15. Different signal transduction pathways that either decrease or increase axonal branching. Adapted from Kalil and Dent, 2014.

2.7. Filopodia

The major cellular structure involved in growth cone turning and axonal branching is- the filopodia. Filopodia are slender finger-like projections that are the primary chemosensory structures involved in growth cone guidance (Zheng *et al.,* 1996; Rajnicek *et al.,* 2006). They act as sites of integration for extracellular cues (Robles and Gomez, 2006; Wolf *et al.,* 2008; Ensslen-Craig and Brady-Kalnay, 2004), signal transduction molecules (Guillou *et al.,* 2008; Ridley, 2006) and cell adhesion molecules like integrins and cadherins (Steketee and Tosney, 2002; Galbaraith *et al.,* 2007) which is crucial for persistence in directed motion.

Formation of filopodia

The most widely accepted model for filopodia formation is the convergent elongation model (CEM) (Svitkina *et al.,* 2003; Yang and Svitkina,2011; Mattila and Lappalainen 2008) (Figure 2.

16). Based on studies from the leading edge, in this model, a branched lamellipodial actin networks nucleated by Arp2/3 give rise to filopodia. A subset of branched actin filaments is protected from and converge together via barbed end clustering proteins such as Ena/VASP, myosin X (Berg and Cheney, 2002; Tokuo *et al.,* 2007) and fomins, which are then rapidly elongated by actin elongators ena/VASP and formins and bundled by fascin. The membrane is deformed by curvature sensing protein such as inverse BAR (I-BAR) domain proteins like IRSp53 which bind to phosphatidylinositol-rich membranes and deform them (Mattila *et al.,* 2007). Then the 10-30 elongating filaments generate enough force to push the deformed membrane and form a protrusion.

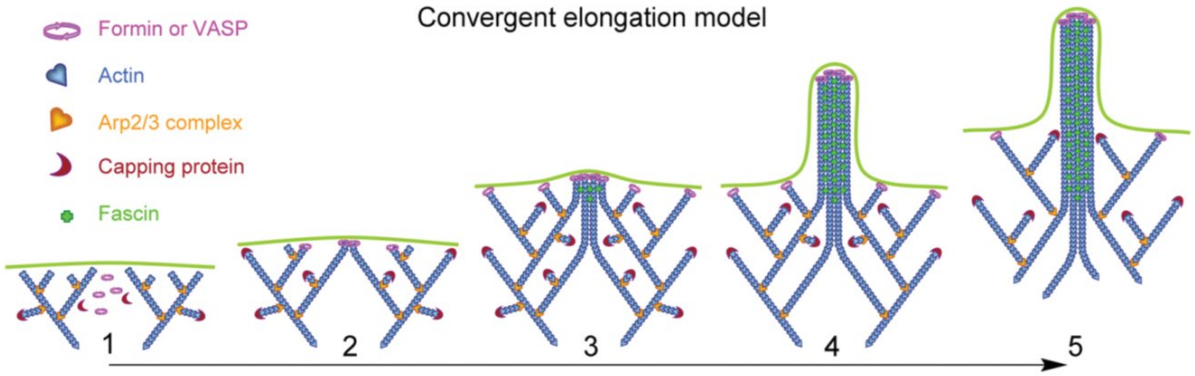

Figure 2. 16. Progressive steps in the Convergent elongation model of filopodia formation.

2.8. Formins

2.8.1. Domain structure and classification

Formins are large (120- 220kDa) proteins identified by their conserved formin homology domains FH1 and FH2 (Castrillon and Wasserman, 1994; Higgs, 2005; Grunt *et al.,* 2008). They are involved in nucleation (Pruyne *et al.,* 2002; Sagot *et al.,* 2002) and elongation (Evangelista *et al.,* 2002; Kovar *et al.,* 2003) of actin filaments. For both, nucleation and elongation the substrate required by formins is profilin-actin monomers and not solely actin. The FH1 domain interacts with profilin (Kaiser *et al.,* 1999, Kovar *et al.,* 2003) and the FH2 domain forms a doughnut

shaped dimer that sits on the barbed end of the polymerizing filament (Mosely *et al.,* 2004; Sagot *et al.,* 2002b).

In mammals, there are 15 different formins known till date and these are subdivided into 7 classes based on differences in their FH2 domains (Figure **2. 17**) (Higgs and Peterson, 2006; Higgs, 2005; Campellone and Welch, 2010): a)Diaphanous (Dia) sub- family containing formins mDia1,2 and 3, b) formin-related proteins in leukocytes (FRL) 1 and 2, c) Dishevelled-associated activators of morphogenesis (DAAM) 1 and 2, d) formin-homology domain proteins (FHOD) 1 and 3, e) Formin (FMN) 1 and 2, f) Delphilin, and g) inverted-formin (INF) 1 and 2.

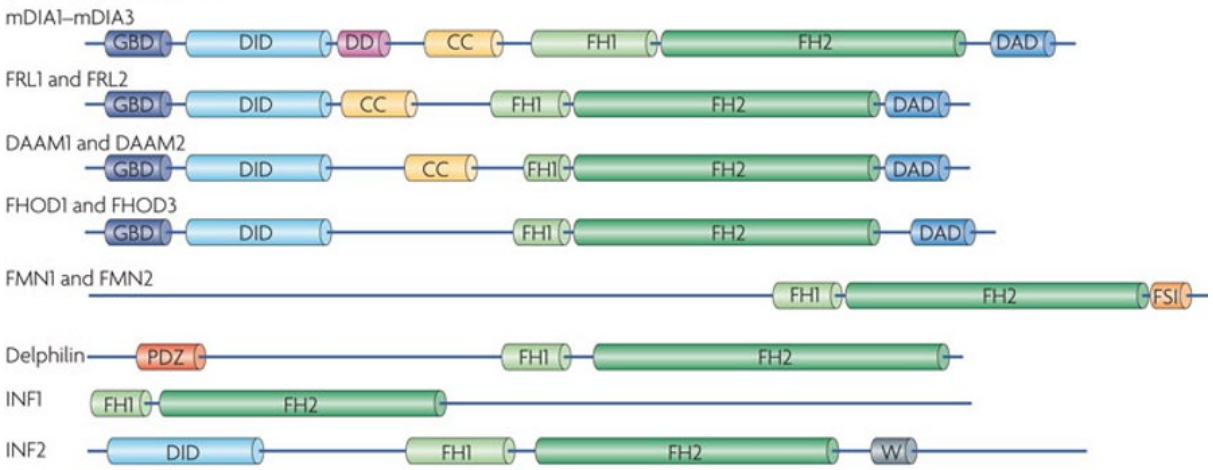

Figure 2. 17. Different classes of mammalian formins. Adapted from Campellone and Welch, 2010.

2.8.2. Mechanism of formin-mediated actin nucleation and elongation

The mechanism of nucleation and elongation follows several steps (Chesarone *et al.,* 2009; Campellone and Welch, 2010): 1) **release of auto-inhibition**. Some formins (known as Dia related formins (DRFs)), are downstream affectors of RhoA and contain an N- terminus Rho binding region (RBD) and Dia inhibitory domain (DID). The C- terminal consists of Dia autoregulatory domain (DAD) domain. The DID and DAD domain bind to each other and autoinhibits the proteins (Lin and Higgs, 2003; Nezami *et al.,* 2006). This autoinhibition is relieved by binding of Rho to RBD domain (Watanabe *et al.,* 1999; Rose *et al.,* 2005). Another mechanism of releasing auto-inhibition is seen in FHOD1, whereby phospohorylation of FHOD1 near its DAD domain by Rho-associated protein kinase (ROCK), activates it (Takeya *et al.,* 2008). 2) **activation**. After

the release of auto-inhibition, formins are activated by homo-dimerization (Li and Higgs, 2005; Mosely *et al.,* 2004; Harris *et al.,* 2004) and disruption of dimerization by deleting the lasso sub-domain abrogates nucleation and elongation activity of formins (Mosely *et al.,* 2004). 3) **nucleation**. Although all formins nucleate actin their FH2 domain, the efficiency with which they do so, varies drastically. Some weaker formins like Bni1 recruit additional actin binding protein to aid their nucleation. As such the mechanism of nucleation by formins is poorly understood (Chesarone *et al.,* 2010; Goode and Eck, 2007). 3) **elongation**. Formins form linear unbranched filaments of actin by being processively bound to the barbed end of an elongating actin filament. This activity of formins is known as processive capping (Kovar et al, 2004; Higashida *et al.,*2004) and protects the barbed end from capping proteins (Pruyne *et al.,* 2002; Harris *et al.,* 2004). During processive elongation, the FH2 dimer exists in two alternating conformations. The first is the "closed conformation" wherein both the FH2 dimers are bound to the terminal actin subunit, thus effectively blocking monomer addition. The second conformation is the "open conformation" wherein one of FH2 dimer steps towards the barbed end either before or after actin monomer addition. The FH2 dimers undergo iterative cycles of alternating between the two states to resemble a "stair- stepping" mechanism of actin elongation (Dong *et al.,* 2003; Brandt *et al.,* 2007; Paul and Pollard, 2009) (Figure **2. 18**).

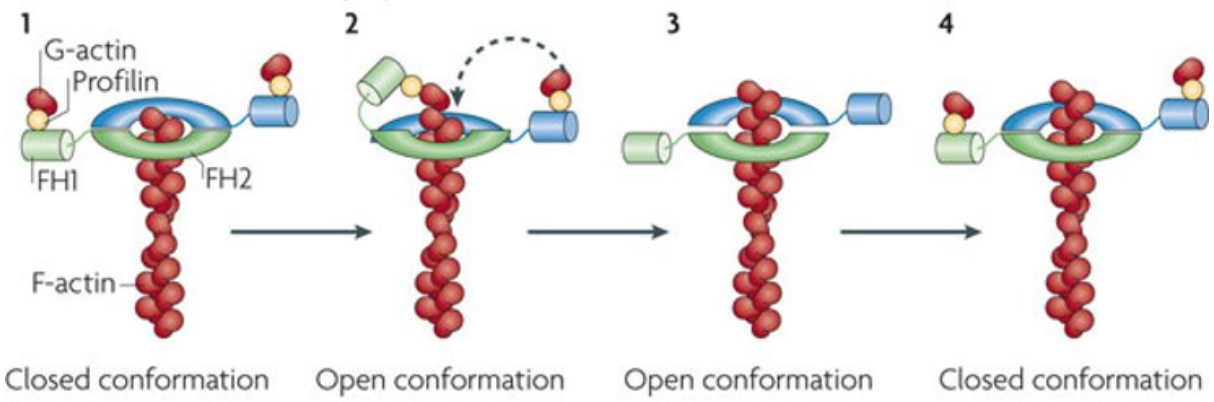

Figure 2. 18. Shows formin-mediated elongation of actin through stair-stepping mechanism. Adapted from Campellone and Welch, 2010.

Interaction of formins with microtubules

2.9. Formin 2 (Fmn2)

Fmn2 is non-Diaphaneous related formin (DRF) and a member of the sub-family of FMNs. Domain analysis of orthologs of Fmn2 from *drosophila*, chick, mouse and human shows highly conserved FH2 domain (Figure **2. 19**).

```
human          RKQPIEPCRPMKPLYWTRIQLHSK-----------------------------------
mouse          RKQLIEPCRPMKPLYWTRIQLHSK-----------------------------------
chick          -KHVIEPSRPMKPLYWTRIQLHSK-----------------------------------
drosophila     RKSAVNPPKPMRPLYWTRIVTSAPPAPRPPSVANSTDSTENSGSSPDEPPAANGADAPPT
                *  ::* :**:*******   :

human          RDSSTSLIWEKIEEPSID-CHEFEELFSKTAVKERKKPISDTISKTKAKQVVKLLSNKRS
mouse          RDSSPSLIWEKIEEPSID-CHEFEELFSKTAVKERKKPISDTISKTKAKQVVKLLSNKRS
chick          RDSSASLVWEKIEEPSID-YHEFEELFSKTAVKERKKPISDTITKTKTKQVVKLLSNKRS
drosophila     APPATKEIWTEIEETPLDNIDEFTELFSRQAIAPVSKPKELKVKRAKS---IKVLDPERS
                 .:.. :* :***..:*  .** ****: *:   .** . .:.::*:   :*:*. :**

human          QAVGILMSSLHLDMKDIQHAVVNLDNSVVDLETLQALYENRAQSDELEKIEKHGRSSKDK
mouse          QAVGILMSSLHLDMKDIQHAVVNLDNSVVDLETLQALYENRAQSDELEKIEKHSRSSKDK
chick          QAVGILMSSLHLDMRDIQHAVVNLDNSVVDLETLQALYENRAQSDELEKIEKHSKASKEK
drosophila     RNVGIIWRSLHVPSSEIEHAIYHIDTSVVSLEALQHMSNIQATEDELQRIKEAAGGDIP-
               : ***:  ***:   :*:**: ::*.***.**:** : : :* .***::*:: . ..

human          ENAKSLDKPEQFLYELSLIPNFSERVFCILFQSTFSESICSIRRKLELLQKLCETLKNGP
mouse          ENAKSLDKPEQFLYELSLIPNFSERVFCILFQSTFSESICSIRRKLELLQKLCETLKNGP
chick          ENAKSLDKPEQFLYELSLIPNFSERVFCILFQSTFSESICSIHRKLELLQKLCETLKNGS
drosophila     -----LDHPEQFLLDISLISMASERISCIVFQAEFEESVTLLFRKLETVSQLSQQLIESE
                    **:***** ::***.  ***: **:**: *.**:  : **** :.:*.: * :.

human          GVMQVLGLVLAFGNYMNGGNKTRGQADGFGLDILPKLKDVKSSDNSRSLLSYIVSYYLRN
mouse          GVMQVLGLVLAFGNYMNAGNKTRGQADGFGLDILPKLKDVKSSDNSRSLLSYIVSYYLRN
chick          GVMQVLGLVLAFGNYMNGGNRTRGQADGFGLDILPKLKDVKSSDNSRSLLSYIVSYYLRN
drosophila     DLKLVFSIILTLGNYMNGGNRQRGQADGFNLDILGKLKDVKSKESHTTLLHFIVRTYIAQ
               .:  *:.::*::*****.**: *******.**** *******.:.  :** :**  *: :

human          -FDEDAGKEQCLFPLPEPQDLFQASQMKFEDFQKDLRKLKKDLKACEVEAGKVYQVSSKE
mouse          -FDEDAGKEQCVFPLAEPQELFQASQMKFEDFQKDLRKLKKDLKACEAEAGKVYQVSSAE
chick          -FDEDAGKEQCIFPLPEPQDLFQASQLKFEDFQKDLRKMKKDLRVCETEAAKVYQLSLEE
drosophila     RRKEGVHPLEIRLPIPEPADVERAAQMDFEEVQQQIFDLNKKFLGCKRTTAKVLAASRPE
                 .*..   :  :*:.** :: :*:*:.**:.*::: .::*.:  *:  :.**   *  *

human          HMQPFKENMEQFIIQAKIDQEAEENSLTETHKCFLETTAYFFMKPKLG---EKEVSPNAF
mouse          HMQPFKENMEQFISQAKIDQESQEAALTETHKCFLETTAYYFMKPKLG---EKEVSPNVF
chick          HLQPFKDSMEQFISQAKIDQENEEKSLTEAHKSFLETAAYFCMKPKMG---EKEVSPHSF
drosophila     IMEPFKSKMEEFVEGADKSMAKLHQSLDECRDLFLETMRFYHFSPKACTLTLAQCTPDQF
                ::***..**:*:  *. .    . :* * :. ****  :: :.**       : :*. *

human          FSIWHEFSSDFKDFWKKENKLLLQERVKEAEEVCRQKKGKSLYKIKPRHDSGIKAKISMK
mouse          FSVWHEFSSDFKDAWKKENKLILQERVKEAEEVCRQKKGKSLYKVKPRHDSGIKAKISMK
chick          FNIWHEFSSDFKDFWKKENKLILQERVKEAEEVCRQKKGKSLYNIRPKHDSGIKAKISMK
drosophila     FEYWTNFTNDFKDIWKKEITSLLNELMKKSKQAQIESRRNVSTKVEKSGRISLKERMLMR
               *. * :*:.**** **** . :*:* :*::::.  :.: :   ::.     .:* :: *:

human          T---
mouse          T---
chick          I---
drosophila     RSKN
```

Figure 2. 19. Comparative analysis of Fmn2 (FH2 to end) in *drosophila*, chick, mouse and human.

2.9.1. Biochemical characterization of Fmn2

Knowledge of biochemical characterization of Fmn2 comes mainly from studies done in the *drosophila* ortholog *Cappucino* (Capu), which reveals that the tail domain at the C-terminal of Capu (1029-1059 a.a.; lacking the DAD domain) has low-affinity binding to actin monomers and filaments through charged electrostatic interactions owing to a stretch positive amino acids at the

end. The tail plays a role in actin nucleation without affecting elongation rates. Removal of the tail reduced actin bundling and processivity (Viczarra *et al.,* 2011, 2014; Roth-Johnson *et al.,* 2014).

2.9.2. Cooperative interaction with Spire

Interactions with another actin nucleator, Spire, has been demonstrated for both *in-vitro* and *in-vivo* for Capu (Quinlan *et al.,* 2007; Manseau and Schüpbach, 1989) and mouse Fmn2 (Montaville *et al.,* 2014, 2016; Viczarra *et al.,* 2011; Zeth *et al.,* 2011). This interaction is mediated by the N-terminal kinase noncatalytic C-lobe domain (KIND) of Spire and C-terminal tail domain of Fmn2 (now referred to as Fmn2-Spire-Interaction (FSI) domain). The two KID domain bind to the two FH2 domain of Fmn2 and inhibits actin nucleation by Fmn2 but enhances nucleation by Spire. Once actin is nucleated by Spire, Fmn2 is recruited and activated by Spire (independent of Rho-GTPase) and facilitates its association with the barbed end. Fmn2 -Spire interaction leads to phases of rapid elongation and arrested growth of the actin filament through a "ping-pong" mechanism wherein Fmn2 and Spire alternatively displace each other from the elongating barbed ends.

2.9.3. Diverse cellular functions

Fmn2 has been implicated in the process of oocyte maturation in mouse (Leader *et al.,* 2002; Kwon *et al.,* 2010) and oogenesis in *drosophila* (Quinlan, 2013) embryos. Interestingly, Fmn2 has been marked as a potential oncogene in leukemia (Charfi *et al.,* 2019; Jin *et al.,* 2016) and, in contrast, silencing of Fmn2 has been co-related with colorectal cancer (Li *et al.,* 2018) and causes cell-cycle arrest by inhibiting the degradation of p21 (Yamada *et al.,* 2013). Fmn2 has widely ranging roles in endocytosis (Lian *et al.,* 2016), lysosomal degradation (Lian *et al.,* 2018) and long-range vesicular transport (Schuh 2013; Tittel *et al.,* 2014).

2.9.4. In the Central Nervous System (CNS)

Initially, Fmn2 was discovered as a protein that was highly expressed in the developing and adult CNS of mice and humans (Leader and Leder, 2000), as confirmed by transcriptome analysis of tissue-specific expression of about ~3,500 genes in humans (Fagerberg *et al.,* 2014). Over the years, several case studies in humans have implicated Fmn2 in cognitive impairment such as mental retardation (Perrone *et al.,* 2012) and intellectual disability (Almuqbil *et al.,* 2013; Law *et al.,* 2014) and sensory processing dysfunction (SPD) (Marco *et al.,* 2018). Further studies in mice have

identified high expression of Fmn2 in hippocampus (Leader and Leder, 2000; Law *et al.,* 2014; Agís-Balboa *et al.,* 2017). There was reduced synaptic density (Law *et al.,* 2014) and accelerated age-dependent memory impairment (Agís-Balboa *et al.,* 2017; Peleg *et al.,* 2010). The mechanism of function of Fmn2 in the developing nervous system was first elicited by Saharabudhe et al. in 2016. Fmn2 was found to important for axonal path finding *in-vivo* (Figure **2. 20**) and at the cellular level Fmn2 knocked down growth cones had impaired motility and directionality.

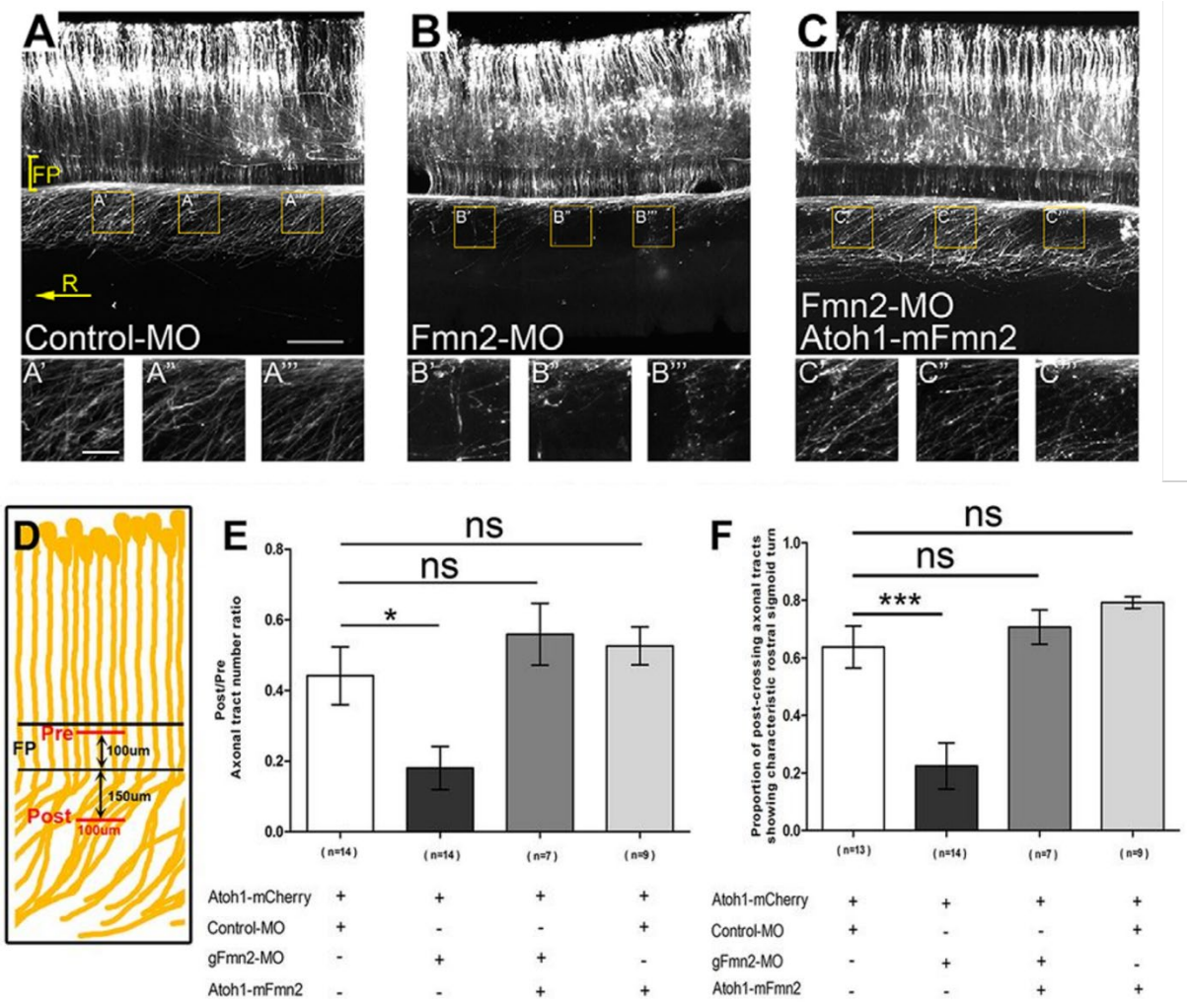

Figure 2. 20. Fmn2 knockdown affected axonal pathfinding *in-vivo*. Figure from Sahasrabudhe *et al.,* 2016.

Chapter 3

MATERIALS AND METHODS

3.1. Primary neuronal culture

Preparation of glass coverslips

Round glass coverslips (Bluestar Ltd.) of size no.1 and 22mm diameter were used for culturing neurons. The coverslips were first boiled at 60 °C for 4-5 hours in 1N HCl in a glass beaker. They were then rinsed thoroughly to remove an HCl and soaked in 70% ethanol and stored at 4 °C for future use.

