Role of FMN2 in Axonal Branching

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Certificate

This is to certify that this dissertation entitled “Role of FMN2 in Axonal Branching” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study carried out by Sooraj S Das at Indian Institute of Science Education and Research under the supervision of Dr.Aurnab Ghose, Associate Professor, IISER Pune during the academic year 2018-2019.

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Declaration

I hereby declare that the matters embodied in the report entitled “Role of FMN2 in Axonal Branching” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Aurnab Ghose and the same has not been submitted elsewhere for any other degree.

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Abstract

Axonal branching process is an essential mechanism for forming complex neural circuits by facilitating in the formation of multiple synaptic connections with multiple target-fields. Even though the axonal branching mechanism is still poorly understood, several potential candidates might have a role in the axonal branching process, but our attention went to FMN2 protein due to its increased expression level in the nervous system of mammals and few studies implicating FMN2 with intellectual disabilities. FMN2 is actin nucleation and elongation protein from a well-known Protein family called Formins. There are studies confirming FMN2 being an actin bundling and microtubule interacting protein. These diverse functions with the cytoskeletal system found to be useful in an axonal branching process. From this project, actin nucleation and elongation activity of FMN2 was found to be regulating the protrusive activity. The actin patch majorly governs initiation of a protrusion and FMN2 was found to be influencing actin patch dynamics also. Depletion of FMN2 in neurons significantly reduced the lifetime and area of actin patch in the axon shaft and by overexpressing the FMN2 in neurons displayed a distinguishable increase in actin patch lifetime. Still, there is a lot to be understood about axonal branching mechanism from the perspective of FMN2. Hence this project is going ahead in search of that.

Table of Contents

Abstract iv

Chapter 1 Introduction 1

1.1 Axonal Branching 1

1.2 Types of Axonal Branching 2

1.2.1 Arborization 2

1.2.2 Growth Cone Bifurcation 3

1.2.3 Collateral Branching 3

1.3 Cytoskeletal Regulation of Collateral Branching 4

1.4 The Formin family 9

1.5 FMN2 Structure and Neurodevelopment 11

1.6 Objectives 12

Chapter 2 Materials and Method 13

2.1 Materials 13

2.2 Plasmids and Morpholinos 13

2.3 Methods 13

2.3.1 Preparation of 10x PBS 13

2.3.2 Preparation of 4x PHEM 14

2.3.3 Sterilization of 22mm Circular Coverslip 14

2.3.4 Preparation of Culture Plates 14

2.3.5 Keeping Eggs for Incubation 15

2.3.6 Preparation of Electroporation Cuvettes 15

2.3.7 Dissection and Culturing of Chick Spinal Neurons 15

2.3.7.1 Media Prepared 15

2.3.7.2 Procedure 15

2.3.7.3 For Non-Transfected Cultures 16

2.3.7.4 For Transfected Cultures 16

2.3.7.5 Electroporation Parameter 16

2.3.8 Fixing and Staining of Primary Neuron Culture 16

2.3.9 Imaging 17
2.3.10 Quantification parameter and methods 18
2.3.10.1 Protrusion analysis 18
2.3.10.2 Patch Area Analysis 18
2.3.10.3 Patch Dynamics Analysis 18
2.3.11 Statistical Analysis 19
Chapter 3 Results 20
3.1 Reduction in Protrusion Activity in Axon Shaft upon Knockdown of FMN2 20
3.2 Increased Protrusion Activity upon Overexpression of FMN2 23
3.3 Protrusion Activity is Rescued by Morpholino Resistant FMN2 FL 25
3.4 Protrusion Activity is not getting Rescued by Morpholino Resistant FMN2 I2A 26
3.5 Protrusion Activity may be Rescued by Mutant Lacking the Tail Region of FMN2 26
3.6 Neurons have Increased Proportion of Long Protrusions in 48 hr Incubation Period 29
3.7 Observable Decrease in Mature Branches in Neurons Grown in vitro For 48 hrs following FMN2 Knockdown 30
3.8 Recognizing the Actin Structures that Facilitate Collateral Branching 31
3.9 FMN2 Co-Localizes with the Actin Patch/Trail that Facilitates Collateral Branching 33
3.10 Fmn2 Influences Actin Patch and Trail Dynamics 35
Chapter 4 Conclusion and Discussion 39
4.1 Conclusion 39
4.2 FMN2 have a Role in Maintaining the Actin Patch 39
4.3 Influence of FMN2 in Transition Stage of Filopodia Initiation 40
4.4 FMN2 in the Maturation of a Filopodia 40
4.5 A model for FMN2 function in Axonal Branching 41
4.6 Distribution of Protrusion along Axon 42
4.7 Protrusion Length and FMN2 42
4.8 Future experiments 43

Reference

List of Figures

Figure 1.1 Multiple connections with multiple targets 2
Figure 1.2 Types of Axonal Branching 3
Figure 1.3 Stages of Axonal Branching and Downstream Mechanism 5
Figure 1.4 Filopodia Formation and Maturation into a Branch 8
Figure 1.5 Formin Domains and Activity Over Actin 10
Figure 1.6 FMN2 expression in Mammals 12
Figure 3.1 Observable Reduction in Protrusions in Axon in FMN2 Depleted Neurons 21
Figure 3.2 Knockdown of FMN2 Decrease Protrusive Activity 22
Figure 3.3 Observable Increase Protrusion Activity in FMN2 Overexpressed Neuron. 24
Figure 3.4 Overexpression of FMN2 Increase Protrusive Activity 25
Figure 3.5 Observable Recovery in Protrusion Density due to Nucleation and Elongation Activity of FMN2 27
Figure 3.6 Nucleation and Elongation activity of FMN2 Rescue Protrusive Activity In Axon Shaft 28
Figure 3.7 Neurons have Increased Proportion of Long Protrusions in 48 hr Incubation Period 29
Figure 3.8 Decrease in Protrusion Initiation and Maturation Activity in FMN2 Depleted Neurons Cultured in vitro for 48 hrs. 30
Figure 3.9 Types of Actin Structures that Facilitate in Axonal Branching 32
Figure 3.10 FMN2 Colocalize with Actin Patch and Trail 34
Figure 3.11 Lifetime of Actin Patch is Directly Proportional to FMN2 Expression Level 36
Figure 3.12 Depletion of FMN2 Disrupted Actin Patch Dynamics 37
Figure 3.13 No Significant Changes for Actin Trail in gFMN2 Depleted Neurons 38
Figure 4.1 A model for FMN2 function in Axonal Branching 42

Chapter 1
INTRODUCTION

1.1 Axonal Branching

Neurodevelopment is an intricate process, which depends upon the external and internal factors that mold the nervous system. To form complex neural circuits, multiple synaptic connections needed to be established among billions of neurons (Kalil and Dent 2014) (Fig 1.1A). For the establishment of multiple synaptic connections with multiple targets, branches that arise from the axon shaft are essential (Gallo 2011; Armijo-Weingart and Gallo 2017). These branches then form highly branched arbors which make synaptic contact with multiple targets (Kalil and Dent 2014) (Fig 1.1B). This process of forming branches from the axon shaft is known as axonal branching. Location of axonal branching and subsequent guidance is regulated by extracellular cues and neural activity. Extracellular cues provide positive and negative regulation of axon branching by activating specific signaling pathways. Cues that promote axonal branching can be termed as branch promoting factors (eg: NGF and BDNF) and cues that inhibit branching are termed as branch inhibitory factors (eg: EphrinA) (Danzer et al. 2002; Ketschek and Gallo 2010; Yates et al. 2001). Combination of these factors determines the location of branching, guidance to the target, size, and structure of the arbor (Luo and Flanagan 2007; McLaughlin and O'Leary 2005; Kalil and Dent 2014; Livet et al. 2002). Neural activity of a neuron or its adjacent neurons will influence the branch addition, retraction or morphology (Reh and Constantine-Paton 1985; Gosse et al. 2008; Hua et al. 2005). There are several mechanisms that cooperate to maintain axonal branching with high precision and efficiency. Axonal branching is not just limited to the developmental stage. Nerve tissues could be damaged by traumatic injury or degenerative diseases (Onifer et al. 2011; Angelov et al. 2006). During such nerve injury or neurodegeneration, axonal branching facilitates whole or partial functional recovery by re-establishing the network contacts or by rewiring the network. (O'Leary et al. 1990). Although we have a grasp on a basic signaling pathway and a subset of external and internal factors that involved but the understanding regarding the nitty-gritty of the downstream pathways and players remain elusive. There are several questions needed to be asked regarding this. Through this project, I have attempted to expand the

current understanding of axonal branching process with respect to the role of FMN2 in different stages of an axonal branching process.