Coating of Glass-bottom dishes

Plastic dishes of 35mm (Laxbro Ltd) were used for neuronal cultures. A hole was drilled at the bottom of the dish using a driller of 12mm radius. The edges of the hole were smoothened and covered with 22mm glass coverslips using silicon adhesive (Dow Corning 3145 RTV). The dishes were kept at room temperature overnight or at 60 °C for 1 hour for the glue to dry and harden. The glass- bottom dishes were then sterilized with ethanol and kept under UV for 15-20 minutes before coating. As neurons are poorly adhesive to plain glass, the dishes were coated with poly-L-lysine (PLL) (Sigma) at concentration 1mg/ml for 1 hour at 37 °C to augment the adherence of negatively charged neurons through non- specific charged interactions of the positive charges of PLL. Then dishes were washed extensively in phosphate buffered saline (PBS, pH-7.4) to remove any unattached PLL as free PLL is toxic to the cells. To promote axonal outgrowth, the dishes were then coated with more physiologically favourable substrate of either Laminin (Sigma) or Fibronectin (Sigma) at a concentration of 20 µg/ml for 4 hours at 37 °C or overnight at 4 °C.

Dissection and transfection of spinal neurons

For our studies, the chick (*gallus gallus*) embryo was used as the model system. It was established in the lab due to low cost and maintenance, easy availability, well defined developmental stages and short developmental timespan. Furthermore, the neurons acquired from primary cultures of spinal cord and dorsal root ganglion tissues are large and well suited for high resolution imaging.

Chick embryos, purchased from local hatchery (Venkateshwara hatcheries Ltd.), of stage 25-26 (day 5-6) were used for this purpose. The egg was cracked from the broad end to create a window and the embryo was gently taken out into the dissecting medium (L-15 medium (Gibco, HiMedia)

+ 1x penstrep (Gibco)). The embryo was placed on its back and the head was detached from the torso. The vertebral column between limbs was isolated and placed in a clean dish. Then the spinal tissue was taken out by cutting vertebral column open. The spinal tissue was then chopped into smaller pieces and transferred into a tube for trypsinization with 500µl of 0.05%Trypsin-EDTA (Lonza, HiMedia) at 37 °C for ~20 minutes. After trypsinization the dissociated tissue was centrifuged at 3000rpm for 3 minutes to change the medium for transfection.

For transfection, the cells were suspended in Optimem® (Gibco) media. 5-20µg of plasmid and 100µM of morpholino are added to the mix and gently tapped for mixing. The cell suspension is then transferred into the electroporation cuvette and pulsed according to the following parameters:

Poring pulse:

- Voltage (V) – 125 or 150
- Pulse length (msec) - 5
- Pulse interval (msec) - 50
- Number of pulses - 2
- Decay rate (%) - 10
- Polarity - +

Transfer pulse:

- Voltage (V) - 20
- Pulse length (msec) - 50
- Pulse interval (msec) - 50
- Number of pulses - 5
- Decay rate (%) - 40
- Polarity - +/-

Additionally, 400µl of culture medium (L-15 medium + 10% fetal bovine serum (FBS) (Gibco) + 1x penstrep (Gibco)) is added to the cuvette and then plated on 2-3 coated glass dishes for incubation at 37 °C for 24-48 hours.

Morpholino treatment

100µM of morpholinos were electroporated in neuronal cultures to knockdown Fmn2. Their details are as follows.

Name	Sequence	Location	Mode of action
Standard control	5'-CCTCTTACCTCAGTTACAATTTATA-3'	none	Translation Blocking
gFmn2#2 MO	5'-CCATCTTGATTCCCCATGATTTTTC-3'	ATG (start)	Translation Blocking

The efficacy of knockdown (~70%) was characterized both in-vitro and *in-vivo* and has been shown in Sahasrabudhe *et al.,* 2016.

Plasmid Constructs

Neurons were transfected with various plasmid DNA constructs for different purposes. pCAG-GFP and pCAG-mcherry were used to screen for morpholino positive neurons. Tractin, EB3 and tubulin constructs were used to visualize actin and microtubule dynamics, respectively. Mouse Fmn2 constructs were used for rescues. Full length (FL) *gallus* Fmn2 was used for localization experiments.

S.NO.	PLASMID NAME	PLASMID BACKBONE	SOURCE	ADDGENE VECTOR DB NO	INSERT NAME/SOURCE	GENE BANK ACC NO	PRIMERS USED FOR CLONING	REGION THAT IS CLONED WITH MODIFICATIONS MADE INDICATED	MUTATIONS MADE
1	pCS2-GFP-Tubulin	pCS2	Gift from Dr. Engel, Centre for Organismal Studies , Heidelberg	NA	tubulin	NM_006082	NA	NA	NA
2	Ngn-EB3-GFP	Ngn	Gift from Dr. Mary Halloran, University of Wisconsin-Madison, USA.	NA	EB3	NA	NA	NA	NA
3	Ngn-Tag-RFP	Ngn	Gift from Dr. Mary Halloran, University of Wisconsin-Madison, USA.	NA	Tag	NA	NA	NA	NA

4	pCAG-GFP	pCAG	ADDgene	11150	-	-	-	-	-
5	pCAG-mCherry		Cloned	632524	mcherry/pmcherry c1	-	Fwd 5'ATATATACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGG3' Rev 5'ATATATGCGGCCGCTTTACTTGTACAGCTCGTCCATGCCGCC3'	cloned by ligation between AgeI and NotI	none
6	pCAG-EB3-GFP and pCAG-EB3-mCherry		Cloned	NA	EB3/pEGFP-EB3	NM_012326	Fwd - 5'GCAAAGAATTCTGCAGTCGACGGTACCCGCCACCATGGCCGTCAATGTGTACTCC3' Rev- 5'CCATGGTGGCGACCGGTGGATCCCGGGTTAAGTACTCGTCCTGGTCTTCTTG3'	complete cds, cloned by Homologous recombination between KpnI and XmaI	none
7	pCAG-gFmn2-FL-GFP and pCAG-gFmn2-FL-mCherry		Prepared in the lab by Ajesh Jacob and Abhishek Sahasrabudhe	NA	gFMN2	KU711529.1	NA	NA	NA
8	pCAG-mFmn2-FL-GFP		cloned	NA	mFmn2/ Gift from Dr. Philip Leder, Harvard Medical School	NM_019445	Fwd- 5'AGTCGACGGTACCCGCCACCATGGGGAACCAGGATGGGAAG 3' Rev- 5'GGTGGCGACCGGTGGATCCCGGGtCGTTTTCATGCTTATCTTCGCTTTAATC 3'	complete cds, cloned between KpnI and XmaI	none
9	pCAG-mFmn2-ΔFSI-GFP		Cloned	NA	pCAG-mFmn2-FL-GFP	NM_019445	Fwd- 5'GCAAAGAATTCTGCAGTCGACGGTACCCGCCACCATGGGGAACCAGGATGG 3' Rev 5'CCATGGTGGCGACCGGTGGATCCCGGGCCTTCTGCCTACACACCTCC3'	1-4662, cloned by Homologous recombination between KpnI and XmaI	none
10	pCAG-mFmn2-I1226A-GFP		Cloned	NA	pCAG-mFmn2-FL-GFP	NM_019445	Fwd- 5'CAAAAGGTCACAAGCAGTAGGAGCTCTAATGTCTAGTCTGCATTTAGATATG 3' Rev 5'CATATCTAAATGCAGACTAGACATTAGAGCTCCTACTGCTTGTGACCTTTTG 3'	complete cds, cloning by Site Directed mutagenesis (SDM)	I1226A
11	pCAG-Tractin-GFP		Cloned	NA	tractin		Fwd 5'ATGGCGCGACCACGGGGCGCGGGGCCCTGCAGCCCCGGGTTGGAGCGGGCTCCGCGCCGGAGCGTCGGGGAGCTG3' Rev 5'CCCCCTGCGGCCGCTGCGGCGGCGACTGCGGCGCAGCGCGCTTCGAAGAGCAGGCGCAGCTCCCCGACGCTCCGG3'		None

Table 1. Details of the plasmids used in the work described in this thesis.

Blebbistatin experiments

The Transfected neuronal cultures were incubated for 24 hours. The drug was added at a concentration of 30µM in culture medium and incubated at 37 °C for 15 minutes. The live imaging was done between 15 to 45 minutes of adding the drug.

Fixing and staining neuronal cultures

To preserve and visualize both actin and microtubules in the growth cones for STED imaging, after 24 hours of incubation, the neurons were pre-extracted for 90 seconds in 0.4% glutaraldehyde and 0.2% triton-X 100 in 1xPHEM (60mM PIPES (Sigma), 25mM HEPES (Sigma), 10mM EGTA (Sigma), and 4mM MgSO4·7H20) buffer. Then the cultures were fixed in3% glutaraldehyde (Sigma) in PHEM buffer for 5 minutes at 37 °C. The cultures were then washed with 0.5%BSA (HiMedia) in 1x PBS for 5 minutes. This was repeated twice, and rest of the protocol was performed with PBS buffer. Thereafter, the cultures were permeabilized with 0.5%triton X-100 for 10 minutes. After two washes, the glutaraldehyde was quenched with 5mg/ml sodium borohydride for 5 minutes. Blocking step was done with 10% BSA for one hour. Primary antibody for total microtubule (anti-α-tubulin, DM1a, Sigma) and detyrosinated tubulin (EMD Millipore, AB3201) was used at a concentration of 1:3000 and 1:500, respectively and incubated at 4 °C overnight. Secondary antibodies of anti-mouse 568 and 405 for total tubulin and anti-rabbit 488 for detyrosinated tubulin were added for 1 hour at concentration of 1:1000 and 1:500, respectively. Finally, Phalloidin-633 was used at concentration of 1:100 for 1 hour to images actin for STED experiment. The stained cultures were mounted in Mowiol-DABCO medium (2.5% 1, 4-Diazabicyclo-octane (DABCO) (Sigma), 10% Mowiol 4-88 (Sigma), 25% glycerol (Sigma) and 0.1M Tris-HCl, pH-8.5).

For triple staining of Fmn2, microtubule and actin, the growth cones were fixed in 4% paraformaldehyde and 0.25%glutaraldehyde in 1X PHEM buffer for 20 minutes at RT without any pre-extraction. After 3 washes with 1XPHEM, the cultures were permeabilized with 0.1% triton X-100 for 10 minutes. Blocking was done in 3%BSA in 1XPHEM buffer for 1 hour. Primary antibodies for Fmn2 (anti-CRQ, lab generated) (Sahasrabudhe *et al.,* 2016) and total tubulin (DM1a) were used at a concentration of 1:200 and 1:3000, respectively. Incubation with

primary antibody was done overnight at 4 °C. Secondary antibody for Fmn2 was anti-rabbit 488 at a concentration 1:500 and for total tubulin was anti-mouse 405 at a concentration of 1:1000 for 1 hour at RT. Phalloidin-568 (Invitrogen) was used at a concentration of 1:100 for 1 hour at RT before mounting in 80% glycerol. The same procedure was used to fix and stain axons to visualize actin and microtubule.

3.2. Microcontact printing

Microcontact printing is a form of lithography that uses patterns to form a monolayer of the surface on a substrate. Using this method, direct patterning of biomolecules can be achieved without any loss of biological activity. Patterning of proteins on the substrate surface, for the purposes of this study served as well-defined haptotactic cues (substrate bound, adhesion-based cue) for studying neuronal guidance. There are several steps to this process, which starts with preparation of the silicon wafer. The wafer is then used as master plate to make the PDMS stamps. These PDMS stamps containing the micropattern are then inked with the substrate solution and used for printing on glass.

Preparation of the master plate

The master stamp was created on silicon using traditional photolithography technique (Figure 3. **1**). In this process the silicon wafer is spin coated with a light-sensitive polymer- the photoresist. The pattern is then transferred using an optical mask, which is aligned to the coated wafer and then exposed to UV. After exposure, the wafer is chemically developed to remove the UV-exposed part of the photoresist. The wafer is then etched to remove the uppermost layer of silicon to create depth in the pattern. The depth is tightly controlled by the rate and time of etching. After etching the wafer is stripped off the photoresist mask. For this study, the master plate with the pattern as shown in Figure 3. **2** was commercially manufactured through Bonda Technology Pte. Ltd., Singapore.

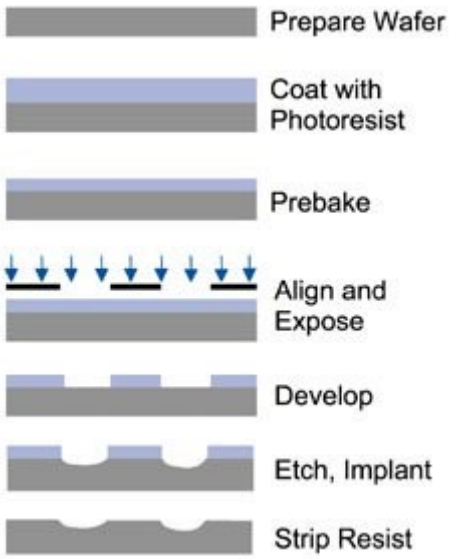

Figure 3. 1. Steps involved in photolithography technique to create the master stamp containing the micropattern. Reproduced from Mack, 2006.

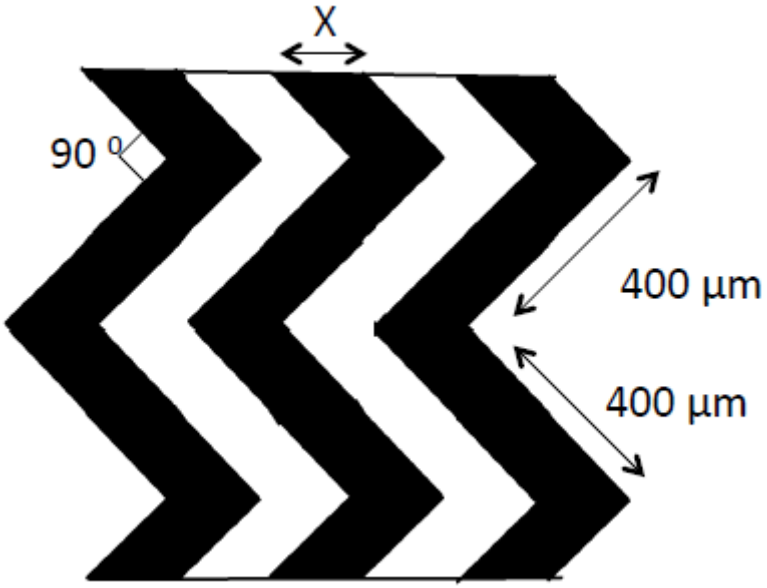

Figure 3. 2. The striped pattern used for micropatterning ECM proteins on glass for the turning assay. depth 10µm

Silanization of the master plate

The master plate was silanized to prevent it from adhering to PDMS and to clean it as well. To silanize the plate, a beaker with a few drops of trimethylsilane was placed in a vacuum desiccator along the master. The vacuum was turned on for 5-10 minutes in order to form tricholoromethyl silane vapours. Once, the vapour was formed, the vacuum was switched off and the master plate

was kept in the sealed desiccator overnight for silanization. Next day, the silanization was completed by curing the plate at 70 °C for 30 minutes. The silanized plate was sealed with parafilm to prevent dust.

Preparation and inking of PDMS stamps

Stamp casting

A 100ml solution of elastomer was prepared by mixing the PDMS and curing agent (Sylgard™ 184, Dow Corning) in the 10:1 ratio. The solution was mixed with a glass pipette for about 5 minutes. The resulting solution was centrifuged ad 3000 rpm for 5 minutes to remove the bubbles. The PDMS solution was slowly poured over the master plate and cured at 60 °C for 2 hours. The resulting PDMS stamps containing the microstructures were peeled off carefully.

Inking the stamps

The stamps were cleaned with isopropanol and air-dried. 100µl of protein solution containing Laminin (10µg/ml), Fibronectin (10µg/ml) and BSA (2.5 µg/ml), was added to each stamp. The stamps were incubated for 20-25 minutes at room temperature (RT) for adsorption of the solution. The excess solution was then aspirated and washed once with PBS. The stamps were then air-dried and printed onto the Poly-L-Lysine (0.1 mg/ml) coated glass dishes. The printing was done by carefully placing the inverted stamp and gently pressing it for one minute before removing it cautiously, so as to avoid double printing of the pattern (Figure 3. **3**). Once the stamp was removed, PBS was added to the dish immediately.

Stamps were reused for multiple cycles of printing by cleaning them via sonication in 1%SDS for 15 min followed by another 15 min in 70% ethanol. The stamps were stored in isopropanol until next use.

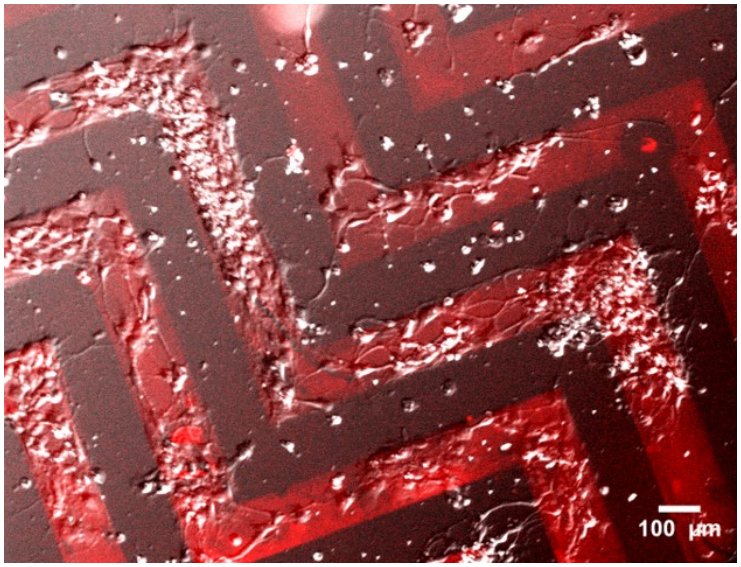

Figure 3. 3. Neurons (after 48 hours) growing on striped pattern microprinted on PLL- coated glass coverslips.

3.3. Zebrafish injections

Wild-type TU strain zebrafish maintained at 28.5℃ in a recirculating aquarium (Techniplast) were crossed to obtain eggs. The eggs were injected with 25 pg Ngn1: EB3-GFP, 25 pg Ngn1: TagRFP-CAAX (Gift from Dr Mary Halloran) and 0.125mM morpholino (Gene Tools, LLC) in a 2 nL volume in the cytoplasm at 1 cell stage.

Morpholino sequences:

Name	Sequence	Location	Mode of action
Standard control	5'- CCTCTTACCTCAGTTACAATTTATA -3'	none	Translation Blocking
gFmn2 MO	5'- ACAGAAGCGGTCATTACTTTTTGGT -3'	ATG (start)	Splice Blocking

The efficacy of morpholino treatment was confirmed by observing the band sizes of the cDNA obtained from the RNA of the fishes injected with control and Fmn2 morpholino (Dhriti Nagar, Unpublished Data). After injections, the embryos were maintained at 28.5℃ in E3 medium containing 0.002% methylene blue. Injected embryos were screened for mosaic expression of GFP and RFP, dechorionated and mounted laterally on glass slides. We used vacuum grease (Dow

Corning) to make a well on the glass slide in which the zebrafish embryo was positioned laterally in E3 medium containing 2% methylcellulose (Sigma) and 0.03% MS-222 (Sigma).
The zebrafish injections and standardization of splice-blocking Zf Fmn2 morpholino were done by Dhriti Nagar. While, the imaging was done by both of us and analysis was done by me.

3.4. Image Acquisition

Imaging for fixed cultures

The images for **triple** staining and quantifying detyrosinated/total tubulin ratio in growth cones were taken using Zeiss LSM 780 confocal system was used with 63X, 1.4 NA oil objective and GaAsp detector. For **axonal branching** studies, Zeiss LSM 710 system was used with 63x, 1.4 NA objective. In every system, the pixel size was kept at about ~100nm.

STED images were taken using the Leica TCS SP8 system with a 100X, 1.4 NA oil objective. A continuous wave (CW) of 660nm was used to deplete microtubules stained with Alexa Flour 568 and the 775nm was used to deplete actin labelled with phalloidin 633. HyD detector with a time gating of $0.5 \leq tg \leq 6$ ns was used for image acquisition. The pixel size was kept at <85 nm. Images were post-processed by deconvolution using the Hugyens Professional software (version 17.04.0p6 64b, Scientific Volume Imaging). Default settings with 15 iterations was employed during deconvolution.

Live imaging

Live imaging of **growth cones** was done after 24 to 36 hours of incubation. Neurons transfected with moderate amounts of EB3 were chosen for imaging. Widefield imaging was done on the Olympus IX-81 system (Olympus Corporation, Tokyo, Japan) with 100x, 1.4 NA Apo oil immersion objective and Hamamatsu ORCA-R2 CCD camera. Time- lapse images were acquired using the RT Xcellence software with time interval of 2s for 100 frames at 37 °C. Time lapse images of tractin-GFP were acquired every 2s for 150 frames. For turning, end point images after 36 hours were taken using a 40X, 1.3 NA oil objective on the Olympus system. For **colocalization** experiments of Fmn2 and actin, imaging was done using the Oxford Nanoimager system with HILO (High Inclination and Laminated Optical sheet) imaging setup using the 100X 1.4 NA

objective. Dual- colour images were acquired with exposure time of 500ms at 37 °C. ***In-vivo* live-imaging** of EB3 and Mnx1-GFP line in zebrafish embryos was done using the Zeiss LSM 780 multiphoton system using the 63X oil objective. For EB3, the images were acquired at 1.5-2s time interval for 100 frames and for Mnx1-GFP line, end point single images were captured.

3.4. Image analysis

Microtubule morphology in the growth cone

Neurons positive for pCAG-GFP or pCAG- mcherry were considered positive for morpholino transfection and hence for imaging. For quantification of microtubule morphology in the growth cone, the classification followed as reported in Tanaka and Kirschner, 1991.Microtubules that formed loops with the distal ends of microtubule being bent at 90° or more, were classified as looped. Microtubules that were spread out through the growth cone with some of them being curved or sinuous were marked as splayed and microtubules that remained straight and tightly packed with no curved microtubules were recognized as bundled.

Microtubule stability analysis using detyrosinated/total microtubule ratio

For detyrosinated and total microtubule measurements, the images for control and Fmn2KD were acquired with the same exposure settings. Using ImageJ, the total microtubule channel was thresholded manually and was used as a mask to limit the area for measurement of detyrosinated tubulin in the growth cone. The fluorescence was quantified using the formula, Corrected fluorescence = Integrated Density – (Area of selected cell X Mean fluorescence of background readings). The intensity measurement obtained for detryosinated tubulin was normalized to total tubulin intensity.

Actin-microtubule alignment and filopodia analysis

For quantifying microtubule alignment to actin, the deconvolved phallodin channel showing actin bundles was used as reference to identify the start of microtubule alignment as microtubules are guided along actin bundles as they invade the filopodia (Sabry *et al.,* 1991; Zhou *et al.,* 2002). The length of alignment of microtubule to actin was measured the using the segmented line tool of ImageJ. For growth cone filopodia number and length measurements, only protrusion greater

than 2µm were considered for analysis. And microtubule length higher than 0.5 µm from the base of filopodia was considered as an entry into the filopodia.

EB3 tracking, visualization and analysis

EB3 comets in the growth cone were analysed using *plusTipTracker 1.1.4* version (Matov *et al.,* 2010; Applegate *et al.,* 2011) on MATLAB 2010b (mathworks). The images were first background subtracted in ImageJ using rolling ball radius of 50, to enhance the contrast. Detection of the comet was done via the *plusTipGetTracks* GUI with following settings (Stout *et al.,* 2014; Biswas and Kalil, 2017): detection method = anisotropic gaussian; PSF sigma = 1 to 1.5 and Alpha value= 0.005 to 0.02, depending on the most faithful detection of comets across several frames of each movie. Tracking of the comets were done using the following parameters: Search radius (Range) = 3 to 12; minimum sub-track length (frames) = 3; break non-linear tracks was unchecked; Maximum Gap Length (frames)= 11; Maximum shrinkage factor (relative to growth speed) = 0.8; Maximum angle (°C) forward= 40 and backward = 20; fluctuation radius = 3.5. The post-processing parameters used were Frame rate (s) =2 and pixel size (nm)= 64. Growth speed of comet tracks was visualized using the growth speed range from 0 to 20 µm/min in all frames using the *plusTipSeeTracks* GUI. The EB3 comet tracking in the filopodia was done manually to avoid any false detection due to movement of the filopodia itself. Comets that emerged from the microtubule dense region were tracked using point tool of ImageJ, from the central domain to the peripheral or filopodial region, if they appeared in at least 3 continuous frames. The coordinates of the comet position were saved as ROI's and exported to Microsoft excel for calculating growth speed. Comets that disappeared were observed for minimum 3-5 continuous frames to check for rescue. Only unambiguous comet entry into filopodia were considered for dwell time analysis. Simultaneous multiple comet entries into the filopodia were tracked for growth speed and excursion depth analysis along with manual counting of number of comets entered per min per filopodia but excluded from dwell time analysis. Number of comets in each frame of the movie was extracted from the "movieinfo" file generated from *plusTipTracker* using a small code in MATLAB (mathworks);

```
a=[100,1];
```

```
for i=1:100

a(i,1) =(length(movieInfo(i,1).xCoord));

end
```

The median value of comet number obtained from 100 frames of each movie was normalized to the area of the growth cone. The area of the growth cone was a mean of 10 measurements obtained manually using the freehand selection of ImageJ in every 10th frame.

For analysis of EB3 comets obtained from *in-vivo* imaging in zebrafish, kymographs were generated using the segmented line tool of ImageJ and velocities were extracted using the velocity measurement tool.

Turning analysis

For quantifying the percentage of neurons that turned or crossed, only neurons growing on stripes and those that interacted with the border were considered.

Axonal protrusion density and distribution analysis

Only neurons with the complete axon visible, were considered for analysis. Protrusion shorter than 0.5µm were discarded. For distribution analysis, the length of the axon was divided into 10 equal segments. And the number of protrusions in each segment was quantified manually and plotted against the percent distance from the growth cone.

Actin patch area and dynamics

First frame of each tractin-GFP movie was utilized to calculate the patch area. The image was auto-thresholded using the suitable thresholding algorithm in ImageJ. The area of an actin patch was measured manually using the freehand selection tool. For analysing patch dynamics, the images were first bleach and drift corrected using the Bleachcorrection and StackReg plugin of ImageJ, respectively. Kymographs from these time-lapse images were generated using the KymoClear 2.0 plugin. Segmented lines were drawn manually on each kymograph to mark the patches. Patch speed and lifetime was calculated using the velocity- measurement tool plugin.

3.5. Graphical representation and statistical analysis

All the graphical representation and statistical analyses were performed using GraphPad Prism 5. Graphical representations were plotted using scatter dot plot with middle line showing the mean and error bars indicating the standard error of mean (SEM). Number of data points quantified in each graph have been indicted either in the figure legends. Mann-Whitney U test was employed for comparing two groups. When comparing more than two groups, non-parametric one-way ANOVA or Krusal-Wallis test was used.

Chapter 4

ROLE OF FMN2 IN ACTIN-MICROTUBULE COORDINATION DURING AXONAL PATHFINDING

All the *in-vitro* experiments and analyses were performed by me. The *in-vivo* zebrafish injections were done by Dhriti Nagar, the imaging involved both of us and the analysis was done by me.

4.1. Results

Fmn2 has been implicated in neurodevelopmental cognitive disorders in humans (Leader and Leder, 2000; Fagerberg *et al.,* 2014; Almuqbil *et al.,* 2013; Law *et al.,* 2014; Agís-Balboa *et al.,* 2017; Peleg *et al.,* 2010). However, the mechanistic details of Fmn2 function in neurodevelopment remain poorly understood. The first study shedding light into this matter showed that deficiency of Fmn2 resulted in defects in axonal pathfinding *in-vivo* (Sahasrabudhe *et al.,* 2016). Axonal guidance occurs through the growth cone machinery at the tip of the axon. The growth cone senses the environment through chemosensory structures, the filopodia. The growth cone motility and directionality are governed by the underlying cytoskeleton rearrangements. And indeed, the study also found that reduction of Fmn2 affected both growth cone speed and directionality in-vitro. Since growth cone navigation is dependent on coordination between actin and microtubules (Dent and Gertler, 2003; Lowery and Vactor, 2009; Buck and Zheng, 2002; Geraldo and Gordon-Weeks, 2009), this thesis investigated the mechanisms of actin-microtubule crosstalk via Fmn2.

4.1.1. Fmn2 influences microtubule organization and exploration in the growth cone

To assess the influence of Fmn2 on microtubules in neurons, chick primary neuronal cultures were prepared using laminin coted plates and incubated for 24-48 hours. In these neurons, Fmn2 was depleted at the protein level using a specific Fmn2 translation blocking morpholino and the knock down (KD) was characterized using two different approaches of western blotting and immunofluorescence both in-vitro and *in-vivo* in Sahasrabudhe *et al.,* 2016 and efficacy of knockdown in neuronal cultures achieved was ~50%. The Fmn2 depleted growth cones were observed for any changes in the microtubule organization. Microtubules (MT) adopt different modes of organization based on different stages of growth cone motility and are classified as (a) looped (Figure 4. **1** A); when most microtubules are bent at an angle of 90^0 or more and this morphology is correlated with growth cone stall. The rate of forward translocation of microtubules

exceeds the rate of forward movement of growth cone which causes the microtubules to buckle against the compressive force of the barrier. (b) splayed (Figure 4. **1** B); when microtubules are spread out in the growth cone with a mixture of straight and curved microtubules during forward motion. The rate of polymer growth and translocation equals the rate of forward movement of the growth cone (c) bundled (Figure 4. **1** C); when most of the microtubules are straight and tightly packed occurring before growth cone collapse (Tanaka and Kirschner, 1991, 1995; Gordon-Weeks, 2004). We observed that the splayed organization is more frequent with 55%. But, when Fmn2 levels are reduced, the bundled organization became more frequent with drop in proportion of looped organization (Figure 4. **1** D). The area occupied by microtubules, as measured by total tubulin staining, remained unchanged (Figure 4. **1** E). The increase in bundled organization is co-related with reduced growth cone speed, as shown earlier (Sahasrabudhe, *et al.,* 2016). Moreover, the percentage of filopodia invaded by microtubules, in Fmn2 knocked-down growth cones, was almost halved with only 24±6% (standard error of mean) filopodia showing microtubule presence compared to about 41±6% in control growth cones (Figure 4. **1** F). This indicates that diminishing levels of Fmn2 influences exploratory behaviour of dynamic MT in a developing neuron.

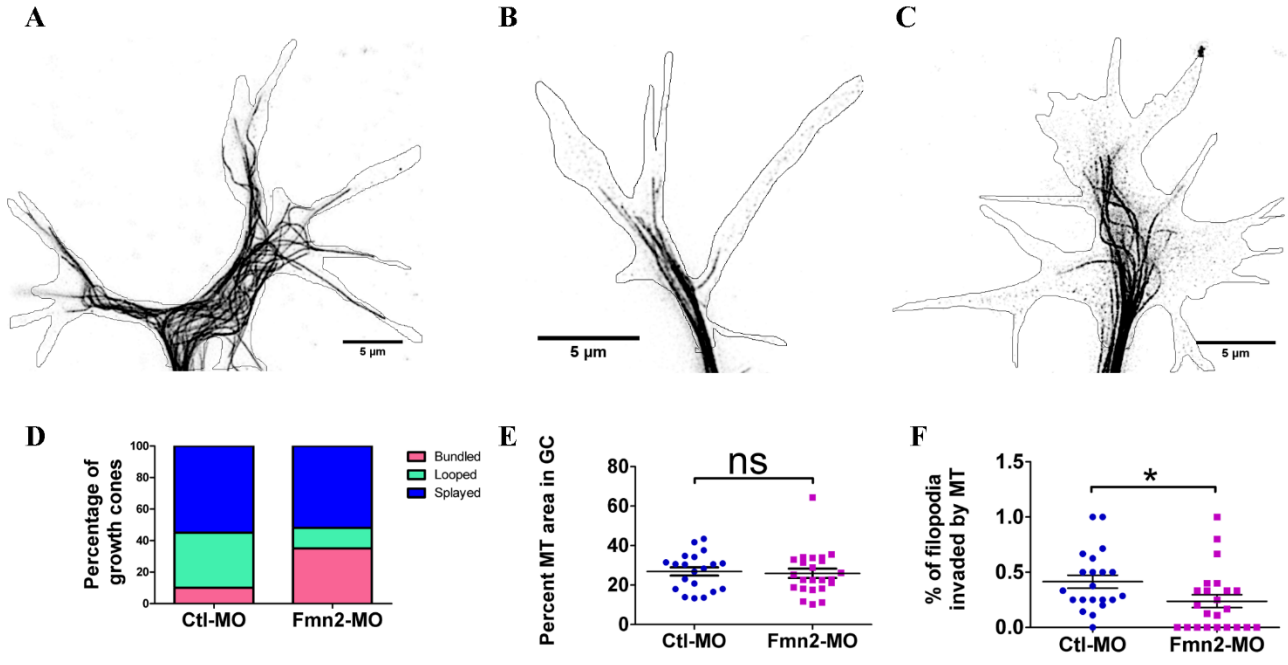

Figure 4. 1. Microtubule morphology in the growth cone. A. Looped **B**. Bundled **C**. Splayed morphology **D**. percentage of growth cones showing these three morphologies. n=20 for Ctl-MO and n=23 for Fmn2-MO. **E**. percentage of area occupied by microtubules normalized to growth cone area; n=20 for Ctl-MO and n=23 for Fmn2-MO; *P<0.05, ns P>0.05; Mann-Whitney test.

4.1.2. Fmn2 KD affects microtubule stability in the growth cone

The above data demonstrated that Fmn2 influenced microtubule organization in the growth cone. This preliminary observation prompted us to look further into microtubules dynamics and stability in the Fmn2 knockdown neurons. Microtubule stability in the growth cone was measured by two methods: 1) using ratio of detyrosinated/total tubulin in growth cone. Microtubules undergo multiple post-translational modifications, including detyrosination. Detyrosinated microtubules are more long-lived and less dynamic with much lower turnover rate (Webster *et al.,* 1987; Kreis, 1987). In neurons, detyrosination confers stability to microtubules from cold-induced or drug-induced depolymerization due to differential binding of MAPs (Microtubule-associated proteins) (Peris et al, 2009; Bass *et al.,*2016). We depleted Fmn2 and evaluated the extent of microtubule detyrosination using specific antibodies against detyrosinated and total tubulin (Figure 4. **2** A and A´, B and B´). We found that Fmn2 depeletion, reduced the proportion of detyrosinated microtubules in the growth cone (Figure 4. **2** C).

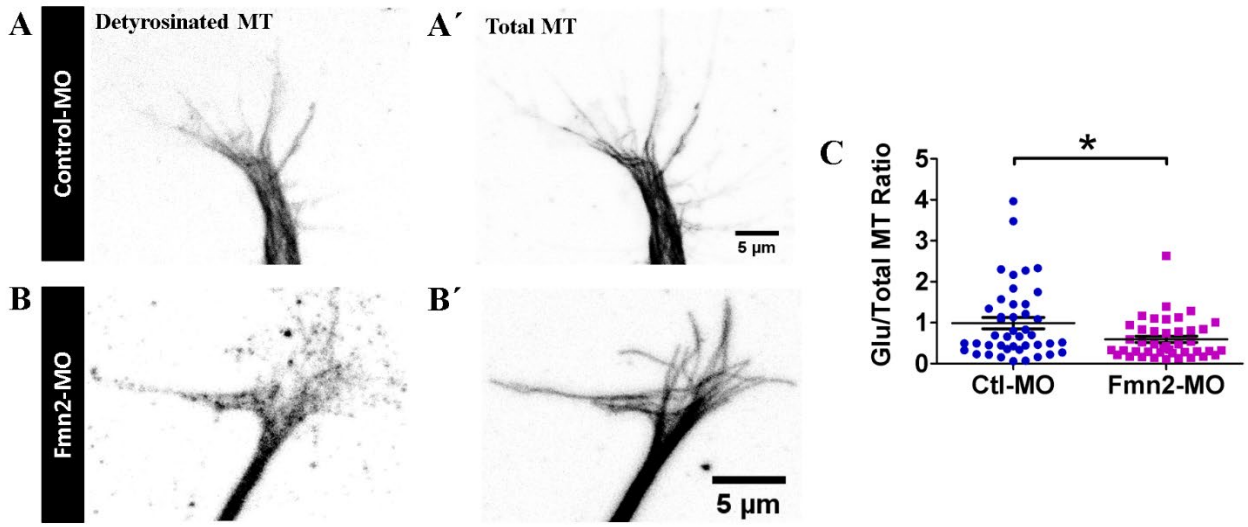

Figure 4. 2. Microtubule stability in the growth cone. A and **A´** shows staining of detyrosinated and total MT in control neurons (n=43), respectively. **B** and **B´** shows staining of detyrosinated and total MT in Fmn2-MO neurons (n=42), respectively. **C.** shows quantification of detyrosinated MT normalized to total MT; *P<0.05; Mann-Whitney test.

Along with microtubule organization, microtubule dynamics was evaluated as well by live-imaging of EB3-GFP (Figure 4. **3** A) and quantified using the plusTipTracker algorithm (Figure 4. **3** B and C) (Matov *et al.,* 2010; Applegate *et al.,* 2011; Stout *et al.,* 2014; Biswas and Kalil, 2017). It was found that even though the number of EB3 comets per growth cone was similar (Figure 4. **3** D), the mean growth speed of microtubules in the Fmn2 KD growth cones was higher (8.489 ±0.5866 μm/min) compared to control with mean speed of 6.294 ± 0.4532 μm/min (Figure 4. **3** E). The microtubule growth lifetimes (7.636 ± 0.5920s in control and 7.833 ± 0.3860s in Fmn2 KD) were similar (Figure 4. **3** F). On the other hand, the distance covered by the polymerizing microtubule was higher when Fmn2 was reduced (1.173 ± 0.3272 μm), in contrast to 0.8728 ± 0.06591 μm in control conditions (Figure 4. **3** G). Furthermore, the overall dynamicity, i.e., the collective displacement of all EB3 tracks over their collective lifetimes, was increased with depletion of Fmn2 (7.450 ± 0.4370 μm/min and 5.669 ± 0.3945 μm/min in Fmn2-MO and control-MO, respectively) (Figure 4. **3** H). Apart, from reducing the levels of stable microtubules knockdown of Fmn2 also increased the dynamicity of microtubules.

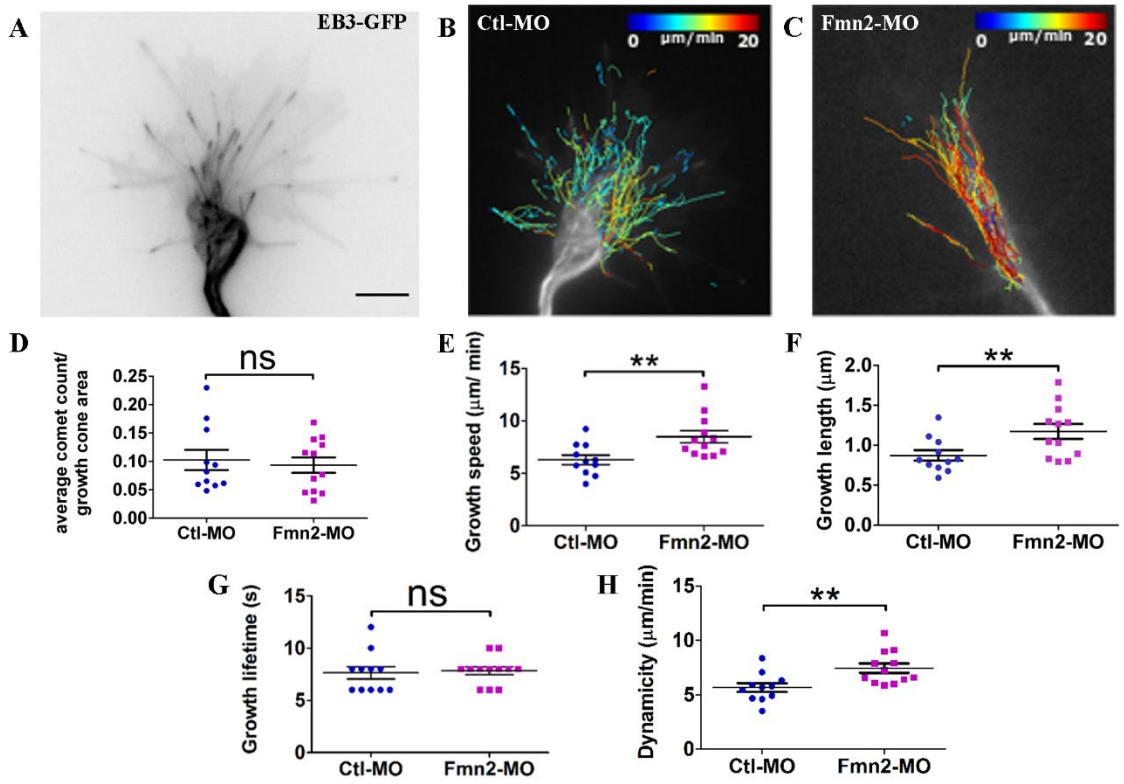

Figure 4. 3. EB3 dynamics in growth cones in-vitro. **A**. shows EB3 comets in a growth cone. **B**. shows speed of EB3 comet tracks in control and Fmn2-MO(**C**) growth cones. **D**. average number of comets in a growth cone normalized to GC area in Ctl-MO (n=11) and Fmn2-MO (n=12). Quantification of (**E**) growth speed (**F**) growth length (**G**) growth lifetime and (**H**) dynamicity from n=2917 tracks from Ctl-MO (11) and n=2426 tracks from Fmn2-MO (12) growth cones; **P<0.01, ns P>0.05; Mann-Whitney test; scale bar- 5µm.

4.1.3. Actin-microtubule alignment in the growth cone

Endogenous staining of Fmn2, microtubules and actin revealed that Fmn2 in filopodia is most prominent when the actin intensity is also quite high and these actin-Fmn2 rich filopodia commonly have MT innervation (83.89 ± 5.342%) (Figure 4. **4** E). In a motile growth cone, the exploratory microtubules invade the peripheral domain and are guided along actin bundles (Sabry *et al.,* 1991; Williamson *et al.,* 1996; Scheafer *et al.,* 2002; Zhou *et al.,* 2002). Considering the fact that Fmn2 decorates the actin bundles all along filopodia and that on Fmn2 depletion there is reduced MT presence in filopodia, we chose to focus on whether Fmn2 affected MT dynamics, specifically, in the filopodia. This interaction involves alignment at the bases of filopodia and affects later dynamics of MT. Thus, using super-

resolution microscopy, we quantified the alignment of microtubules to filopodial actin bundles in the growth cone (Figure 4. **5** A and B). Percentage of microtubules aligned to actin bundles in lamelllipodial and peripheral zones (Figure 4. **5** A´´´ and B´´´) in control growth cones was 62±5% and in case of Fmn2 depletion the percentage was reduced to 32±5% (Figure 4. **5** C). Moreover, the microtubules that did align followed actin bundles for reduced lengths compared to control (Figure 4. **5** D). As a consequence, the percentage of microtubules innervating the filopodia were also reduced to 15% compared to 30% in control (Figure 4. **5** E), even though the number of microtubule ends were comparable (Figure 4. **5** F) and ultimately resulted in reduced filopodial lengths in Fmn2-MO (Figure 4. **5** G). These results suggest that Fmn2 facilitates the alignment of microtubule to actin and its subsequent presence in the filopodia.

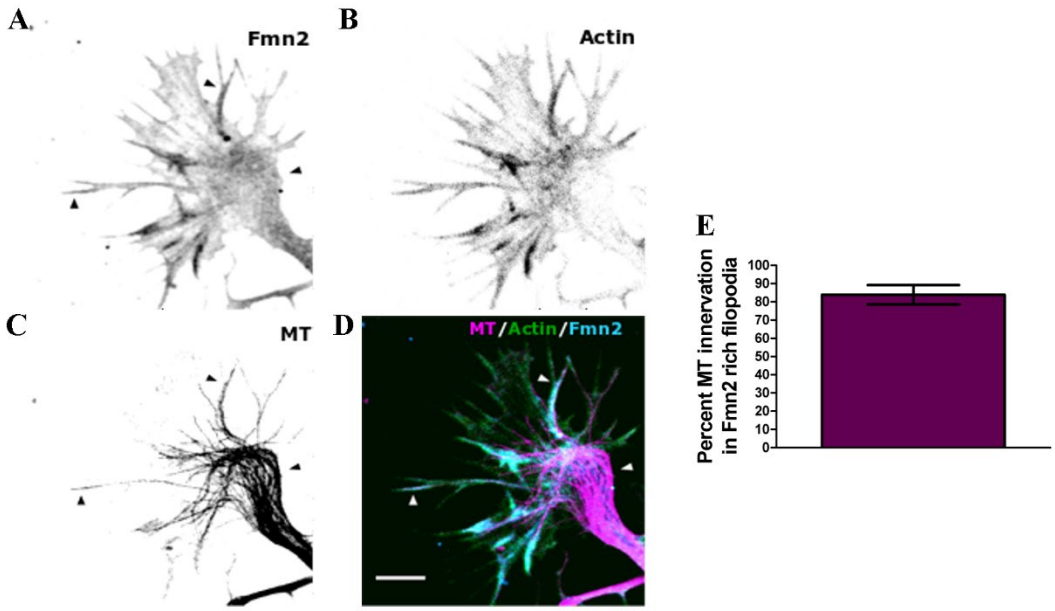

Figure 4. 4. Fmn2, MT and Actin. shows a growth cone stained with **A.** Fmn2, **B.** microtubule and **C.** actin D. Merged images of Fmn2(in green), microtubules (in red) and actin (in cyan). Fmn2 is enriched in the Microtubule which has entered the filopodia shows proximity to Fmn2 in the filopodia (marked by arrowheads). Scale bar- 5µm

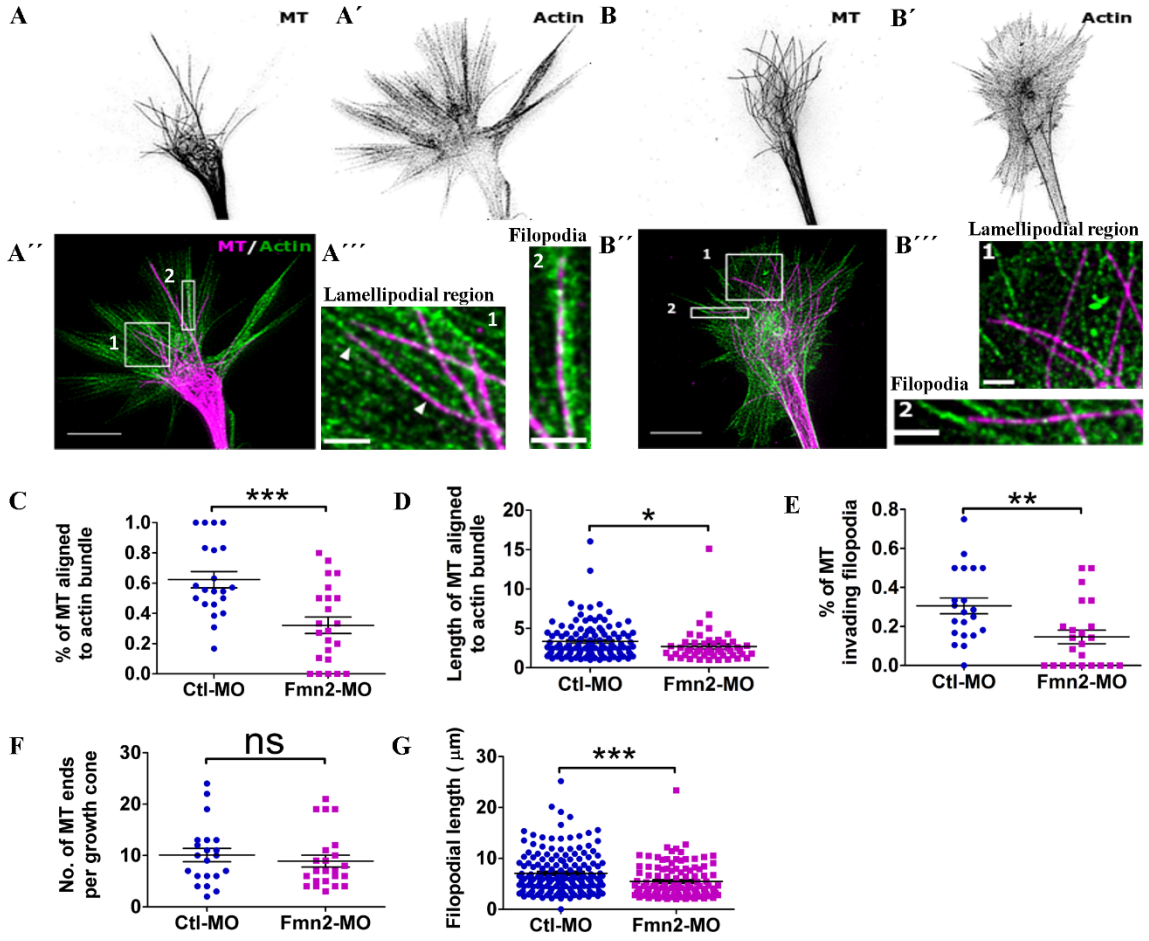

Figure 4. 5. Actin-microtubule alignment in the transition and peripheral zones. **A** and **B** show microtubule, **A´** and **B´** show F-actin, **A´´** and **B´´** show merged actin-MT image of Ctl-MO and Fmn2-MO growth cones, respectively. **A´´´** Inset showing alignment of actin and MT in the transition and peripheral zone in control and in Fmn2-MO in **B´´´**. **C**. quantification of the percentage of MT aligned to actin bundles in Ctl-MO (n=21) and Fmn2-MO (n=23). **D**. quantification of the length for which MT follows the actin bundles in Ctl-MO (n=121) and Fmn2-MO (n=50). **E**. Quantification of percentage of MT invading filopodia and **F**. showing quantification of number of MT ends in each growth cone in Ctl-MO (n=21) and Fmn2-MO(n=23).G shows comparison of filopodial lengths between Ctl-MO (n=168) and Fmn2-MO (n=118); ***P<0.001, **P<0.01, ns P>0.05; Mann-Whitney test; scale bar- 5µm, 1 µm.