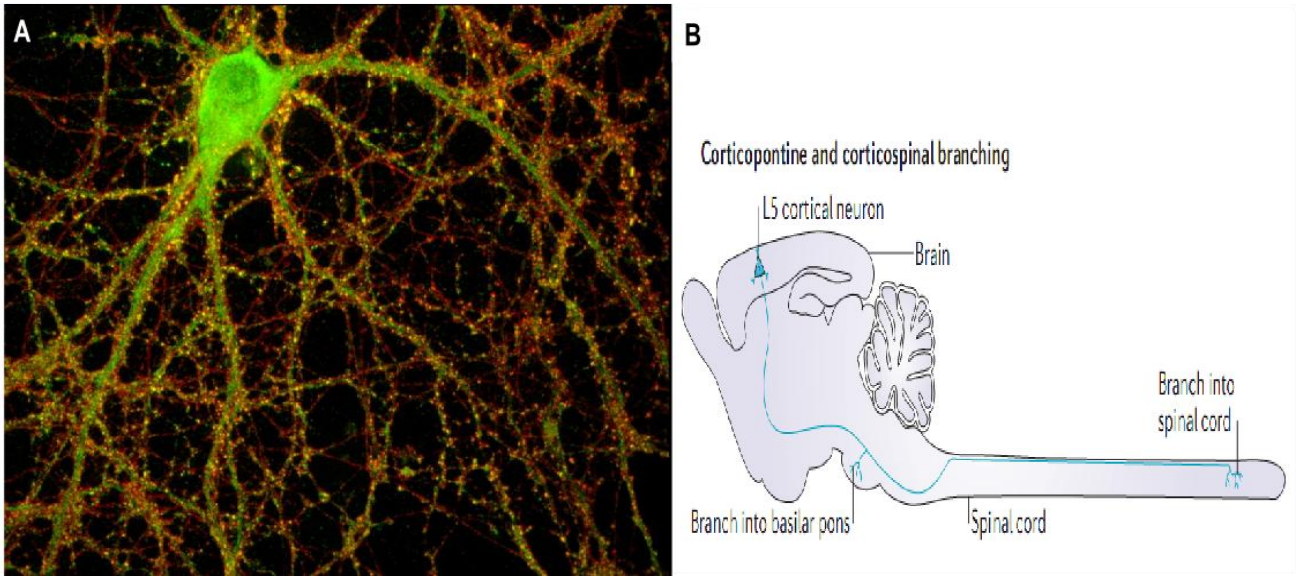

Fig 1.1 **Multiple Connections with Multiple Targets**: (A) Hippocampal neuron with multiple neurites and numerous synaptic connections (yellow). This signifies the magnitude of complexity in a neural network. Credit: Lisa Boulanger, Department of Molecular Biology (B) Cortical neuron forming branches at basilar pons and spinal cord. This is a simple example showing us that neurons tend to make connections with multiple target fields. (Figure reproduced from Kalil and Dent 2014.)

1.2 Types of Axonal Branching

On the basis of morphology, complexity, and function, an axonal branching process is divided into three types, Arborization, Bifurcation, and Collateral Branching.

1.2.1 Arborization

Arborization is the process of higher order branch formation at the terminal region of axon shaft (Fig 1.2A). Arborization occurs only after the axon has reached the specific target field. This branching process is the resultant of specific target driven signaling by the neurotrophic factors such as NGF, NTF3 etc (O'Leary et al. 1990; Krylova et al. 2002; Lentz et al. 1999). This type of axonal branching is common in sensory neurons in the DRG (Dorsal Root Ganglion) while in the target field (Katow et al. 2017). It is structurally effective in covering the targets field such as peripheral tissue of the skin or spinal motor neurons. This will enable effective sensing of the cues from the target tissue for better neural network input.

1.2.2 Growth Cone Bifurcation

This is the simplest axonal branching process in terms of morphology. Bifurcation develops due to an asymmetric growth cone extension, were the activity of the leading edge of the growth cone is repressed and lateral edges of the growth cone take control of the migration (Sainath and Granato 2013) (Fig 1.2B). Generally, this type of branching does not facilitate arborization in the target field. Instead, it facilitates in development of the basic network structure through axon guidance by ensuring distant multisynaptic connectivity. Branching of DRG sensory neurons while innervating the spinal cord is a good example of growth cone bifurcation-based branching (Schmidt et al. 2007).