4.1.4. Fmn2 knockdown destabilizes microtubules in the filopodia

Guidance of microtubule by actin bundles is thought to involve lateral interactions which decelerates the polymerizing microtubule (Figure 4. **6** A). This hypothesis was evaluated using EB3 dynamics and quantified as shown in Figure 4. **6** B in control. But due to loss in actin-

microtubule alignment such deceleration is not observed in Fmn2 KD neurons (Figure 4. **6** C). In fact, the growth speed of EB3 comets within filopodia of Fmn2 depleted growth cones were significantly higher than those observed in control-MO treated growth cones (Figure 4. **6** D) (9.72 ± 0.52 µm/min and 7.31 ± 0.29 µm/min for Fmn2-MO and control-MO, respectively). However, the dwell times of EB3 comets in Fmn2-MO treated filopodia were much shorter (86.40 ± 7.58s in control and 40.91 ± 5.347s in Fmn2-MO) suggesting that lateral interactions between MT and actin were mediated by Fmn2 and stabilized microtubules in the filopodia (Figure 4. **6** E). The number of microtubule entries into a filopodia was significantly higher when Fmn2 was depleted (Figure 4. **6** F), even though the excursion depth of the EB3 comets within filopodia showed no difference, suggesting little change in hindrance to MT growth (Figure 4. **6** G). Higher number of entries into a single filopodia was also indicative of increased dynamicity of MT in Fmn2 depleted growth cones.

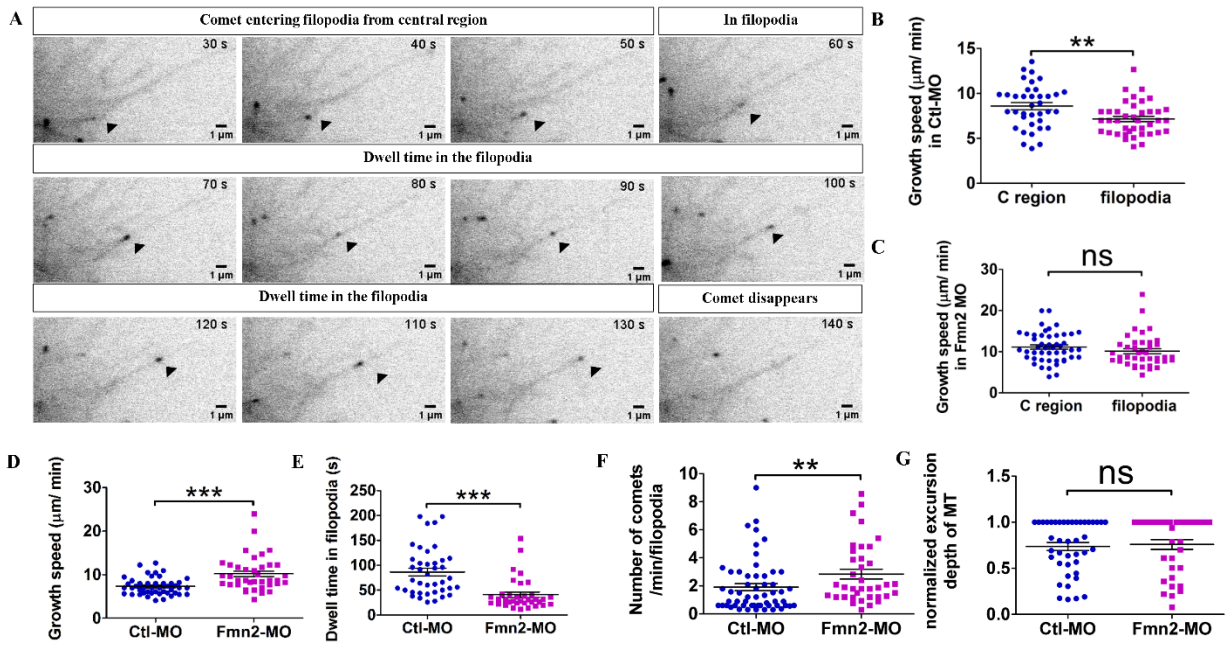

Figure 4. 6. MT destabilization in the filopodia. **A.** shows frames of a time lapse movie of EB3 comets starting in the central region and entering the filopodia (marked with arrowhead). The time the comet stays in the filopodia is quantified as dwell time. **B.** quantification of speed as the comet enters filopodia in Ctl-MO (n=37 in C-region and n=40 in filopodia) and in Fmn2-MO (n=48 in C-region and n=40 in filopodia) in **C. D.** growth speed in filopodia in Ctl-MO (n=46) and Fmn2-MO (n=38). **E.** Dwell time in filopodia in Ctl-MO (n=40) and Fmn2-MO (n=35) **F.** Number of comets entering every filopodia every minute in Ctl-MO (n=56) and Fmn2-MO (n=37) **G** Excursion depth travelled by comet in filopodia normalized to the length of the filopodia in Ctl-MO (n=46) and Fmn2-MO (n=38). .; ***P<0.001, **P<0.01, ns P>0.05; Mann-Whitney test.

4.1.5. Effect of Fmn2 knockdown on tubulin-GFP dynamics

Since EB3 being a "+"-tip binding protein falls off once the microtubule catastrophes, this destabilization of microtubules in the filopodia was also confirmed by measuring catastrophe using tubulin-GFP construct (Figure 4. 7 A). In line with EB3 observations, dwell time of microtubules in Fmn2-MO filopodia was significantly reduced compared to that in control-MO filopodia (Figure 4. 7 B). Moreover, the latency period for the first catastrophe event was also much shorter (4.00 ± 0.61s for Fmn2-MO and 9.30 ± 1.42s for Control-MO) (Figure 4. 7 C). During shrinkage, the mean catastrophe speed in Fmn2 depleted filopodia was 14.13 ± 2.08 µm/min, which was significantly higher than the control filopodia with speed of 9.866 ± 0.76 µm/min. (Figure 4. 7 D).

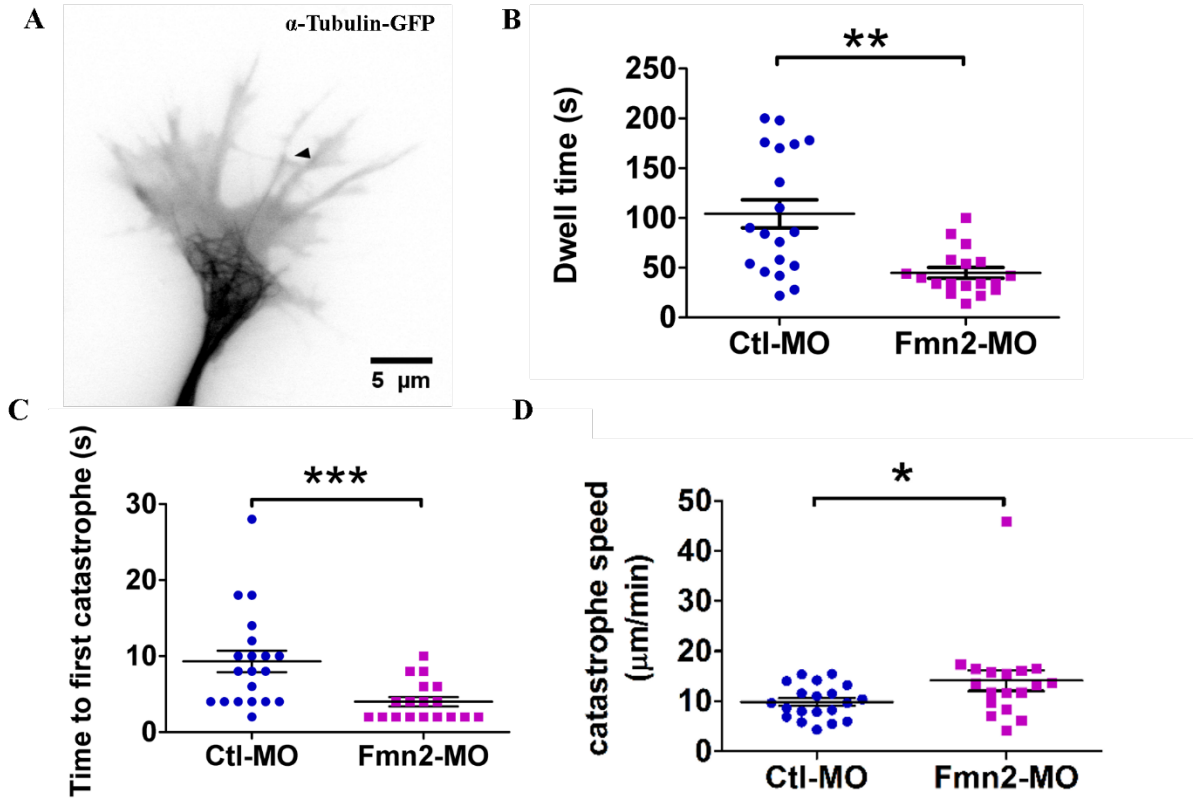

Figure 4. 7. Tubulin dynamics in the filopodia. **A**. Shows a microtubule labelled by tubulin-GFP in a filopodia. **B.** Catastrophe speed **C**. Time to first catastrophe and **D.** Dwell time of a microtubule quantified in Ctl-MO (n=20) and Fmn2-MO (n=18) filopodia; ***P<0.001, **P<0.01, * P<0.05; Mann-Whitney test.

4.1.6. Movement of microtubule in the filopodia

As the polymerizing microtubule is associated with filopodial actin bundles, they are subjected to the influence of retrograde movement of the actin bundles and exhibit a waggling behaviour in the filopodia (Liu *et al.,* 2010) (Figure 4. **8** A). Figure 4. **8** B shows a kymograph of an EB3 comet in a control-MO treated filopodia display a retrograde movement event and pause, even though the microtubule is polymerizing as is evident by the presence of EB3 at the tip. Thus, if retrograde flow were to be absent then the waggle would be gone, and movement of the microtubule would appear much smoother (Figure 4. **8** A′)**.** This is exactly what we observed when we reduced retrograde flow using blebbistatin (Figure 4. **8** B′ and C).

Now, when Fmn2 was knocked down, the retrograde flow increased in the central region but was unaffected in the filopodia (Individual data sets on gels from Sampada Mutalik, PhD thesis and on glass from Ketakee Ghate, unpublished data). Thus, even in the presence of retrograde flow, much smoother trajectories of microtubule in filopodia were observed (Figure 4. **8** A′′ and B′′) and quantified (Figure 4. **8** D)**.** This indicated that smoother movement of microtubules was due to loss of physical coupling between actin and microtubule rather than an indirect effect of reduced stearic hindrance due to a smaller number of actin bundles.

In effect, Fmn2 knockdown destabilizes the microtubule in the filopodia due to loss of interaction between microtubule and actin.

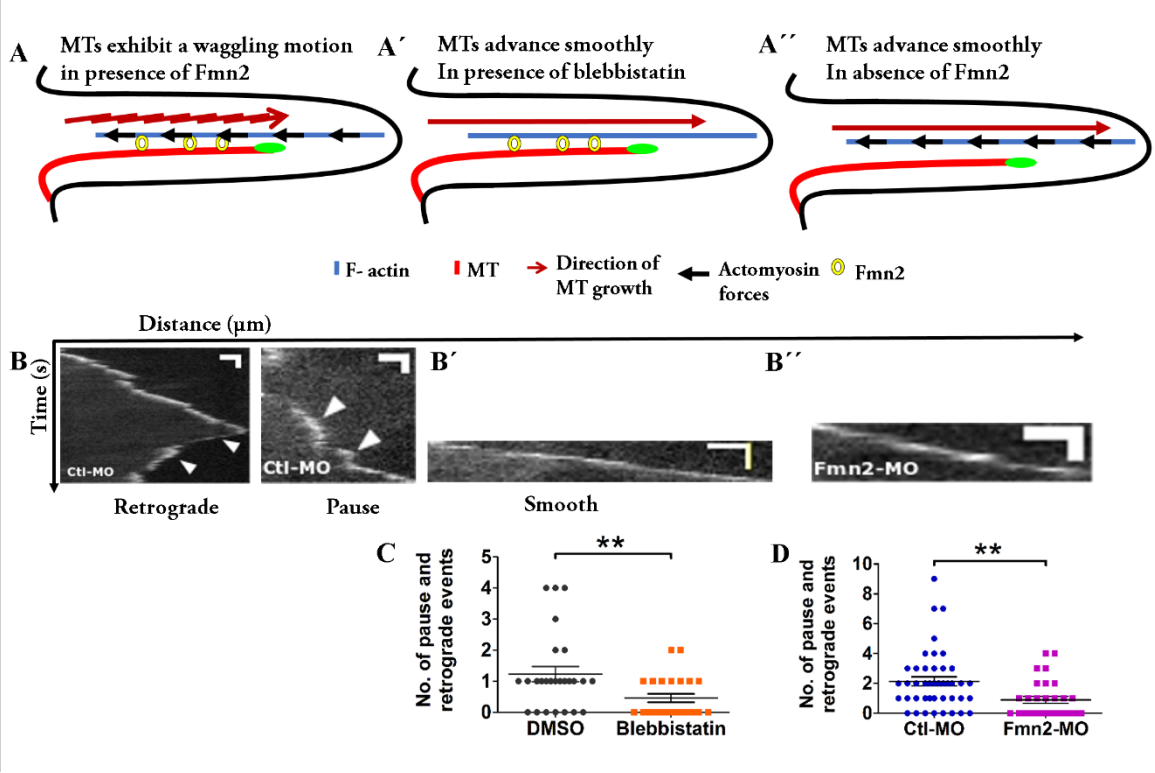

Figure 4. 8. Microtubule movement along actin bundles. A., A´ and A´´ show schematic of MT movement in control, blebbistatin treated and Fmn2-MO neurons. The control filopodia show jagged movement of MT with retrograde and pause events due to coupling with actin which are pulled by actomyosin forces. As retrograde flow is stopped using a drug blebbistation, the movement of MT become smooth, as even though actin and MT are coupled there is no backward pull on actin. And when Fmn2 is knocked down, the coupling between MT and actin is affected and hence the smoother trajectories. The representative kymographs of these conditions are shown in **B., B´ and B´´. C.** Quantification of retrograde and pause events for blebbistatin treatment (n=26, DMSO and n=24, blebbistatin) and in **D** for control (n=43) and Fmn2-MO (n=30) neurons; **P<0.01, Mann-Whitney test; Scale bar- 10µm, 10s.

4.1.7. Microtubule and actin crosslinking occur through FSI domain of Fmn2

The FSI domain of the Cappuccino (*drosophila* orthologue of Fmn2; Capu) has been shown to be involved in both actin and microtubule binding (Viczarra *et al.,* 2014; Roth-Johnson *et al.,* 2014) and thus, this domain is likely to have prominent role in interpolymer crosslinking. The binding of this domain with both polymers, occurs through electrostatic interactions (Roth-Johnson *et al.,* 2014) and it is charge conserved in vertebrates including chick and mice (Figure 4. **9** A). Furthermore, since the biochemical data from the lab (Priyanka Dutta) also confirmed these interactions using chick FSI domain, we, therefore, made FSI-deletion construct of the mouse ortholog of Fmn2 (Figure 4. **9** B). The mouse ortholog is resistant to

the anti-chick Fmn2 morpholinos used in this study and the full-length expression of this gene rescued the waggling behaviour of microtubule in filopodia as shown in Figure 4. **9** C. But the Δ_FSI mutant was unable to rescue the waggle of microtubule as demonstrated (mean retrograde and pause events of control-MO is 2.54 ± 0.41, 0.9231 ± 0.19 for Fmn2-MO, 2.44 ± 0.43 for mFmn2_FL rescue and 0.69 ± 0.16 for mFmn2 Δ_FSI mutant rescue), suggesting that the FSI domain is indeed involved in coupling actin and microtubule. We also examined other parameters like growth speed (Figure 4. **9** D) and dwell time (Figure 4. **9** E) of microtubule in the filopodia and discovered that consistent with the rescue of waggling behaviour, the morpholino-resistant full length Fmn2 was able to rescue the increase in MT polymerization speed and reduced EB3 dwell time upon Fmn2 depletion. However, Δ_FSI failed to rescue these. Therefore, it appears that Fmn2 couples actin and microtubule, via FSI and thereby stabilizes the microtubules.

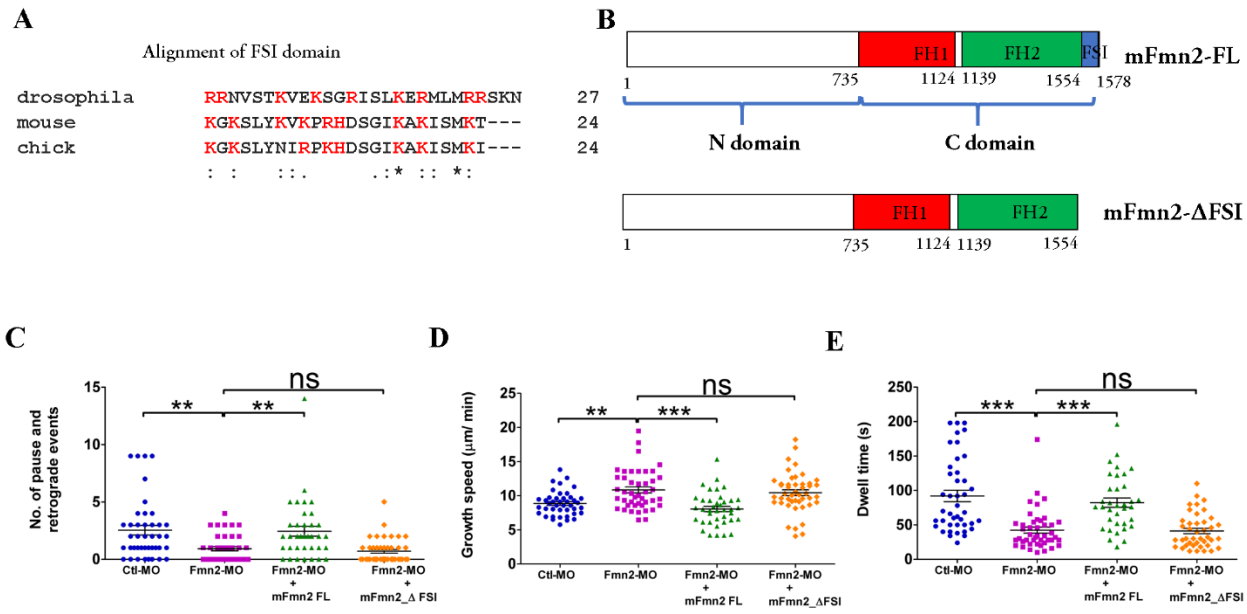

Figure 4. 9. FSI domain links actin and microtubule. A. shows the sequence alignment of FSI domains from *drosophila*, chick and mouse. The positively charged amino acids (required for electrostatic interactions) are marked in red. **B.** schematic shows the domain of morpholino resistant mouse Fmn2 with full length (FL) and ΔFSI mutant. **C**. Plots show pause and retrograde events, **D**. growth speed and **E.** dwell time was rescued by morpholino- resistant mouse Fmn2 but not when the FSI domain was lacking. (Ctl-MO, n=41; Fmn2-MO, n=39; mFmn2-FL rescue, n = 36; mFmn2_Δ_FSI, n= 43; for graph C. (Ctl-MO, n=42; Fmn2-MO, n=44; mFmn2-FL rescue, n = 39; mFmn2_Δ_FSI, n= 46; for graph D. (Ctl-MO, n=42; Fmn2-MO, n=44; mFmn2-FL rescue, n = 35; mFmn2_Δ_FSI, n= 41; for graph E; ns P .0.05; **P<0.01; ***P<0.001; Krusal-Wallis test followed by Dunn's multiple comparison test.

4.1.8. Microtubule presence and stability impacts filopodial lengths and stability

Till now, we gained an understanding of the mechanism of Fmn2 and MT interaction and its influence on MT dynamics and stability. We found that Fmn2 promotes MT stability, particularly, in the filopodia. To examine functional consequences upon destabilization of filopodial MTs, we calculated filopodial lengths with and without microtubule presence in both control and Fmn2 KD growth cones. We found that, in both cases, microtubule presence co-related strongly with filopodial lengths (Figure 4. **10** A). However, since, overall filopodial lengths were reduced in Fmn2-MO (Saharabudhe *et al.,*2016), a contributing factor could be reduced proportion of peripheral MT leading to a decrease in the proportion of filopodia with MT presence (Figure 4. **1** F). Filopodial stability was directly influenced by microtubule presence, as measured by entry of EB3 comets into the filopodia (Figure 4. **10** B). But mere entry was not enough. As suggested by analysis of EB3 entries in Fmn2 depleted filopodia, microtubules that were able to enter the filopodia were de-stabilized and it is this reduced microtubule stability which contributed to decreased filopodial lifetimes.

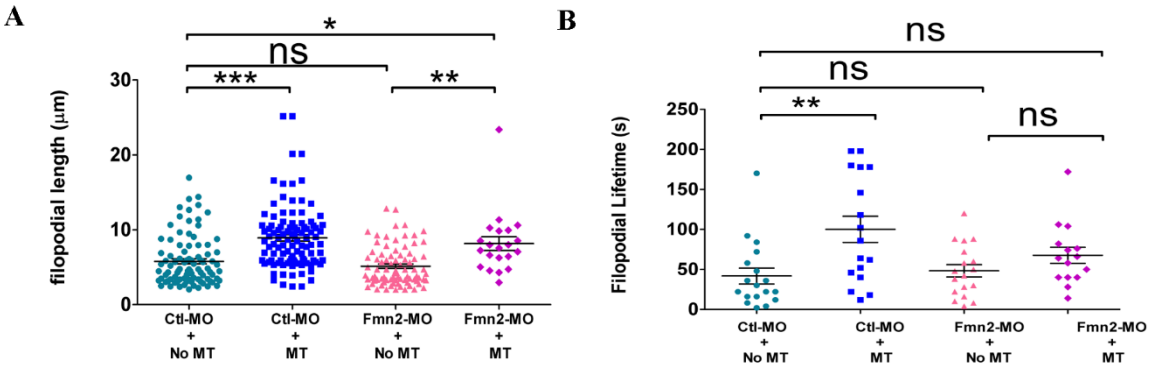

Figure 4. 10. MT presence and stability impacts filopodial elongation and stability. A. Shows filopodial lengths with or without microtubules (Ctl-MO+ No MT, n=92; Ctl-MO+ MT n= 104, Fmn2-MO+ No MT n=83 Fmn2-MO+ MT n=21). **B.** shows filopodial lifetimes with or without EB3 entry (Ctl-MO+ No MT, n=18; Ctl-MO+ MT n= 17, Fmn2-MO+ No MT n=18 Fmn2-MO+ MT n=15). ns P >0.05; *P<0.05, **P<0.01; ***P<0.001; Krusal-Wallis test followed by Dunn's multiple comparison test.

4.1.9. Fmn2 knockdown leads to defects in growth cone turning

As a moving growth cone encounters a sharp border of adhesive cues, the growth cone turns away from the border and during this turning, microtubules are captured and stabilized asymmetrically one side of the growth cone (Nader *et al.,* 2008; Nader *et al.,* 2012; Buck and Zheng, 2002). Therefore, to confirm our understanding of the functional consequences of Fmn2 mediated actin-microtubule crosstalk and its consequential stabilization of microtubule in the filopodia, we employed a haptotactic turning border assay (Liu *et al.,* 2010; Turney and Bridgman, 2005; Jean *et al.,* 2012). We used microcontact printing to create alternate border of fibronectin +laminin (ECM) and poly-l-lysine (PLL) and plated neuron on these substrates and found that although both surfaces are permissive for neuronal growth, neurons growing on ECM preferred not to cross-over into PLL at the borders. In control neurons, ~73% neurons turned as they reached the border (Figure 4. **11** A and C). But when Fmn2 was depleted, only ~32% turned while the majority crossed the border (Figure 4. **11** B and D). Thus, revealing that the Fmn2-MO neurons clearly showed defect in sensory guidance as they were able to move but were unresponsive to the cue.

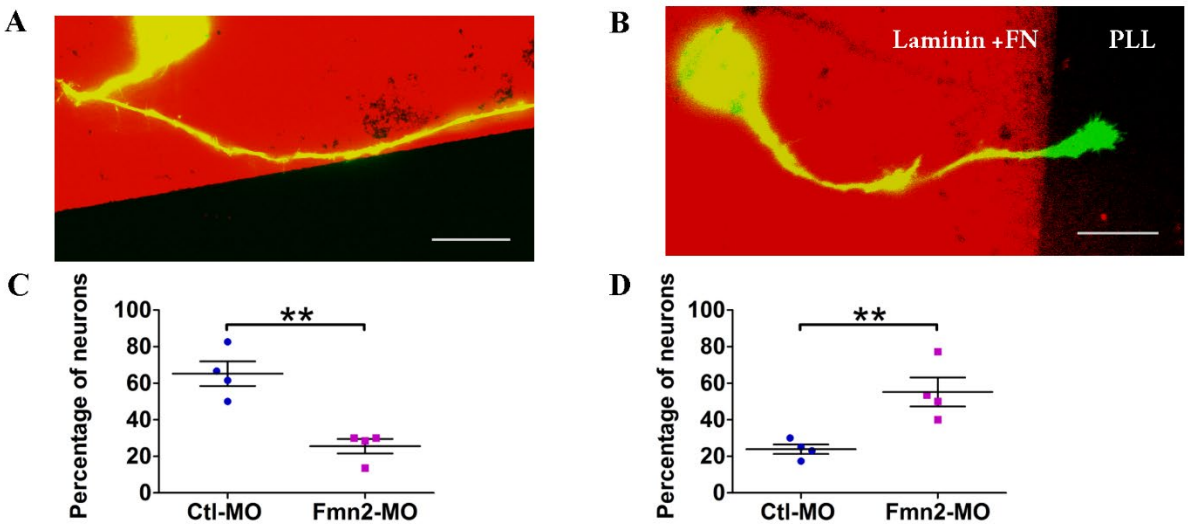

Figure 4. 11. Defects in growth cone turning. A. shows a representative image of a control neuron which turned as it reached the border between PLL and Laminin + FN.**B**. shows a representative image of Fmn2-MO neuron which does not turn as it reaches the border and crosses over. The quantification of the same are shown in graphs **C** and **D**. (Ctl-MO=68, Fmn2-MO=78). Scale bar -20 µm; **P<0.01; Unpaired t test.

4.1.10. Fmn2 knockdown affects microtubule dynamics in-vivo

To confirm the effect of Fmn2 knockdown on microtubule dynamics *in-vivo*, Fmn2 was depleted using splice-blocking morpholinos in Rohon-beard (RB) neurons of zebrafish. The extending peripheral RB neurons were imaged using *ngn* promoter driven EB3-GFP at 24-28 hfp (Figure 4. **12** A). EB3 marks the polymerizing or "+"- end of growing microtubules and kymographs generated from EB3 labelled comet tracks in growth cones were quantified (Figure 4. **12** B). We discovered that mean growth speed of microtubules was higher in growth cones depleted of Fmn2 KD (6.7 ±0.2774 µm/min) compared to control (5.7 ±0.2419 µm/min) (Figure 4. **12** C). Along with higher speed, track lifetime of these comets was also shortened (Figure 4. **12** D). Thus, depletion of Fmn2 affected microtubule stability *in-vivo* as well.

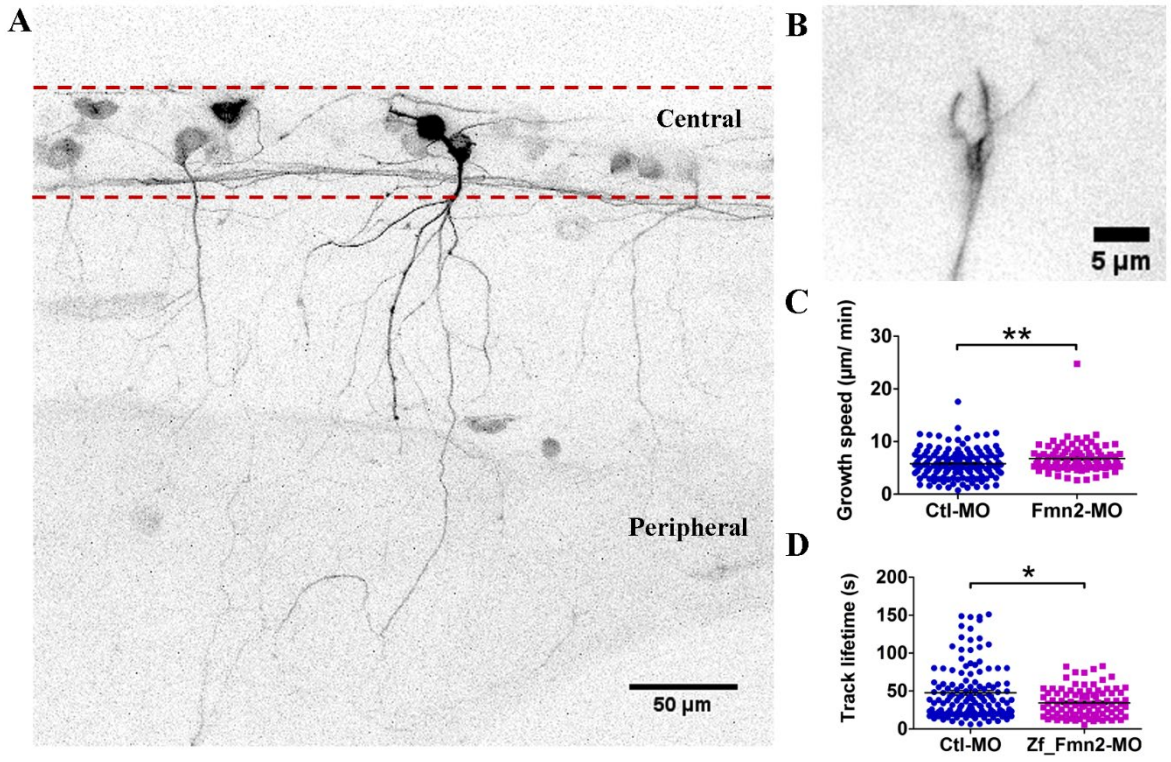

Figure 4. 12. EB3 dynamics in growth cones *in-vivo*. A. Sensory RB neurons of zebrafish embryo expressing EB3-GFP. The dotted lines mark the spinal cord. Only peripheral RB neurons were quantified. **B**. shows a peripheral growth cone displaying EB3 comets *in-vivo*. **C** and **D**. shows quantification of growth speed in **C** and track lifetime **D** in n=134 comet tracks from Ctl-MO (no. of GC =13) and n=95 tracks from Fmn2-MO (no. of growth cones =12) growth cones; **P<0.01, *P<0.05; Mann-Whitney test.

4.2. Discussion

The present study investigates the interaction of Fmn2 and microtubule dynamics in the context of axon guidance. We found that depletion of Fmn2 altered the microtubule organization in the growth cone, reducing microtubule stability. Fmn2 decorated the actin bundles and co-localized with microtubules in the peripheral and central region of the growth cone. We also found that, Fmn2 facilitated the association of actin bundles with microtubule in the lamellipodial and peripheral zone. The loss of this alignment led to higher growth speed and shorter dwell times of microtubule even though the distance travelled in the filopodia were no different. It also led to increased catastrophe speed and much reduced time before the first catastrophe. Coupling of actin and microtubule produces a waggling movement of microtubule which is lost when the retrograde pull on actin is reduced by using blebbistatin. And the de-coupling of two, due to knock down of Fmn2, abolished the waggle even in the presence of retrograde flow. The waggling behaviour of microtubule could be rescued using the morpholino-resistant full-length mouse ortholog of Fmn2.We also found that coupling of two cytoskeletal elements occurs through FSI domain of Fmn2 in-vitro. and loss of FSI domain was not able to rescue the waggle of microtubule and neither the growth speed nor dwell time in the filopodia. As a result, de-stabilization of microtubules in Fmn2 knocked down filopodia also led to shorter filopodial lifetimes. Ultimately, the loss of coordination between the two cytoskeletal elements led to defects in sensory guidance and growth cone turning. Finally, we confirmed that reduction of Fmn2 levels in zebrafish embryos led to higher growth speeds and shorter track lifetime of dynamic microtubules *in-vivo*.

Fmn2 influences MT organization and stability

Microtubule organization is causal to growth cone motility. Microtubules become bundled just before undergoing a membrane collapse and forming a new axon (Tanaka ad Kirschner, 1991). So, the presence of a more bundled form in Fmn2 depleted growth cone indicates that this a consequence of poorly spread growth cones and increased retrograde flow (Sahasrabudhe et al, 2016). These changes in organization of microtubules had originally been attributed as a passive response of microtubules to constriction of the membrane caused by actin cytoskeleton. But the bent or curved microtubules that give rise to looped or splayed morphology could also be due to

post-translational modification (acetylation) of microtubules (Portran *et al.,* 2017; Xu *et al.,* 2017). Acetylation provides protection against mechanical breakage and FH1, FH2 domains of multiple formins have been revealed to directly induce acetylation (Thurston *et al.,* 2012). This raises the possibility, that apart from actin mediated compression, the more straightened microtubule in bundled form could be due to reduced proportion of acetylated microtubules after Fmn2 knockdown. Microtubule stability, as marked by PTMs such as detyrosination (Schulze *et al.,* 1987; Webster *et al.,* 1987), was also reduced. This decreased proportion of detyrosinated microtubules also demonstrated that depletion of Fmn2 decreased microtubule stability in growth cones.

Fmn2 crosslinks actin-MT

Studies on Capu have been shown to crosslink actin and microtubules *in-vitro* (Rosales-Nieves *et al.,* 2006) and this crosstalk affects cytoplasmic streaming in *drosophila* oocytes (Rosales-Nieves *et al.,* 2006; Dahlgaard *et al.,* 2007). Binding of microtubule to Capu occurs primarily through non-specific charged interactions in the FSI domain, although FH2 domain has also been implicated in this interaction (Roth-Johnson *et al.,* 2014). These domains are conserved from *drosophila* to human (**Error! Reference source not found.**).

Moreover, biochemical studies (Priyanka Dutta, unpublished data) from the lab show that FH2FSI region of *gallus* Fmn2 binds to both actin and microtubule. The co-sedimentation assays demonstrate that the FH2FSI domain also stabilizes microtubule. To test the ability of Fmn2 to crosslink actin and microtubules, fluorescently-labelled pre-polymerised phalloidin stabilised F-actin and taxol-stabilised microtubule filaments with and without FH2FSI or FH2ΔFSI were visualized using TIRF microscopy. In controls lacking Fmn2, the actin and MT filaments were randomly oriented. However, in the presence of FH2FSI, F-actin and microtubule filaments co-aligned to form thick hybrid bundles. On the other hand, the deletion of the FSI domain (FH2ΔFSI) resulted in lack of co-alignment and filaments were randomly distributed as seen in no protein controls.

Co-sedimentation assays were also used to test F-actin-microtubule crosslinking ability of Fmn2. Incubation of FH2FSI with F-actin and microtubules simultaneously resulted in higher amounts of both the cytoskeleton polymers partitioning into the pellet fraction. Incubation with FH2ΔFSI

failed to show similar crosslinking between microtubules and F-actin. These results were confirmed using live-TIRF imaging of actin and microtubule filaments in the presence of Fmn2.

Still, in neurons, other factors like the changes in actin architecture (Colin *et al.,* 2018) or growth cone area (Kiss *et al.,* 2018) do indirectly influence microtubule dynamics. Using STED microscopy, we chose to examine the alignment of microtubule with actin bundles as physical proximity of the two cytoskeletons is the minimum requirement for cross-talk. We find that, depletion of Fmn2 leads to loss of this alignment, thereby leading to more dynamic microtubules in the filopodia. We also find that Fmn2 localizes to the entire filopodia, reaffirming the biochemical observation of actin bundling by Fmn2 (Priyanka Dutta, unpublished data) and Capu (Vizcarra *et al.,* 2014). Actin bundling requires the FSI domain (Vizcarra *et al.,* 2014) which makes this domain a common interaction point with both microtubule and actin. However, we reason that the common FSI domain is initially involved in nucleating and bundling actin and then as the microtubule enters the filopodia, one half of the dimer attaches to the polymerizing microtubule. The other half remains bound to acto-myosin filament and this coupling stabilizes microtubules and causes them to display a waggling behaviour while progressing in the filopodia.

In conclusion, our findings reveal the mechanism of Fmn2 mediated actin-microtubule coordination and its consequences in neurons. This finding has implications in other biological contexts like chromosome segregation, a process that required Fmn2 for the movement of chromatids (Mogessie and Schuh,2017) and biomedical application, including, spinal cord regeneration. Multiple modes of actin-microtubule crosstalk are known to exist in the growth cone (Coles and Bradke, 2015; Dogterom and Koenderink, 2018) and it appears that formins are emerging as a major class of proteins that mediate this cross-talk(Pawson *et al.,* 2008; Bartolini *et al.,* 2008; Gilliard *et al.,* 2011). In the near future, it will quite exciting to examine this cross-talk in distinct biological contexts (Li *et al.,* 2016; Pan *et al.,* 2013; Szikora *et al.,* 2017; Daou *et al.,* 2014; Qu *et al.,*2017). This cross-talk appears to have two independent mechanisms. Formins can interact with microtubules independently of their interaction with actin (Roth-Johnson *et al.,* 2014; Bartolini *et al.,* 2008). As noted in Foldi *et al.,* 2017, owing to the common interaction domains, it has been shown that there is competitive binding of formin to either microtubule or actin (Gilliard *et al.,* 2011; Roth-Johnson *et al.,* 2014) and that formin interaction with actin and microtubule are mutually exclusive. In contrast, our data and others (Szikora *et al.,* 2017; Pawson

et al., 2008; Rosales-Nieves *et al.,* 2006) show that formins interact with actin and microtubule simultaneously, i.e., crosslink them. Both the mechanism(s) might be relevant depending on cell type and signalling specific upstream regulators.

Model for actin- adhesion and microtubule crosslinking in growth cone turning

In the growth cone, parallel actin bundles form filopodia and at its base sits the actomyosin machinery (Bornschlögl *et al.,* 2013; Mallavarapu and Mitchison, 1999). Building on the substrate-attachment model by Chan and Odde in 2008, we hypothesize that in a stationary growth cone, the barbed end of the actin bundles undergo polymerization in the forward direction which is balanced by the retrograde pull due to myosin II activity. While the microtubule distribution is symmetrical, and most microtubules are restricted to central domain by actin retrograde flow. Now, as the growth cone moves or senses a cue there is increase in actin polymerization and bundling by Fmn2 in the chemotactic filopodia (Figure 4. **13** B). There is a concomitant increase in filopodial stability as the filopodia adheres to the substrate and forms point contacts which act as molecular "clutches". The engaged clutch mechanically links to actin cytoskeleton and attenuates the retrograde flow. Retardation in retrograde flow enable a greater number of microtubules to asymmetrically invade the peripheral region. Higher recruitment of Fmn2 to actin bundles increases the probability of actin- microtubule alignment and its subsequent entry into the filopodia. Here, one molecule of the Fmn2 dimer attaches to the incoming microtubule and stabilizes it. The other half remain attached to the actin filament. As Fmn2 crosslinks actin and microtubule and bundles them, the microtubules provide structural support to the filopodia, which, in turn, promotes actin coupling to the clutch, thereby, shifting the balance towards polymerization and growth cone moves forward and/or turns (Figure 4. **13** A).

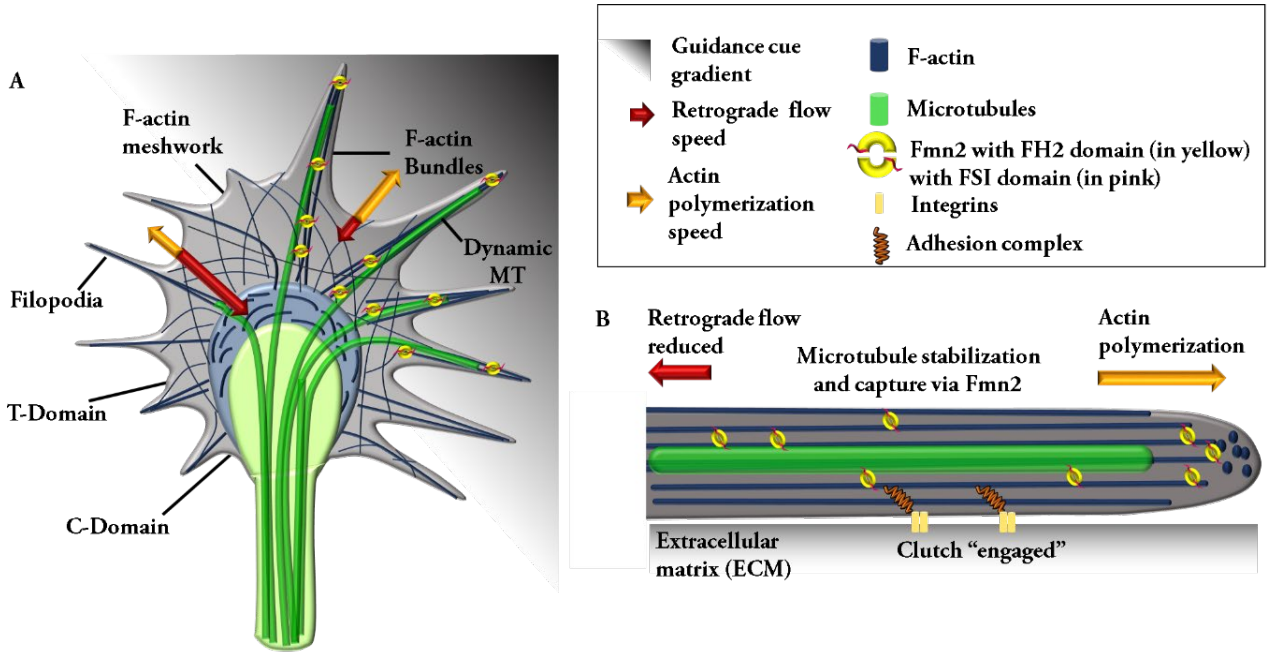

Figure 4. 13. Model for Fmn2 mediated actin-microtubule coordination in axonal pathfinding. A. Shows the cytoskeletal rearrangement and crosstalk mediated via Fmn2 in response to a cue. **B**. Interaction between actin and microtubule in the filopodia

Chapter 5

ROLE OF FMN2 IN AXONAL BRANCHING

All the *in-vitro* experiments were done with Sooraj Das (BS-MS student) as part of his training and 5th year M S thesis. Analysis shown in figure 5.9 involved Divya S. (BS-MS student). The *in-vivo* zebrafish injections were done by Dhriti Nagar and imaging involved both of us.

5.1. Results

Axonal branching is another important developmental process through which the neurons form connections. Collateral branching, wherein the branches appear from the shaft of the axon, is the major form of branching involved in establishing the circuitry (Cohen-cory *et al.,* 2010; Snider *et al.,* 2010). Branch formation begins with an actin patch (Gallo, 2006; Orlova *et al.,* 2007; Ketschek and Gallo, 2010) after induction by extracellular cues (Gallo and Letourneau, 1998; Danzer *et al.,* 2002). Patches are meshwork of actin filaments that are formed mainly through Arp2/3 and matures and undergoes actin filament rearrangement to form a protrusion (Armijo-Weingart and Gallo, 2017; Ketschek and Gallo, 2010). The protrusions are then stabilized and mature into a branch with the entry of dynamic microtubules (Kornack and Giger, 2005; Dent *et al.,* 1999; Ketschek *et al.,* 2015). The microtubules undergo de-bundling at sites of branching before the microtubule invasion (Ketschek *et al.,* 2015; Gallo and Letourneau, 1998). During the formation of a protrusion it is not yet clear how the patches convert to a protrusion as the patches are not protrusive. There is evidence that actin elongators Ena/VASP (Dwivedy *et al.,* 2007) are promote branching but it is not known whether formins contribute this process of branch formation. Owing to the enrichment of Fmn2 in the developing nervous system (Leader and Leder, 2000), we sought to assess the role of Fmn2 in axonal branching.

5.1.1. Fmn2 regulates protrusion density in the axon

An axon initiates the process of collateral branching with a protrusion. To investigate the role of Fmn2 in this process of filopodia formation, we knocked down Fmn2 at the protein level in chick primary spinal neuronal cultures and manually calculated the number of protrusions normalized to the length of the axon. We observed that protrusion density was reduced by almost half (0.1180 ± 0.01647 protrusions/µm) compared to control-MO treated axons (0.1930 ± 0.01953 protrusions/µm) (Figure 5. **1** A, B and C). Conversely overexpression of chick Fmn2 (gFmn2) led to an increased protrusion density in the whole axon after 24 hours (0.1426 ± 0.01824

protrusions/µm in control; 0.2438 ± 0.02002 protrusions/µm in gFmn2-GFP neurons) (Figure 5. **1** D, E and F).

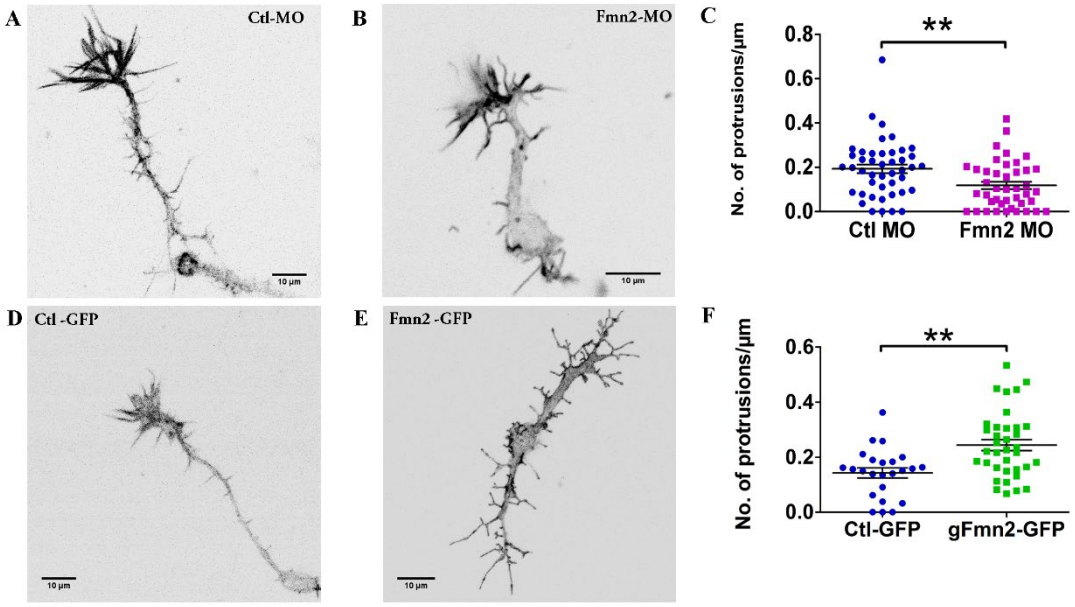

Figure 5. 1 Fmn2 regulates protrusion density. A and **B** show representative images of protrusions in Ctl-MO and Fmn2-MO axons, respectively. **C.** shows the quantification of protrusions per micron in Ctl-MO (n=43) and Fmn2-MO (n=42) neurons. **D** and **E** show representative images of protrusions in Ctl-GFP and Fmn2-GFP axons, respectively. **F.** shows the quantification of protrusions per micron in the same (Ctl-GFP, n=24 and Fmn2-GFP, n=36). **P<0.01, Mann-Whitney test.