1.2.3 Collateral Branching

Collateral branching is initiated by a *de novo* emergence of a filopodium from the axon shaft, in a perpendicular and oblique-angled manner (Fig 1.2C). These filopodia mature into a branch by stabilizing itself and preventing retraction back into the axon shaft. These branches then extend and arborize in the target field. This is the major branching mechanism that facilitates the establishment of a complex neural network. This project is all about shedding light into the mechanism of collateral branching (Gallo 2011).

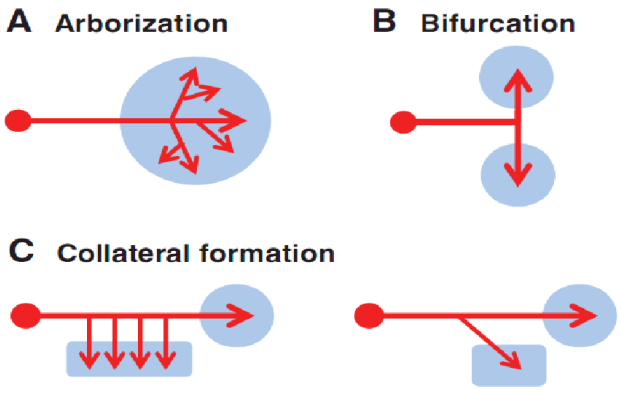

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Fig 1.2: **Types of Axonal Branching**: (A) Arborization make high order branching near the distal end of the axon and it is commonly seen in sensory and motor type neurons. (B) Bifurcation is simple branching through growth-cone splitting forming 'Y' or 'T' shaped daughter branches and this type of branching occurs commonly for axon guidance. (C) Collateral branching is a de-novo initiation of branching from the axon

shaft in a perpendicular or obliquely angled to the axon shaft facing soma. This type of axonal branching facilitates in establishing a complex neural network. (Figure reproduced from Gibson and Ma, 2011.)

1.3 Cytoskeletal Regulation of Collateral Branching

Collateral branching process can be divided into four stages (Armijo-Weingart and Gallo 2017) (Fig 1.3A).

- Actin-patch formation in the axon shaft.
- The emergence of filopodium from the patch.
- Invasion of Microtubule filaments into the filopodium.
- Maturation of filopodium into a branch.

In the initial stage of collateral branching, NGF induces PI3K signaling in the axon shaft (Ketschek and Gallo 2010). This signaling assembles micro domains of PIP_3 in the shaft that initiates the actin patch formation by recruiting proteins like WAVE1, Cortactin etc (Kim et al. 2006). It takes only around the average time of 6 secs for an actin patch formation (DRG neurons from chicken). Swift transport of proteins to the specific location of the axon is not efficient under such time constraint. To achieve such rapid assembly of proteins for the actin patch formation and maintenance, local translation of proteins like WAVE1, ARP2/3 complex, Cortactin etc are crucial. NGF activates local translation pathway through PI3K. PIP3K activates TOR (Target of Rapamycin) which stimulates local protein synthesis in axon shaft (Patel and Hardy 2012; Martelli et al. 2011). Also, PI3K signaling activates Rac1 protein which is well known for the WAVE1 activation (Welch et al. 2003; Chen et al. 2017). WAVE1 is an activator of the ARP2/3 complex (Sweeney et al. 2015). ARP2/3 complex initiates the actin patch formation by nucleating actin filaments as a branch from pre-existing actin filaments (Spillane et al. 2012) (Fig 1.3B). This pre-existing actin filament that facilitates in actin patch formation is known as actin filament seed. Formation of actin filament seed is still not well understood but recent discoveries suggest that Formins and Cordon bleu might have a role in it (Ahuja et al. 2007; Yang and Svitkina 2011). Maintaining an actin patch is a balance between actin polymerization and actin depolymerization

factors (eg: Cofilin) (Hall and Lalli 2010; Spillane et al. 2012). Increase in actin patch lifetime increase the likelihood of filopodium emergence. So to prolong the actin patch lifetime actin filaments should be stabilized. Proteins like Cortactin and Ena/VASP facilitate actin filament stabilization and increase the transition frequency (Rate of filopodia emergence with respect to total actin patch number in a given time) of filopodia emergence (Weaver et al. 2001; Bear and Gertler 2009; Gallo 2011)