5.1.2. Nucleation/elongation activity of Fmn2 is required for protrusion initiation

The loss of protrusion number due to depletion of Fmn2 was rescued by morpholino resistant mouse ortholog of Fmn2 (Figure 5. **3** A and B). We hypothesized that nucleation and elongation of actin filaments by Fmn2 might be important for formation of a protrusion and therefore created a nucleation deficient mutant of mouse Fmn2 by replacing the isoleucine at the 1226th position to alanine. The point mutation was based on data from Cappuccino or *drosophila* Fmn2, wherein mutation at 706th a.a. position led to dramatic decrease in actin assembly without affecting microtubule binding (Quinlan *et al.,* 2007; Roth-Johnson *et al.,* 2014). So, after sequence alignment, it was revealed that the isoleucine at 1706th position is evolutionary conserved (Figure 5. **2**). The resulting mFmn2I1226A mutant was indeed incapable of actin polymerization, as

confirmed by biochemical assays of actin elongation using pyrene-labelled actin and FH2FSI with I2A mutation and wild-type FH2FSI of mouse Fmn2 (Priyanka Dutta, unpublished data). Rescue experiments conducted with the mFmn2I1226A mutant failed to restore the protrusion density in the axon (Figure 5. **3** C and D). Thus, confirming the hypothesis that actin nucleation by Fmn2 is necessary to form protrusion in the axon.

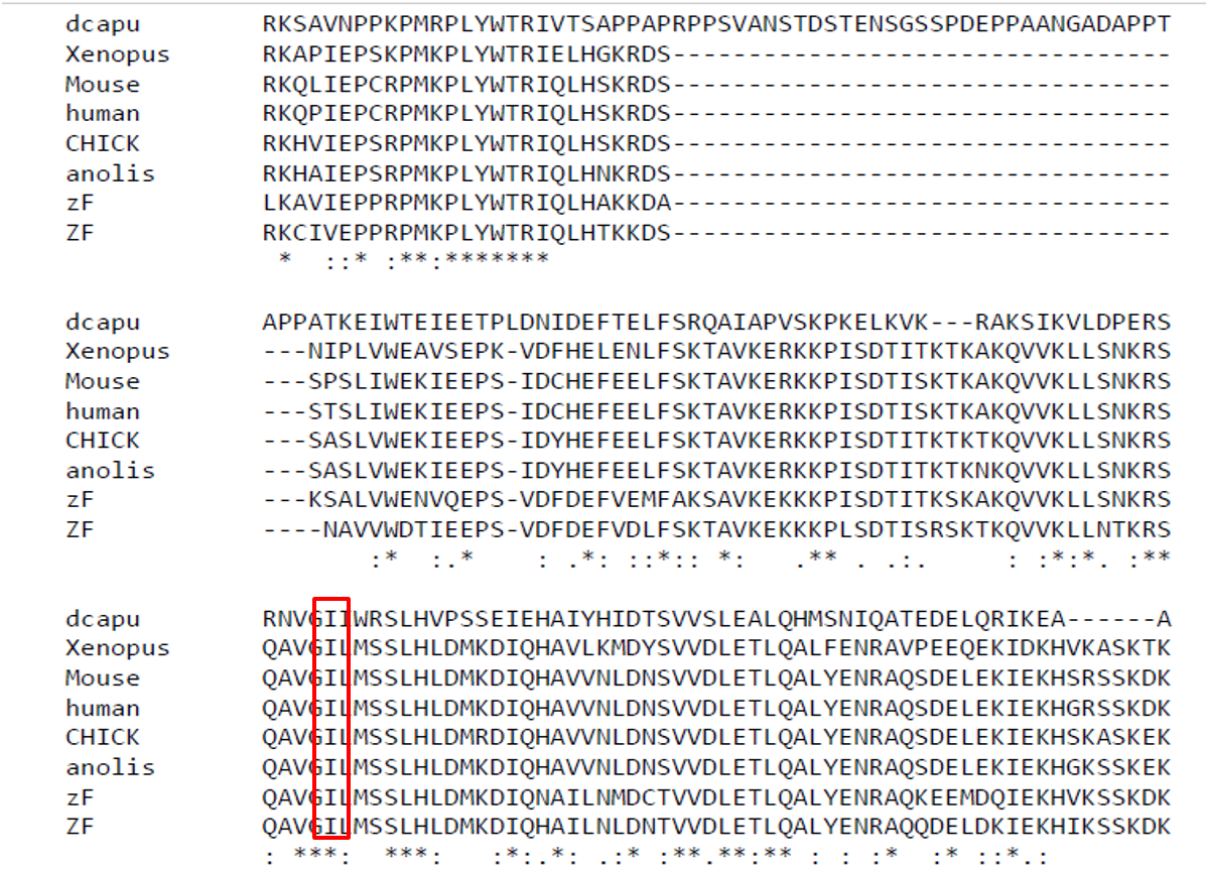

Figure 5. 2. Nucleation/elongation residue in Fmn2. Shows sequence alignment of Fmn2 across multiple species with evolutionary conserved with Isoleucine residue which is critical for actin assembly via Fmn2.

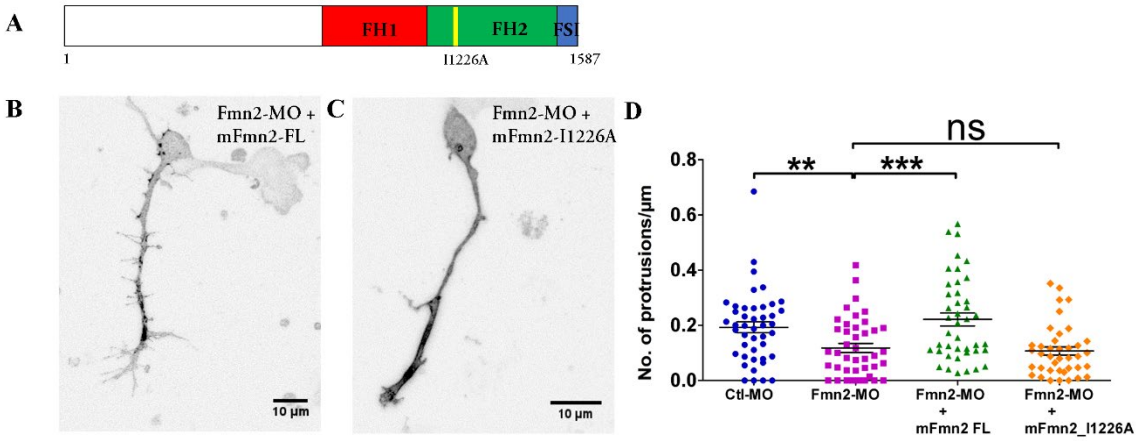

Figure 5. 3. Nucleation activity of Fmn2 is required for forming protrusions. A. Domain organisation of mouse Fmn2 full length with the position of I1226A mutation in FH2 domain. **B**. shows representative image of protrusions in mFmn2-FL rescue. **C.** shows representative image of lack of protrusions in mFmn2-I1226A rescue. **D.** quantification of protrusion density in FL and I1226A rescue. **P<0.001, **P<0.01, ns P>0.05; Krusal-Wallis test followed by Dunn's multiple comparison test.

5.1.3. Distribution of protrusion along the axon

As shown earlier, albeit in a different system (hippocampal neurons), the distribution of filopodial protrusions is not uniform throughout the axon and can be altered based on different signalling mechanisms (Danzer *et al.,* 2002). Hence, apart from examining protrusion numbers, we were curious to know if the distribution of these protrusions along the axon was affected. To quantify this parameter, the axon was divided into 10 equal segments and the number of protrusion(s) in each segment was noted and plotted for each condition. As shown in figure (Figure 5. **4** A), in control-MO neurons the most segments exhibited higher number of protrusions compared to Fmn2 knockdown. The distribution of the number of protrusions was skewed towards the distal end of the axon. Meanwhile, both Fmn2 knockdown and rescue with mFmn2I1226A mutant showed a drastically decreased and relatively homogeneous distribution along the axon. Surprisingly, rescue with mFmn2-FL displayed significantly increased number of protrusions all over the axon with the highest distribution of protrusions the middle region of the axon. This pattern of distribution was also seen in gFmn2 overexpression, wherein the protrusion density was higher in the middle region of the axon as opposed to distal end as seen in control-GFP (Figure 5. **4** B).

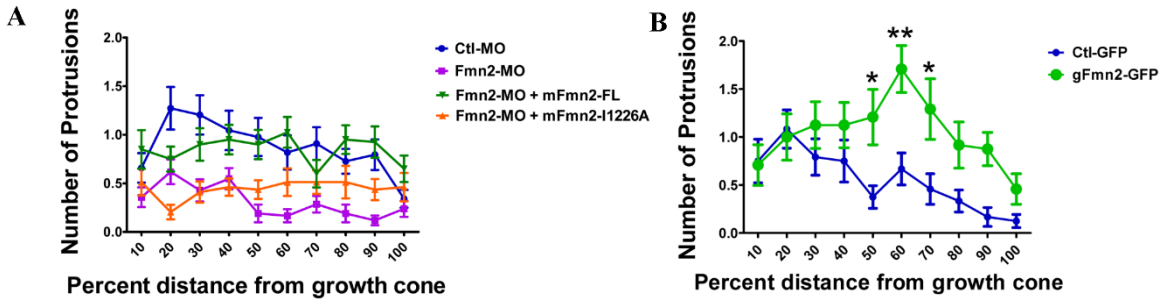

Figure 5. 4. Distribution of protrusions along the axon. A. Shows the distribution of protrusions along the axon in Ctl-Mo(n=44), Fmn2-MO (n=42), mFmn2-FL rescue (n=40) and mFmn2I1226A rescue (n=39). The result of statistical analysis is in Table 2 . **B.** shows the same in Ctl-GFP (n=24) and gFmn2-GFP overexpression (n=24). **P<0.001, **P<0.01, *P<0.05, ns P>0.05; Two-way ANOVA test, matching by coloumn.

Percent distance from the growth cone	Ctl-MO vs Fmn2-MO	Fmn2-MO vs Fmn2-MO + mFmn2-FL	Fmn2-MO vs Fmn2-MO + mFmn2-I1226A	Ctl-MO vs Fmn2-MO + mFmn2-FL	Ctl-MO vs Fmn2-MO + mFmn2-I1226A
10	ns	ns	ns	ns	ns
20	*	ns	ns	ns	***
30	**	ns	ns	ns	**
40	ns	ns	ns	ns	ns
50	***	***	ns	ns	ns
60	*	***	ns	ns	ns
70	*	ns	ns	ns	ns
80	ns	***	ns	ns	ns
90	**	***	ns	ns	ns
100	ns	ns	ns	ns	ns

Table 2. Shows the p-value significance of each treatment, compared to one another. **P<0.001, **P<0.01, *P<0.05, ns P>0.05; Two-way ANOVA test, matching by coloumn.

5.1.4. Actin dynamics and protrusion initiation in the axon

To explore the underpinnings of protrusion initiation and the role of Fmn2 regulating this process, live-imaging using F-tractin-GFP was undertaken. F-tractin is a cellular probe that consists of actin binding domain of at inositol 1,4,5-triphosphate 3-kinase A (ITPKA) (Schell *et al.,* 2001). It was used to visualize F-actin dynamics as it has been shown to have the least amount of effect on actin dynamics in neurons (Patel at al., 2017). Co-localization of tractin-GFP and actin structures using

phallodin staining was done to ensure tractin picks up all structures that are also staining with phalloidin (Figure 5. **5** A). Live imaging of actin dynamics in the axon with F-tractin-GFP revealed two distinct types of actin structures that gave rise to a filopodia. One was **actin patch**- the most predominant actin structure (89.5% , percentage of occurance) which appeared as a roundish blob that was largely stationary, had a long lifetime and gave rise to about 82% of total primary filopodia and 52% of total secondary filopodia (Figure 5. **5** B and Table **3**). The other actin structure that was observed was **actin trail**- a stretch of F-actin that translocated rapidly and gave rise to 18% of total primary filopodia (originating from the axon shaft) and 48% of total secondary filopodia (originating from primary filopodia). The velocity of a trail (10.84 ± 2.850 μm/min) was more than 10-fold higher than a patch (0.8459 ± 0.07003 μm/min), while the lifetime was halved with 16.41 ± 1.599s compared to 30.13 ±2.437s of a patch (Figure 5. **5** C and and Table **3**). Lastly, around 50% of trails originated from a pre-existing patch.

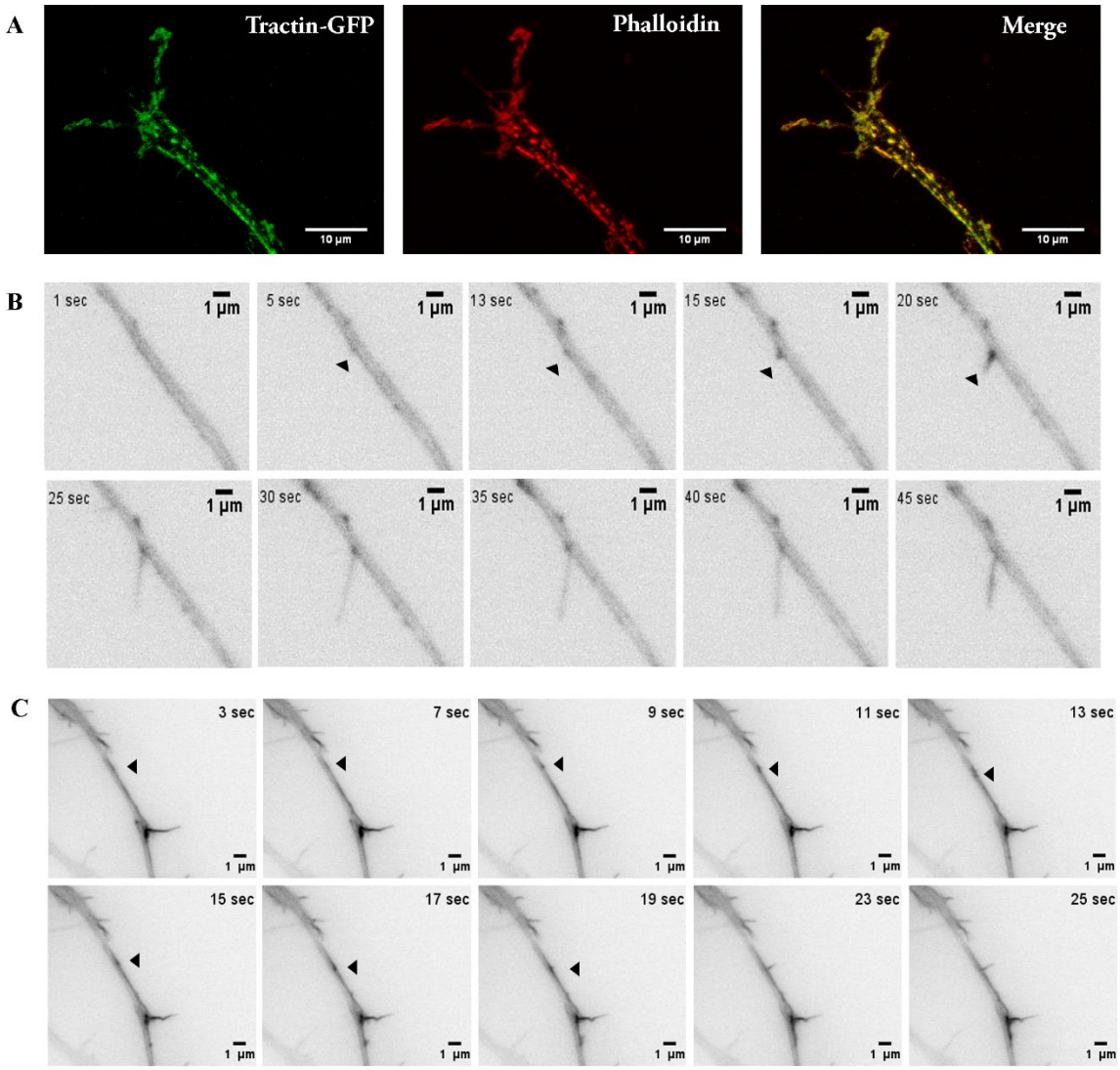

Figure 5. 5. Actin patches and trails in the axon. A. shows an example of a tractin-GFP overexpressed neuron that was stained with phallodin. The merge image shows the co-localization. **B.** Frame of a time-lapse movie showing an actin patch that appears (marked by arrowhead) and give rise to a protrusion and then disappears. **C.** Shows consecutive frames of an actin trail (marked by an arrowhead) moving along the axon before resulting in a protrusion.

Parameter	Patch	Trail
Number analysed	174	38
Percentage of occurrence	89.5	10.5
Number/µm/5 min	1.430361	0.300644999
Percentage that initiates primary protrusion	82	18
Percentage that initiates secondary protrusion	52	48
Mean velocity (µm/min)	0.59598	11.784
Mean lifetime (s)	29.63	10.75

Table 3. Shows the parameters of actin patches and trails in the axon.

This data revealed that every protrusion is preceded by a patch or a trail and suggests that Fmn2 may regulate the dynamics of these structures to influence protrusion density.

5.1.5. Live imaging of Fmn2 in the axon

Overexpression of *gallus* Fmn2 was done to visualize sub-cellular localization of Fmn2 in axons. We found that Fmn2 localized to the base of every protrusion (Figure 5. **6**) and chevron shape. These structures were quite stable and persisted for minutes, well after the protrusion was initiated.

Co-expression of Fmn2- mcherry and tractin-GFP was done to visualize co-localization with actin structures. We found that Fmn2 colocalized to actin patches during the protrusion initiation (Figure 5. **7** B) and accumulated in patches over time (Figure 5. **8** C). We also noticed that the chevron shaped structures were formed before the actin patch (Figure 5. **7** B; Figure 5. **8** A and B; frame 18s for Fmn2 and 22s for tractin), possibly to deform the membrane, followed by an actin patch formation before giving rise to a protrusion.

Co-localization of Fmn2 was also seen with actin trails (Figure 5. **8**). Here too, Fmn2 localizes to a specific sub-membrane region and forms a chevron (8.5s, third frame) and the trail quickly gives rise to a protrusion.

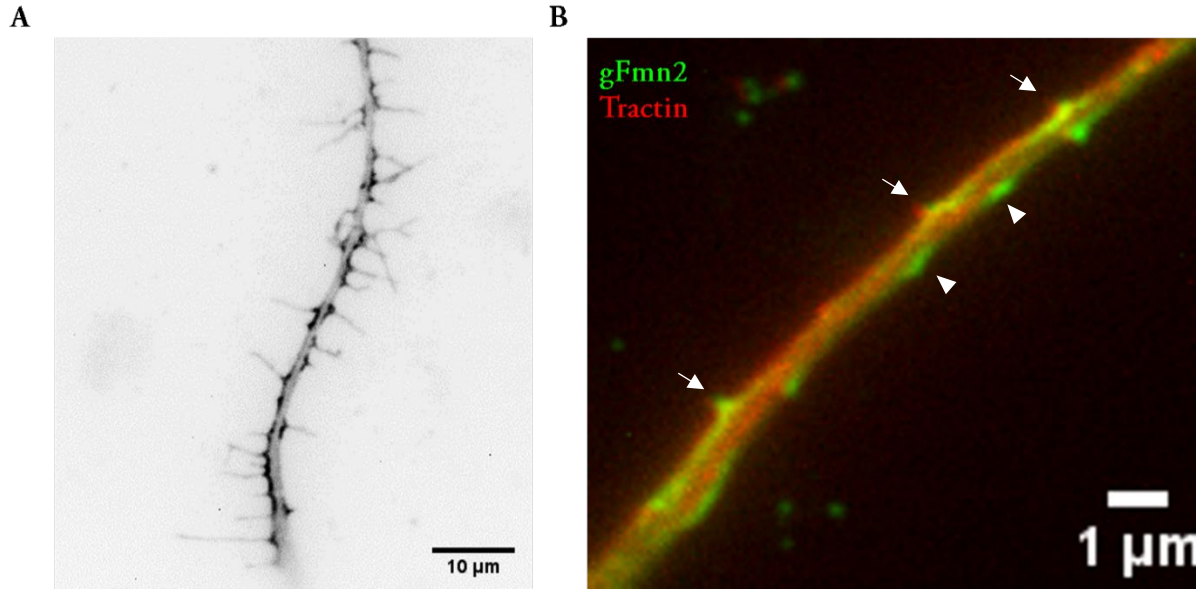

Figure 5. 6. Fmn2 localization in axons. A. shows chevron shaped expression at the base of every protrusion. B. Shows co-localization of Fmn2 and tractin at the base of an emerging protrusion (marked by arrows). Fmn2 localizes to the deformed membrane before actin patch formation (marked by arrowheads).

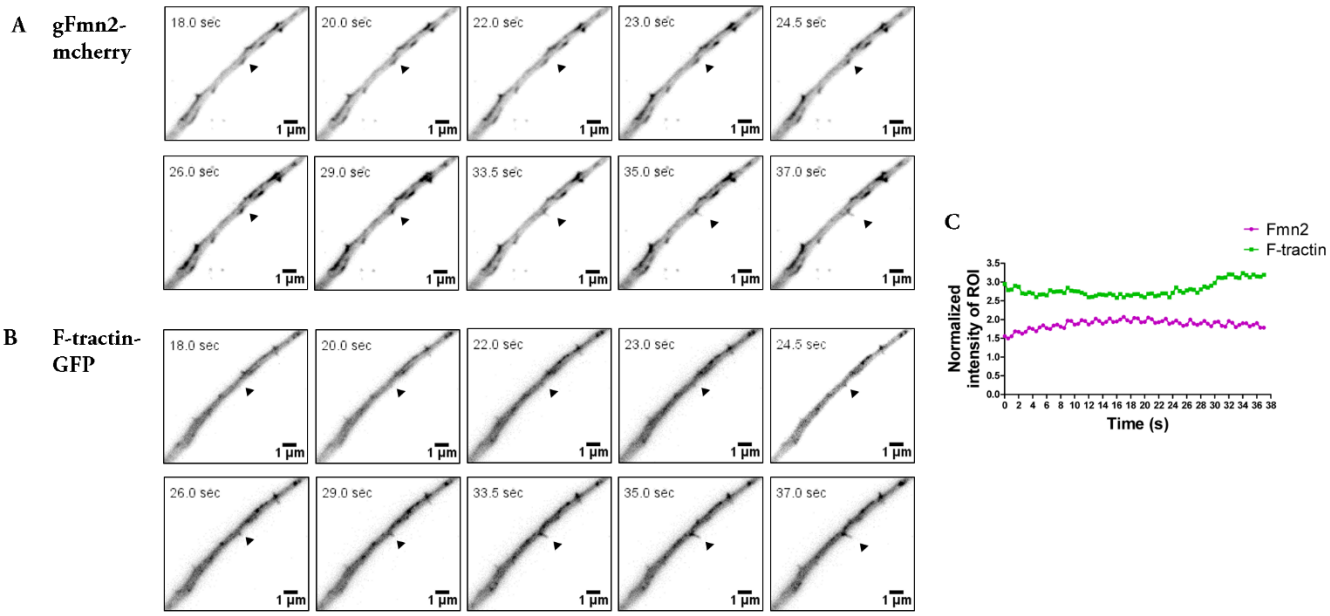

Figure 5. 7. Co-localization of Fmn2 with actin. A and **B** show the frames of live imaging of Fmn2 and tractin together. Fmn2 localizes first just below the membrane and deforms the membrane. Then an actin patch is formed just below the deformation (at 33.5 s) and forms the protrusion. C. shows the normalized intensity plots of Fmn2 (magenta) and F-tractin (green) over 37s of time.