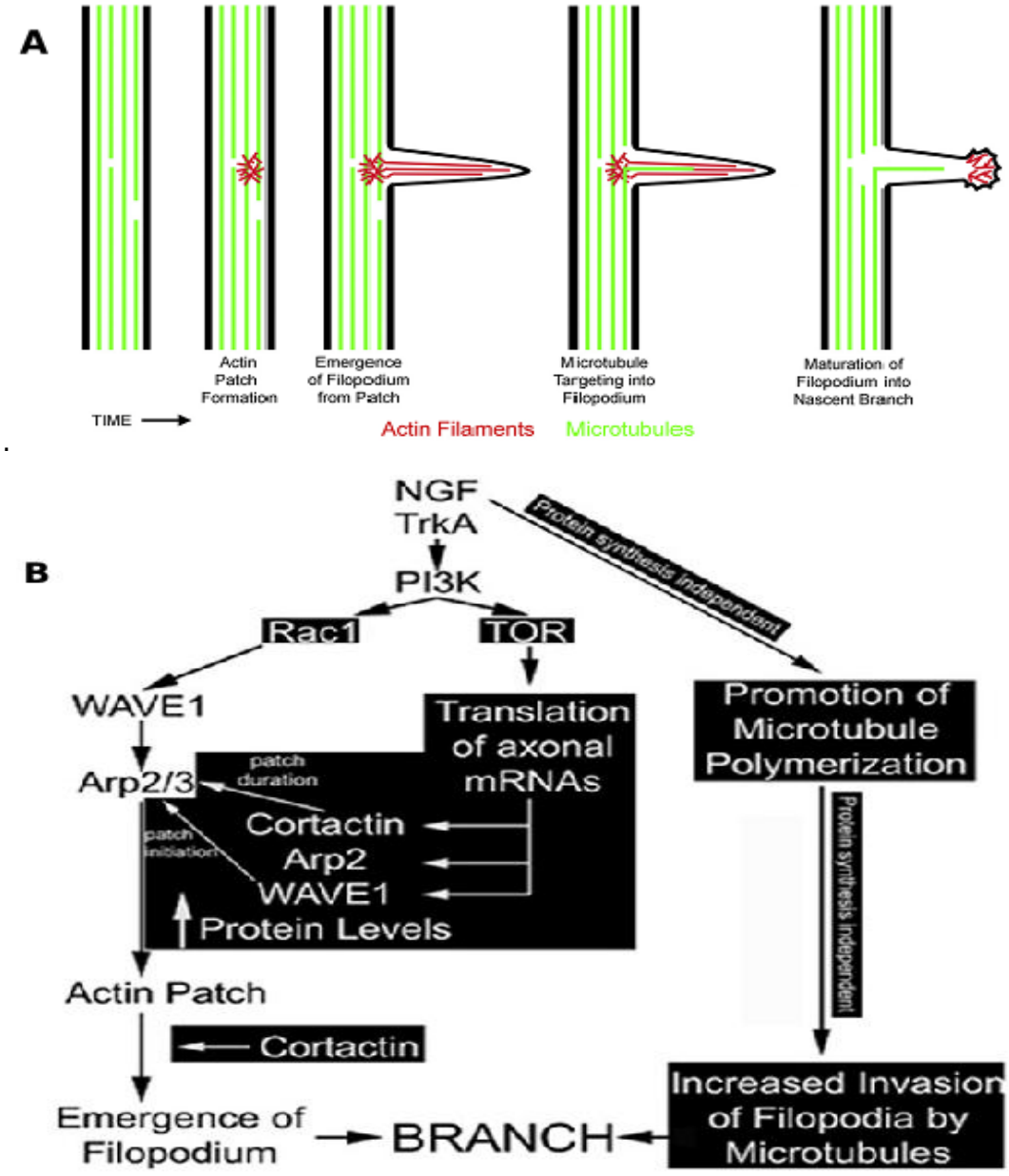


Fig 1.3: Stages **of Axonal Branching and Downstream Mechanism**: (A) This is a simple representation of axonal branching. (Figure reproduced from Armijo-Weingart

and Gallo, 2017.) (B) This is showing the NGF triggered downstream signaling for promoting axonal branching. NGF activates both actin and microtubules for axonal branching and also regulates local translation of regulatory proteins of axonal branching. (Figure reproduced from Spillane et al., 2012.)