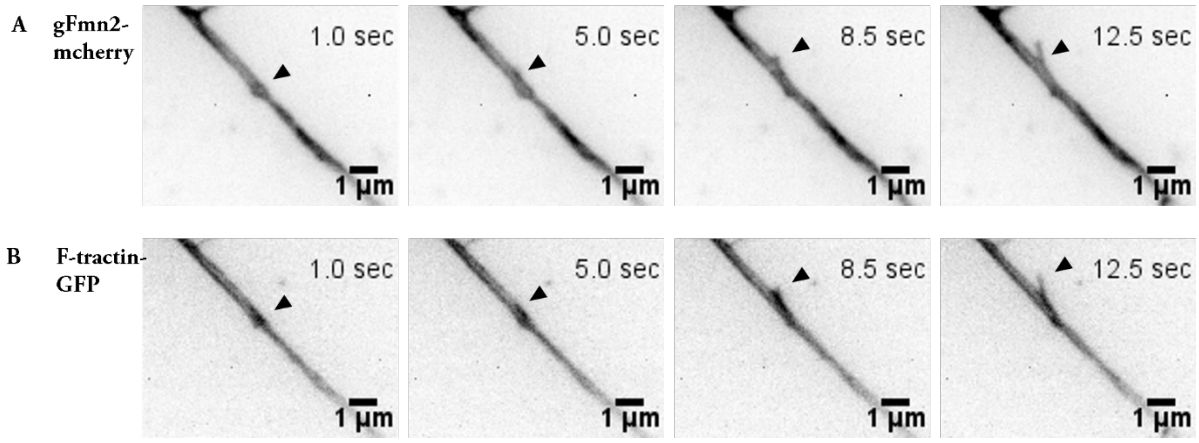

Figure 5. 8. Co-localization of Fmn2 with actin trails. A and **B** show snapshots of Fmn2 and actin trail which moves rapidly before giving rise to a protrusion.

5.1.6. Fmn2 influences actin patch dynamics in the axon

To investigate the role of Fmn2 in regulating the two actin structures, Fmn2 was knocked down and the axons were analysed, and several parameters of patch and trail dynamics were quantified. Analysis from kymographs generated from the time-lapse movies revealed that even though the density of these patches were not different (Figure 5. **9** C), size of the actin patches was significantly reduced with Fmn2 depletion (0.1805 ± 0.01177 µm^2 in Ctl-MO to 0.1519 ± 0.01245 µm^2 in Fmn2-MO) (Figure 5. **9** D). Moreover, the lifetime of these patches was drastically shortened from 53.6 0± 4.821s to 33.93 ± 2.136s (Figure 5. **9** E). During analysis, we also noticed that Fmn2 knockdown caused multiple cycles of patch formation and disappearance taking place in the same spot of an axon, thereby, leading to a "blinking" phenomenon (Figure 5. **9** A and B). These patches, with diminished Fmn2, also showed higher mobility (Figure 5. **9** F), indicating an overall instability of these actin structures.

In addition to regulating patch dynamics, Fmn2 may influence actin trails too as they also exhibited a trend towards shortened lifetime (17.72 ± 2.764s in control and 10.97 ± 1.586s in Fmn2 KD) and higher mobility but the p-value was not significant perhaps due to low numbers (n=38 for control and n=28 for Fmn2). Accordingly, these experiments implicate Fmn2 in regulating actin patches and possibly actin trails.

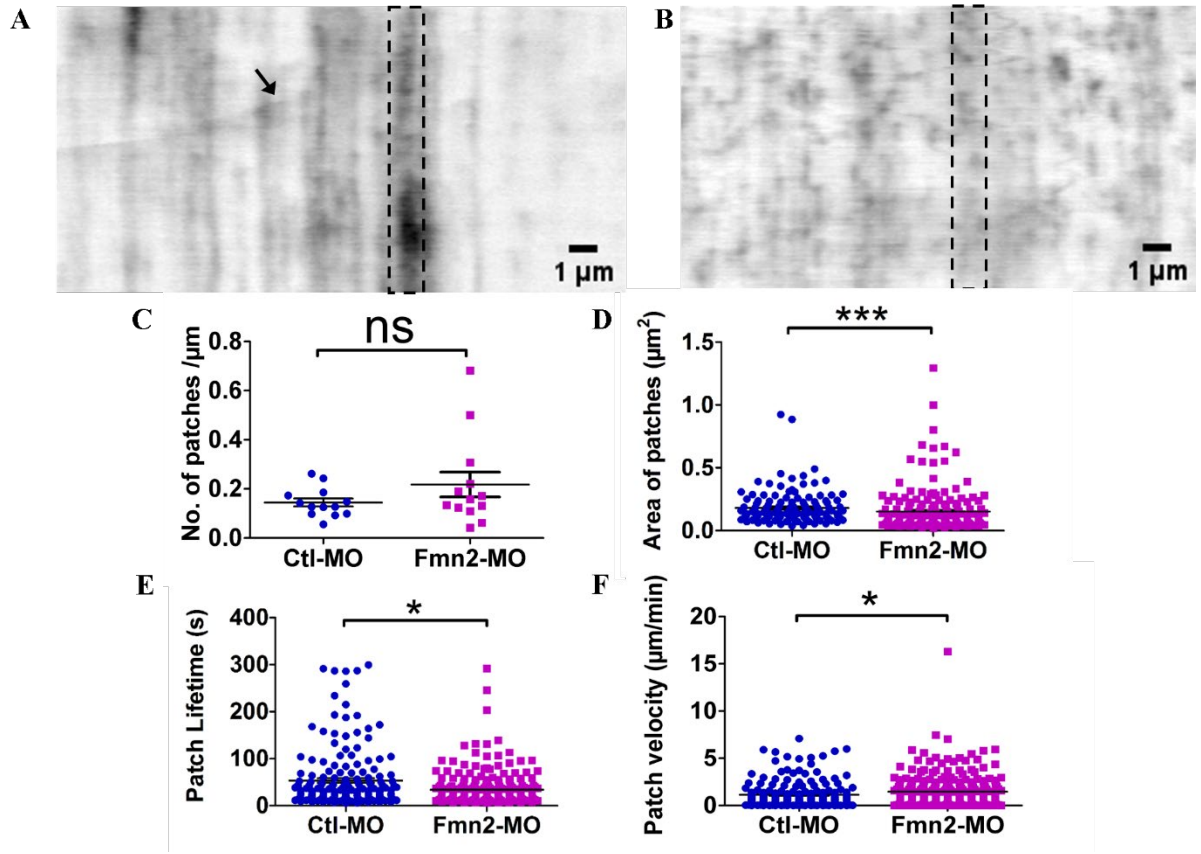

Figure 5. 9. Fmn2 regulates actin patch dynamics. A. shows kymograph of a Ctl-MO neuron expressing tractin-GFP. Dotted box highlights one patch present throughout the imaging time of 300s. The arrow marks the start of an actin trail. **B**. shows representative kymograph of Fmn-MO neuron. The dotted box highlights the "blinking" phenomena of a patch. **C**. quantification of number of patches per µm (Ctl-MO, n=13 and Fmn2-MO, n=13) **D**. quantification of area of patches (Ctl-MO, n=128 and Fmn2-MO, n=189) and **E**. quantification of patch lifetime (Ctl-MO, n=174 and Fmn2-MO, n= 258). **F.** Quantification of patch velocity (Ctl-MO, n=174 and Fmn2-MO, n= 258). **P<0.001, *P<0.05, ns P>0.05; Mann-whitney test.

5.1.7. Fmn2 influences filopodial maturation in the axon

After a protrusion is initiated, a proportion of them undergo elongation and stabilization to form a mature branch. This process is microtubule dependent. It requires targeted entry of dynamic microtubule and its subsequent stabilization into the nascent branch in order to mature (Gallo, 2011; Ketschek *et al.,* 2015). Therefore, to investigate this stage of the process, mature branches from each experimental condition was quantified. The criteria for identifying a branch was such that the protrusion must be >20 microns in length or it must have a growth cone and in either of these cases the protrusion must be innervated by a microtubule (Figure 5. **10** A, B, C and D). After

24 hours of incubation or 1 DIV (days *in-vitro*), the proportion of neurons that had ≥1 primary mature branch was 26%. Depletion of Fmn2, reduced the proportion to less than half at about 8%. This phenotype was restored to control levels in rescue by morpholino-resistant full-Length mFmn2 (27%). And surprisingly, rescue with mFmn2I1226A mutant showed similar proportions of axons with mature branch as control and mFmn2-FL rescue axons (41.3%) (Figure 5. **10** E). Thus, Fmn2 may be involved in two independent functions 1) initiating protrusions and 2) stabilising existing protrusions, which are most likely mediated via distinct domains.

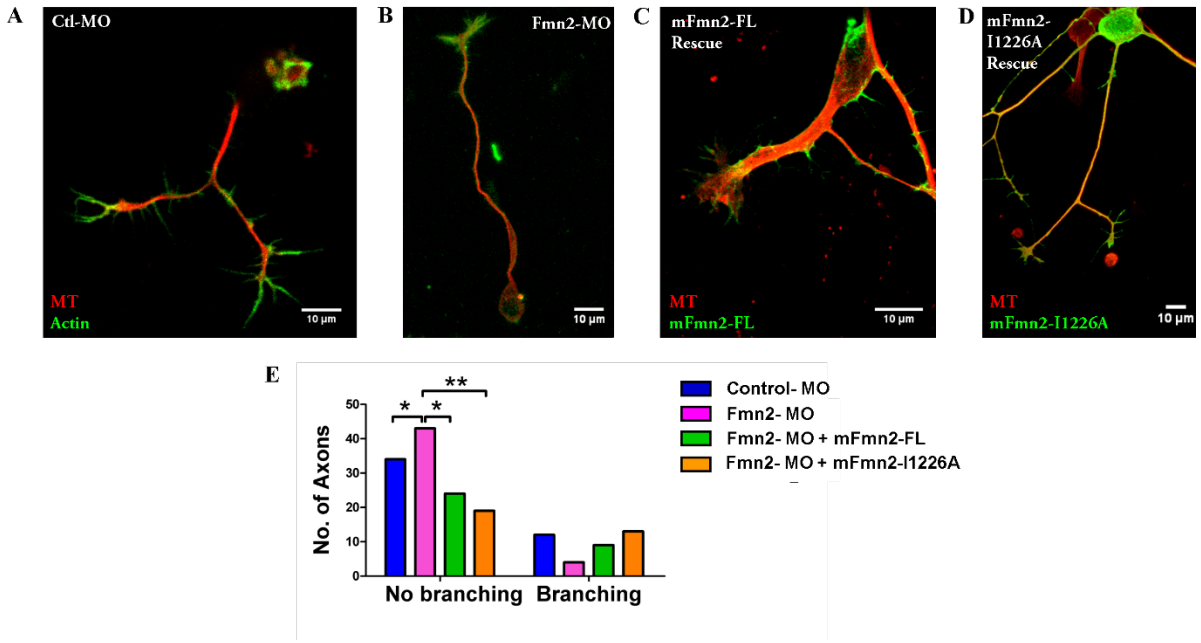

Figure 5. 10. Fmn2 influences branching in axons. A, B, C and **D** show representative images of Ctl-MO, Fmn-MO, mFmn2 Rescue and mFmnI1226A rescue, respectively. **E.** Quantification of branching or no branching in total number of axons (Ctl-MO n= 46, Fmn2-MO n=47, mFmn2 FL rescue n=33, mFmn2-I1226A n=32). **F.** Shows spread of the percentage of axons that display the degree of branching. **P<0.001, *P<0.05; Fisher's Exact test.

5.1.8. Fmn2 influences branching in-vivo

To confirm the role of Fmn2 in branching *in-vivo*, transgenic zebrafish embryos expressing mnx1: GFP were used. Mnx1 protein is expressed in primary motor neurons (PMNs) of a developing embryo. These PMNs are further divided into 3 subtypes: CaP (caudal primary), MiP (middle primary) and RoP (rostral primary) motor neurons (Figure 5. **11** A). The CaP neurons are first to sprout growth cones as early as 18 hours post- fertilization (*hpf*) and traverses ventrally into the ventral median septum till the edge of the axial muscles and then turns dorsally and laterally to project over the axial muscles. The MiP neurons start their growth at about 20 *hpf* and first grow

ventrally, similar to CaP neurons. But, as they reach the horizontal septum, the growth cone halts and another collateral branch from the axon grows dorsally between the surface of the spinal cord and the medial face of the axial muscle. The third subtype, RoP neurons grows similarly till it reaches the horizontal septum. Here, instead of going dorsally as MiP neurons it turns laterally and grows in the horizontal septum (Myers *et al.,* 1986; Eisen *et al.,*1986). The arborization takes about 48 hours. CaP neurons in the control-MO injected fish undergo collateral branching at the distal end of the axon and remain restricted to the individual myotome segment (Sainath and Granato, 2013) (Figure 5. **11** B). Whereas, embryos injected with specific splice -blocking morpholinos against zebrafish Fmn2 (Dhriti Nagar, unpublished data), exhibited drastically reduced branching in CaP neurons. They also displayed delayed axonal outgrowth and disrupted axonal guidance of RoP and MiP neurons (Figure 5. **11** C). These are representative images and the data has not been quantified as the number are too low.

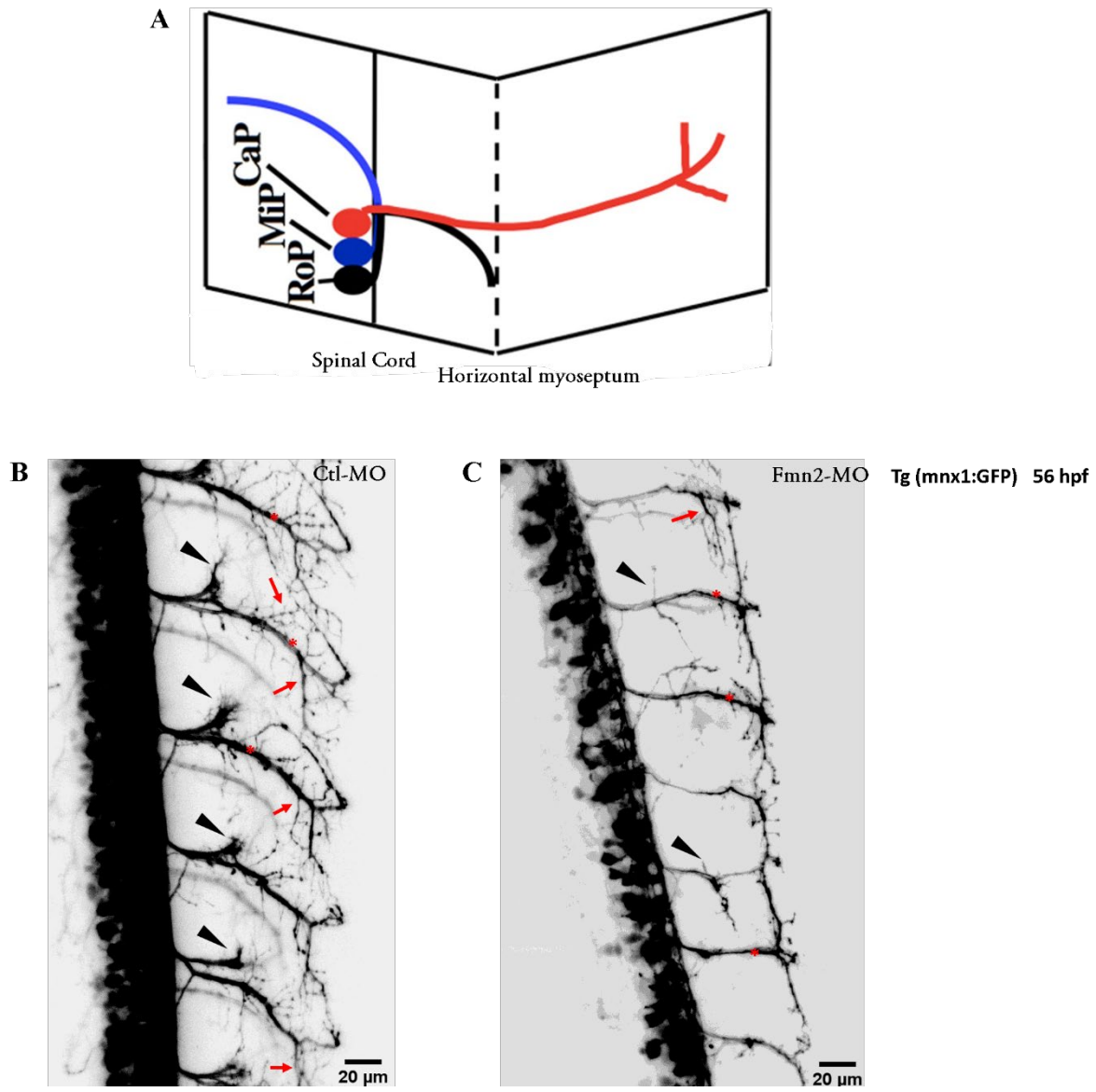

Figure 5. 11. Fmn2 regulates branching *in-vivo*. A. shows the schematic of trajectories of CaP, MiP and RoP neurons. Adapted from Millecamps *et al.,* 2007. **B** and **C.** show images of branching in primary motor neurons of Ctl-MO and Fmn2-MO zebrafish at 56*hpf.* The RoP neurons are marked by an arrowhead. CaP axon shafts are marked by a red asterisk and the axon collateral are marked by a red a red arrow.

5.2. Discussion

Formins has been predicted to be involved in axonal branching, but there is not much evidence seen till now. This study investigated the role of Fmn2 in axonal branching with the purpose of assessing if Fmn2 has a broader role in establishing circuitry other than growth cone navigation. We found that overexpression of Fmn2 led to *de novo* protrusions in the axon. Conversely, knockdown of Fmn2 reduced the number of protrusions quite significantly. This phenotype could be rescued with full length morpholino- resistant Fmn2. Moreover, we uncovered that nucleation/elongation activity of Fmn2 was required for forming such protrusions. We discovered that Fmn2 localized to the base of deformed membranes and preceded the formation of axonal protrusions. To gain a mechanistic understanding, we observed the actin patches involved in formation of these protrusions. Actin patches have been described to initiate axonal protrusions (Loudon *et al.,* 2006; Spillane *et al.,* 2011; Ketschek *et al.,* 2010). Actin trail, on the other hand, is a novel structure that was found to be involved in branching. Furthermore, Fmn2 was visualized to colocalize with both of these structures. But, Fmn2 regulated patch stability by decreasing patch area and lifetime when knocked down and qualitatively increased patch lifetime when overexpressed. Finally, depletion of Fmn2 was shown to decrease branching in neurons both *in-vitro* and *in-vivo*. This phenotype was rescued *in-vitro* by full-length morpholino-resistant Fmn2 and unexpectedly, also by nucleation dead mutant.

Fmn2 localizes to the base of protrusions

One of most striking observations was the presence of a chevron-shaped structure at the base of every protrusion following imaging of Fmn2-GFP transfected axons. The chevron structure, similar to an actin patch, pre-determined a protrusion. But, unlike actin patches, which disappear after a protrusion is formed, these structures appeared quite stable and were present for tens of minutes. It is yet unclear as to the purpose of these structures. Is it to guide microtubules? Or does it act as a filter or barrier of some kind? Or is it present to provide structural support? In literature, only a couple of proteins (of the same family) have been reported to form chevron structures at the base of protrusions. These are Septin -6 and -7 (Hu *et al.,* 2012; Nölke *et al.,* 2016). Septin 6 and 7 appear to sense micron-scale curvatures and were mostly enriched in membrane of positive

curvature such as at the base of branches (Bridges *et al.,* 2016). Moreover, in biochemical studies, Septin complexes crosslinked actin into curved bundles (Marvrakis *et al.,*2014). Another possibility that Fmn2 is recruited by BAR (Bin/amphiphysin/Rvs) proteins such as IRSp53 and SR-GAPs. BAR proteins are a class of proteins both sense and generate curved membranes (Coutinho-Budd *et al.,* 2012). Both IRSp53 (Chung *et al.,* 2015) and SR-GAP 2 and 3 (Wilson *et al.,* 2011; Charrier *et al.,* 2012) have been implicated in brain development. So, Fmn2 may bind to BAR proteins and form a complex that deforms the membrane. Perhaps, it is possible that Fmn2 expression at the base is possibly due to its recruitment to actin bundles, which would aid in subsequent polymerization of actin bundles to deform the membrane and form a protrusion. However, this explanation does not answer why is Fmn2 enriched at positive curvatures. Actin is present all along the cortex of the axon, but Fmn2 appears to be sensitive to curvature of the membrane. Is it because it is recruited by Septin complexes? This hypothesis needs to be tested and could potentially unveil a novel mechanism of formation of protrusions.

Fmn2 regulates actin patch dynamics

During development, branching is evoked by local extracellular cues, such as NGF or Wnt, which ultimately impinge on the underlying cytoskeleton (Kalil and Dent, 2014; Billimoria and Bonni, 2013). The process of branch formation starts with a branched meshwork of actin – an actin patch (Nithianandam and Chien, 2018; Danzer *et al.,* 2002; Mingorance-Le Meur and O'Connor, 2009; Ketschek and Gallo, 2010;). After patch formation, the patch undergoes maturation, increasing in size and intensity. Since, there was no change in patch number upon diminishing Fmn2 levels, we hypothesize that Fmn2 does not affect patch formation. However, significant reduction in patch lifetime and area was observed upon Fmn2 knockdown. This suggests that Fmn2 promotes actin patch maintenance. In fact, when Fmn2 was depleted the patch appeared to "blink" in the same region, suggesting repeated expansion of the patch but difficulty in maturation and/or maintenance. Data from studies in yeast suggest that actin patches are under the control of antagonistic factors and there is a balance between actin assembly and disassembly (Smith *et al.,* 2001; Nakano *et al.,* 2001). This competitive balance is created by proteins nucleating and polymerizing actin patch such as Arp2/3 (Spillane *et al.,* 2011), fimbrin (Nakano *et al.,* 2001), cortactin (Hu *et al.,* 2012) and drebrin (Ketschek *et al.,* 2016). And on the other hand, proteins like myo5p (member of myosin I family) (Smith *et al.,* 2001), myosin II (Loudon *et al.,* 2006),

and Adf1 (actin depolymerizing factor) (Nakano *et al.,* 2001) are involved in destabilization and disassembly of the actin patch. Fmn2 appears to be regulating the patch dynamics by tipping the balance towards actin elongation and stabilizing the F-actin filaments after patch formation.

It could also be possible that Fmn2 tilts the balance owing to its anti-capping activity from the actin filaments. Formins possess anti-capping activity, as they sit on the barbed end to elongate actin (Pruyne *et al.,* 2002; Goode and Eck, 2007) and increase in axonal branching has been observed by inhibiting capping of actin filaments (Menna et al. 2009).

Lastly, although actin patches are the dominant structures that give rise to protrusions, we identified actin trails, which serve as precursors for protrusion formation. Such a structure was first reported in hippocampal neurons and was demonstrated to be formin-dependent (Ganguly *et al.,* 2015). In this study, the trails were found to be important for vesicular transport.

Nucleation/ Elongation activity of Fmn2 is required for protrusive activity

Actin patches are pre-requisite for protrusions but only a proportion of these patches forms a protrusion (Orlova *et al.,* 2007; Ketschek and Gallo, 2010). The process of formation of a protrusion from a patch is less understood. Till now, septin 6 and drebrin has been implicated in this process (Ketschek *et al.,* 2016). It was achieved this by increasing the transition frequency of a patch to protrusion. Actin patches themselves are not protrusive. They are thought to either rearrange themselves from a meshwork to parallel bundles or serve as specialized sites for emergence of bundled actin. The observation that nucleation/elongation dead Fmn2 was not able to rescue protrusion density, appears to suggest that Fmn2 mediated elongation and bundling is required for protrusion formation. Studies have also shown that actin patches are specialized sites, in that, they also function as sites of mitochondrial docking, thereby providing energy for polymerization and local protein synthesis of actin (Ketschek and Gallo, 2010; Spillane et al. 2013).

Possible role of Fmn2 in protrusion maturation

Preliminary analysis of degree of branching show that Fmn2 may possibly regulate collateral branch maturation. As with patches, not all protrusions lead to a mature branch. This frequency of transition from a nascent protrusion to a mature branch was unexpectedly much higher in rescue experiments with nucleation/elongation dead mutant of Fmn2. A possible reason for this could be local increase in the availability of free actin pool, which could direct more actin in existing

protrusions (the ones that were formed despite reduction in overall protrusion density) and enable maturation. Another possibility could be modulation of the microtubule cytoskeleton by Fmn2. Microtubule targeting and subsequent stabilization in protrusion are required for maturation (Chapter 4, Figure 4. **10**). Since, the microtubule binding domain of polymerization mutant of Fmn2 is intact, this interaction could potentially increase the transition frequency of nascent to mature branches. This line of inquiry remains to be tested.

In conclusion, this is the first study to implicate formins in the process of axonal branching. One other recent study has implicated formin 3 in dendritic arborization in nociceptive sensory neurons in *drosophila* (Das *et al.,* 2017). The mechanism of function of formins in this process of branching is not yet clearly elucidated. Formins, in principle, can regulate both nascent protrusion formation and its subsequent maturation owing to its crosstalk with both actin and microtubule cytoskeletons. Furthermore, Fmn2 has also been implicated in vesicular transport (Schuh, 2013) and this process has been demonstrated to regulate axonal branching (Ponomareva *et al.,* 2014). Hence, it will be interesting to explore the interaction of endosomal transport and patch dynamics in the context of branching.

Model for Fmn2 mediated axonal protrusion

In response to an extracellular guidance cue, actin nucleators like arp2/3 and cortactin initiate meshwork of branched actin filaments in the axon (Figure 5. **12**, stage 1). This patch of actin is then co-inhabited by Fmn2, which leads to its maturation/ maintenence either by stabilizing actin filaments or by protecting them from capping proteins. At the same time, Fmn2 localizes to membranes with positive curvature by presumably binding to septin 6 or 7 and cooperate in actin bundling and stabilization (Figure 5. **12**, stage 2). Then the actin patch, just beneath the membrane deformation, acts a specialized region for Fmn2 mediated actin nucleation and elongation. Similar to the growth cone filopodia elongation, polymerization of parallel bundles of actin filament provide the pushing force necessary for protrusion formation (Figure 5. **12**, stage 3). The deformation of the membrane and its bud-like maintenance due to curved actin bundles reduces the force required to push it further to form a protrusion. The protrusion is then stabilized by guided entry of dynamic microtubules and their subsequent stabilization due to Fmn2 mediated actin-microtubule coordination, similar to growth cone filopodia (Figure 5. **12**, stage 4).

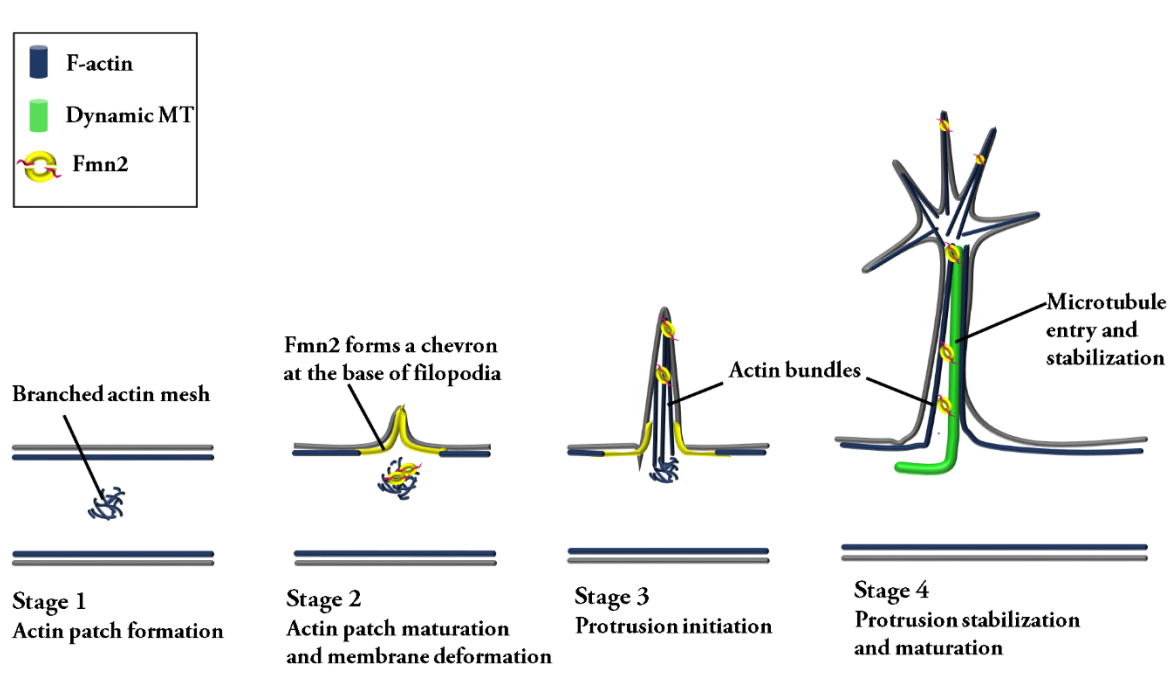

Figure 5. 12. Role of Fmn2 in axonal branching. Figure shows the various stages of axonal branching. Fmn2 contributes to membrane deformation (stage 2) which then leads to a protrusion due to nucleation of actin bundles from an actin patch (stage 3). The nascent protrusion is stabilized by invasion of a dynamic microtubule (stage 4).

Chapter 6

SUMMARY AND FUTURE DIRECTIONS

This thesis investigates the role of Fmn2 in growth cone navigation and axonal branching during neuronal development. As mentioned in the introduction, Fmn2 has been implicated in multiple cognitive disorders but the mechanism of Fmn2 function in neurons remains poorly understood. The work described in chapter 4 is aimed at understanding how Fmn2 regulates actin-microtubule crosstalk- how it occurs and its consequences in growth cone turning. In chapter 4, we investigated the cytoskeletal mechanisms regulating individual stages of axonal branching, from patch formation to a protrusion to finally form a mature branch.

Actin-microtubule coordination in the growth cone

Directed movement of neurons requires coordination between the actin and microtubule cytoskeletons. Actin and microtubule occupy spatially different regions in the growth cone with actin at the transition and peripheral zones and microtubules mainly occupying the central domain, with a few exploring into the periphery. The architecture of these two cytoskeletons give rise to morphology of the growth cone and their remodelling underlies growth cone movement. Here, we probe these two cytoskeletons to understand how guidance mediated movement occurs. We have uncovered a molecule (Fmn2) that mediates the crosstalk in guiding the microtubules along the actin bundles in the filopodia. We show that actin-microtubule alignment occurs from the transition zone and into the filopodia. This coupling, which occurs through the FSI domain of Fmn2, results in stabilization of the microtubule, which in turn leads to filopodial stability and growth cone turning.

Adhesion-based growth cone turning

Growth cone turning involves the actin-adhesion clutch system along with microtubules (Craig, 2018). Actin- mediated clutch provides a mechanical attachment to the ECM (extra-cellular matrix), which enables actin polymerization and leading-edge protrusion and stabilization. At the same time, actin-microtubule coordination is necessary for turning. But not much is known about how these 3 components, namely, actin, microtubules and adhesion molecules interact to bring about growth cone turning. Recent unpublished work has implicated Fmn2 influencing point contacts maturation leading to weaker traction forces in Fmn2 depleted growth cones (Ketakee Ghate, PhD thesis). Point contacts are

integrin-containing, multi-protein structures that form mechanical links between intracellular actin bundles and the extracellular substrate in many cell types (Abercrombie *et al.,* 1975). And indeed, filopodial (containing actin bundles) force generation was also found to be compromised along with reduction is the intensity of tension-dependent maturation signal (phospho-Focal Adhesion Kinase (pFAK) protein) (Sahasrabudhe *et al.,* 2016). So, to elucidate the role of Fmn2 involved in this tension-dependent maturation of point contacts, we employed a technique of measuring tension at sub-cellular levels via a FRET- based vinculin- tension sensor (Grashoff et. al. 2010). Vinculin is one of the prominent residents of focal adhesions and its recruitment to FA's is mechanosensory in nature (Grashoff *et al.,* 2010). We tested the feasibility of this experiment in mouse fibroblasts, NIH3T3. In fibroblasts, point contacts form much stable and long-lasting structures called focal adhesions (FA). Vinculin physically binds to F- actin and acts as a molecular clutch (Thievessen *et al.,* 2013). The vinculin –tension sensor construct consists of mTFP1 and Venus as the FRET pair (Grashoff et. al., 2010) (Figure 6. **1** A). The tension sensor module (TSMod) under low force has high FRET efficiency and when the force across TSMod causes the linker to extend the FRET efficiency decreases. On the contrary, vinculin-tailless construct does not possess the tail and therefore cannot bind to F-actin and consequently cannot sense tension (Adapted from Grashoff et. al., 2010), thus was used as control.

The FRET imaging and analysis protocol is as follows:

1. NIH3T3cells were transiently co-transfected with Vin-TS and actin- RFP and plated on fibronectin (20 µg/ml) coated glass plates 12-16 hours before imaging.

2. The cells were then imaged live 37 degrees before and after drug treatment and the actin – RFP images (excitation: 561nm, emission: 570-725nm), VinTS FRET channel images (excitation: 458 nm, emission: 533–587 nm), and VinTS donor channel images (excitation: 458 nm, emission: 462–501 nm) were obtained.

3. Analysis of the raw images was done in three steps:

- FA segmentation

FA detection and segmentation was done using FA analysis-web server (Berginski and Gomez, 2013). As intensity-based thresholding did not give a good segmentation.

- FA masking

The binary image obtained was converted to a mask using Image J. The mask was made from FRET channel image.

- Ratiometric FRET image

The mask was then applied to the FRET channel and donor channel images and the FRET ratios were generated by taking the ratio of background subtracted intensity values in the FRET channel to the Donor channel.

The FRET protocol using Vinculin-TS was standardized using the ROCK (Rho- associated protein kinase) inhibitor (Y-27632). The ROCK inhibitor inhibits myosin II-based cell contractility which relaxes the cell. Hence, FRET efficiency of vinculin-TS was expected to increase due to low forces in Y-27632 treated cells. As Figure 6. **1** B show, Vin-TS FRET ratio increased in NIH3T3 cells in response to ROCK inhibitor. The normalized mean FRET ratio peaked at 30 minutes after the drug addition and persisted even after an hour (Figure 6. **1** D). The same was done using the vinculin-TL (tailless) construct and there was no significant change in tension at 30 and 60 minutes after drug treatment (Figure 6. **1** C). This was expected as the tailless construct is tension insensitive as it is unable to bind actin and hence change its conformation under tension (Figure 6. **1** D). In future, the FRET based method will be employed in chick neurons.

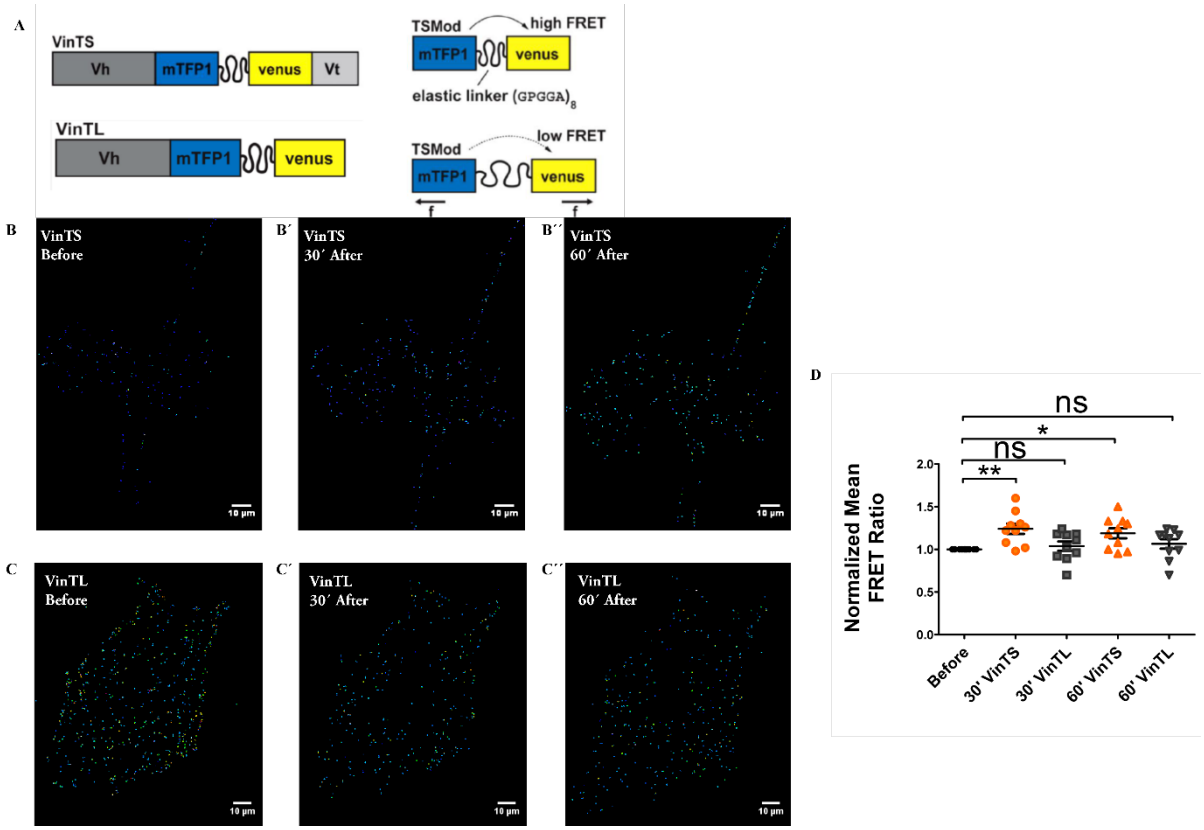

Figure 6. 1. Standardization of FRET using Vinculin Tension Sensor. A. Image from Grashoff *et al.,* 2010 shows vinculin – tension sensor construct with FRET pair mTFP1 and Venus joined by an elastic linker. Vinculin tailless (VinTL) has the FRET pair but is unable to sense as it lacks the tail domain. **B to B´´.** Shows the normalized mean FRET ratio of VinTS before adding the drug and 30 and 60 minutes after. **C to C´´.** shows the same for VinTL construct. **D**. shows quantification of the normalized FRET ratio in these two constructs (VinTS n=10; VinTL n=10). ns P >0.05; **P<0.01; Wilcoxon Signed Rank Test.

Different ECM signalling converges on Fmn2 mediated cytoskeletal regulation in the growth cone

Growth cone motility is also influenced by the ECM (extra-cellular matrix). Due to the engagement of dissimilar integrin receptors and adhesion components, the growth rate of neurons on fibronectin and laminin are quite different (Kuhn *et al.,*1995; Rout, 2013; Gomez *et al.,* 1996). The neurons also show differential sensing and guidance based on neuronal type on these two substrates (Gunderson, 1997; Hynds and Snow,2001). Till now, all the experiments of characterizing the effect of Fmn2 knockdown on growth cone morphology and microtubule organization were done on laminin. Since, we have evidence that Fmn2 also effects adhesions that require integrin-based signalling (Sahasrabudhe *et al.,* 2016; Ketakee Ghate, unpublished data), therefore, we tested on fibronectin. Upon Fmn2 KD, the

growth cone area was reduced on fibronectin (mean area for control was 140.2 ± 14.34 μm^2; for Fmn2 KD was 86.74 ± 9.515 μm^2) (Figure 6. **2** A). Filopodial number and lengths were also reduced (Figure 6. **2** B and C, respectively). The organization of microtubules was affected in a exactly the same manner with bundled form being more frequent with Fmn2 knockdown , indicating instability (Figure 6. **2** D and E). Here too, the filopodial lengths were a function of microtubule presence (Figure 6. **2** F) and a probable reason for shorter filopodia in Fmn2 KD could be a drastic reduction in the proportion of filopodia which were invaded by microtubules (44 ±4% in Control to 26±4% in Fmn2-MO) (Figure 6. **2** G). This data suggests that Fmn2-MT interaction in neurons is conserved across different integrin-based ECM substrates.

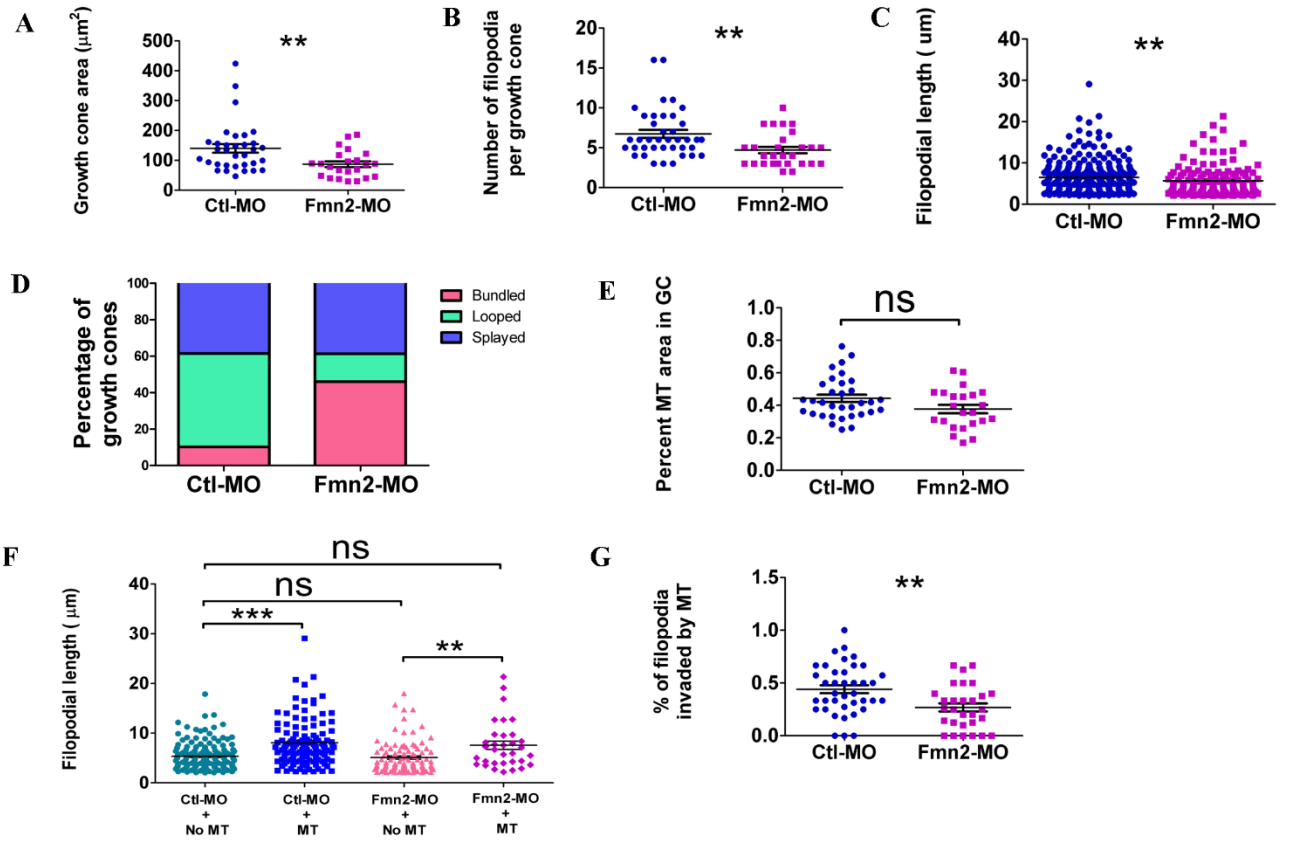

Figure 6. 2. Effects of Fmn2 knockdown on Fibronectin. A. Quantification of growth cone area in Ctl-MO (n=33) and Fmn2-MO (n=23). **B**. number of filopodia per growth cone in Ctl-MO (n=39) and Fmn2-MO (n=28). **C.** Filopodial lengths in Ctl-MO (n=263) and Fmn2-MO (n=132). **D** shows the percentage of bundled looped and splayed organization of MT in the growth cone (Ctl-MO n=39, Fmn2-MO n=26). **E.** Area occupied by MT normalized to total growth cone area Ctl-MO (n=33) and Fmn2-MO (n=23). **F.** Filopodial lengths with MT (Ctl-MO n= 115; Fmn2-MO n=33) and without MT (Ctl-MO n= 148; Fmn2-MO n=98). **G.** Percentage of filopodia invaded by MT in Ctl-MO (n=39) and Fmn2-MO (n=28). ns P >0.05; **P<0.01; ***P<0.001; Mann-whitney test; for F. Krusal-Wallis test followed by Dunn's multiple comparison test.

Axonal branching

Collateral branching is crucial for forming synaptic targets with widely divergent regions in the body. The axon cytoskeleton is packed with stable microtubules running all along its length, with the plus tip at the growth cone. The actin cytoskeleton is sub-membranous and occupies a thin space towards the cortex of the axon. In chapter 4, the branching process in the axon is scrutinized through the lens of Fmn2. We discovered that Fmn2 localizes to the base of protrusions and promotes branching in neurons. Depletion of Fmn2 led to loss of branching and overexpression led to hyper-protrusive activity. We uncovered that the nucleation/elongation activity of Fmn2 is crucial for forming protrusions in the axons. The process of branch formation starts with an actin patch or a trail co-localized by Fmn2. This close physical presence of Fmn2 promotes actin patch stability and maturation but not patch formation. However, how does increased patch stability lead to a protrusion, is still not clear. Therefore, the next immediate goal is to separately investigate the dynamics of patches that give rise to protrusion.

Furthermore, we do not know how does Fmn2 regulate branch maturation. From this perspective, Fmn2 is holds a unique advantage as it binds and bundles both actin and microtubule. Targeted entry and stabilization of dynamic microtubule is required for branch maturation. Preliminary studies of branch maturation show that nucleation/elongation mutant of Fmn2 leads to branching even though the protrusive activity is much reduced. This implies that the few nascent protrusions that are formed in the presence of this mutant have a much higher transition rate to a mature branch than control axons. This also suggests that different domains of Fmn2 regulate different stages of branch formation.

Therefore, studying microtubules dynamics in axonal branching in the absence of FSI domain of Fmn2 is the immediate future goal of this project. Microtubule de-bundling has been shown to be crucial for branch maturation (Bielas *et al.,* 2007; Ketschek *et al.,* 2015). Since Fmn2 bundles microtubule in biochemical assays (Priyanka Dutta, unpublished work), it would be intriguing to explore how Fmn2 influences microtubule dynamics in branch maturation.

Optogenetic control of Fmn2

In conclusion, this thesis dissects the role of Fmn2 in a developing neuron and its functional consequences. Although the functional and biochemical role of Fmn2 have been examined in depth, the upstream regulation of Fmn2 and its interaction with other proteins remains poorly understood. Fmn2 is non-diaphaneous formin which does not have a conserved autoinhibitory domain but capu (Bor *et al.,* 2012b) and mFmn2(Lian *et al.,* 2018) have been shown to undergo autoinhibition.

Although the functional and biochemical role of Fmn2 have been examined in depth, the upstream regulation of Fmn2 and its interaction with other proteins remains poorly understood. Fmn2 is non-diaphaneous formin which does not have a conserved autoinhibitory domain but capu (Bor et al., 2012b) and mFmn2(Lian et al., 2018) have been shown to undergo autoinhibition. To achieve absolute spatio-temporal control of Fmn2, this property of auto-inhibition could be exploited to activate Fmn2 in either a single filipodia in the growth cone or a specific site on the axon. To achieve optogenetic control of Fmn2, two independent methods could be employed to activate Fmn2. As demonstrated in Rao et al., 2014 with mDia1, the C-terminus of Fmn2 will be fused to LOV2-Jα protein. In the dark state, LOV domain will sterically hinder binding of C-terminal Fmn2 to N-terminal Fmn2. But, with exposure to blue light, the Jα helix unwinds to remove steric inhibition. Then the exogenously expressed C-terminus of Fmn2 will competitively bind to N-terminus of Fmn2, leading to its activation. Another way to achieve optogenetic control of Fmn2 is through one of known interactors of Fmn2- Spire 1. The KIND domain of spire is known to recruit Fmn2 to barbed ends and lead to rapid nucleation and elongation of actin filaments (Montaville et al.,2014; Montaville et al., 2016; Quinlan et al., 2007). So, activating the KIND domain of Spire using the LOV2-Jα protein, would lead to recruitment of Fmn2. This method is more advantageous as it would allow spatio-temporal control of endogenous pool of Fmn2.Thus, it would enable asymmetric optogenetic activation of Fmn2 to induce growth cone turning. Moreover, activation at a specific region of the axon could be done to observe if it leads to branch formation at that site, thereby exploring the causal role Fmn2 in these processes.

To achieve absolute spatio-temporal control of Fmn2, this property of auto-inhibition could be exploited to activate Fmn2 in either a single filopodia in the growth cone or a specific site on the axon.

Chapter 7

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