A lifetime of an actin patch could be divided into 3 stages (Gallo 2011) (Fig 1.4A):

- Actin Patch Formation
- Elaboration
- Dissipation

Elaboration is the stage in which the size of the actin patch increases rapidly. This happens when the polymerization rate of actin filaments in the patch exceeds much the depolymerization rate. Recent discoveries indicate that cortactin and Ena/VASP protein play a major role in the elaboration of an actin patch. Cortactin stabilizes the actin filaments. Ena/VASP binds to the barbed end of the actin filament and enhances the actin elongation rate. These attributes to the elaboration of actin patch (Weaver et al. 2001; Bear and Gertler 2009; Gallo 2011).

Most of the actin patches display elaboration but not all of these patches facilitate the emergence of a filopodium. Only 20% of actin patches facilitate the emergence of filopodia (Ketschek and Gallo 2010). We still don't understand the exact trigger for a patch to form a filopodium. However recent discoveries indicate that proteins like Debrin, Ena/VASP, and Formins are essential in maintaining the transition frequency (Ketschek et al. 2016; Lebrand et al. 2004; Mellor 2010; Arasada et al. 2005). To explain the discrepancy in the emergence of filopodia in comparison to actin patch number a model was proposed. This is known as **convergent elongation model** (Yang and Svitkina 2011) (Fig 1.4B). Here the hypothesis is that the actin patch itself cannot make a protrusion on the cell membrane. This is because the actin patch is a meshwork of actin filaments which cannot provide a focused force on to the cell membrane. To deliver such focused force, the actin filaments need to be bundled. The bundled actin filaments then provide a force on to the cell membrane as they elongate. Here the proteins like Ena/VASP and Formins facilitate in elongation of parallel actin filaments that converge from scattered actin filament network established by ARP2/3 nucleation. There is a complex combination of actin elongation factor and capping protein to form such a convergent pack of actin filament to a specific site. These parallel elongating

filaments get bundled by proteins such as Debrin and Fascin. As mentioned earlier these bundled actin filaments provide necessary force for the formation of a protrusion. After the elaboration of the patch and subsequent formation of filopodium, the patch dissipates leaving behind the pointed ends of bundled actin filaments present in the filopodium.

Most of the filopodia that arise from actin patch don't mature into a branch. For a filopodium to mature, initially the filopodium should be permeated by Microtubules. Microtubule in an axon is arranged in bundles of parallel filaments and these filaments are tightly packed. For Microtubule invasion into a filopodium, these bundled microtubules undergo debundling and fragmentations near the location of the filopodium. This process of debundling and fragmentation is not very well understood but recent studies have indicated that the downstream signaling initiated by NGF is responsible for the debundling, fragmentation and subsequent increase in microtubule polymerization (Ketschek et al. 2015; Yu et al. 1994,) (Fig 1.6C). These debundled microtubule filaments invade the filopodium. This invasion of microtubule could be guided by some protein but the actin and Microtubule dynamics and interactions while branching is poorly understood (Pacheco and Gallo 2016; Dent and Kalil 2001). There are studies implicating Septin7 and Drebrin in promoting microtubule localization and organization in axonal filopodium (Ketschek et al. 2016; Hu et al. 2012) (Fig 1.6D). Microtubule-Associated Protein (MAP) prevents the severing of the axonal microtubule bundle and Septin 7 displaces the MAP from the microtubule bundle to promote microtubule debundling. Myosin II somehow prevents microtubule invasion into filopodium and Debrin inhibits Myosin II activity. Along with providing structural stability to the branch, microtubules also act as a tract for necessary proteins and organelle (eg: mitochondria) transportation into filopodium. It also facilitates structural support to the filopodium. After microtubule invasion the filopodia will gain in length and girth and mature into a branch. During maturation, there is an increase in energy expenditure for maintaining the actin-Microtubule structure in a branch. To compensate for the energy expense necessary mitochondria, act as local power-station for the branch (Spillane et al. 2013,).

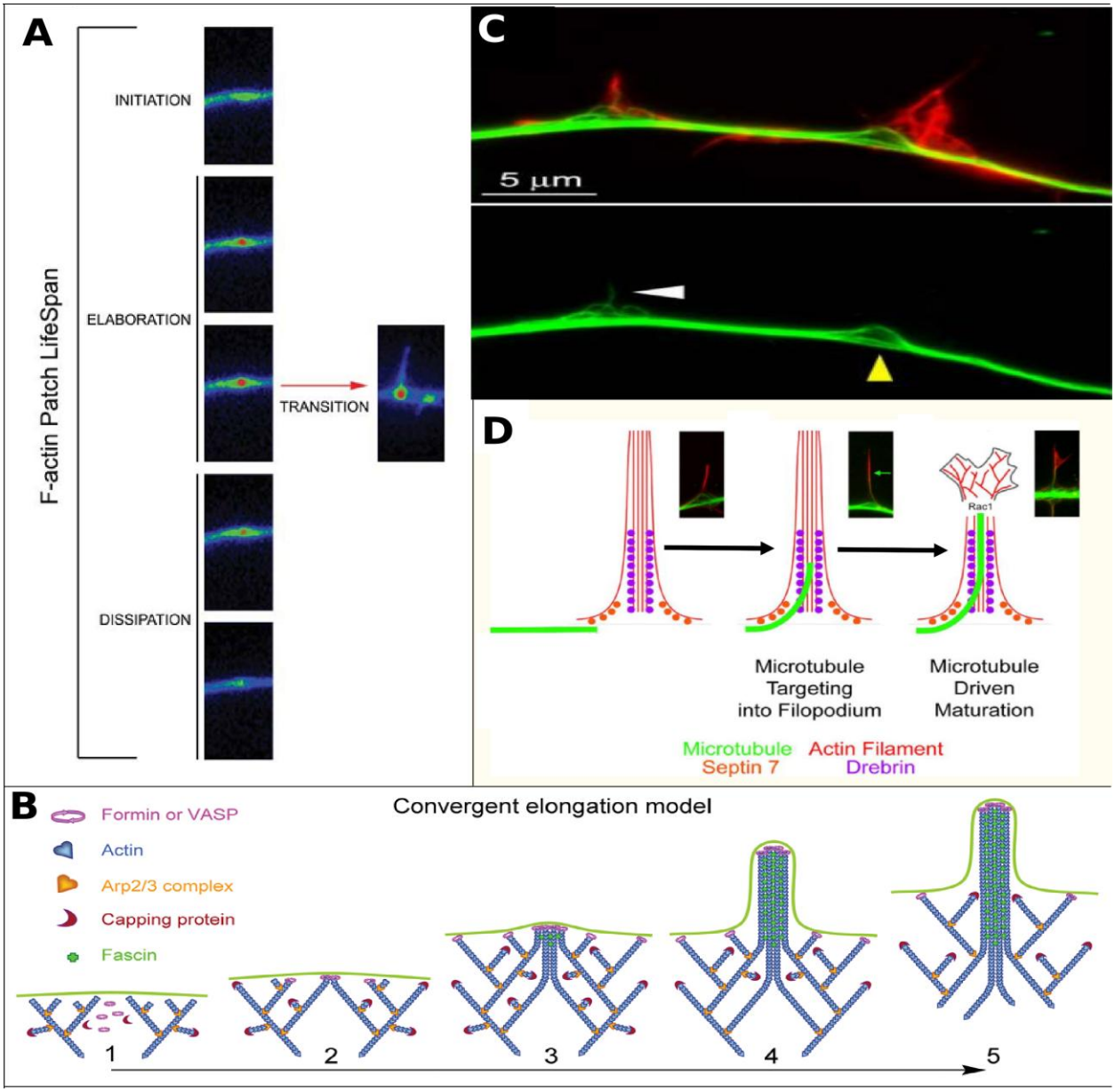


Fig 1.4: **Filopodia Formation and Maturation into a Branch**. (A) This is a representation of an F-actin patch lifespan from live imaging of fluorescently tagged β-actin in axon shaft. This describes 3 stages of an F-actin patch and transition of an F-actin patch during elaboration for the emergence of a filopodium. (Figure reproduced from Gallo 2011.) (B) The basic representation of Convergent Elongation Model. Here it describes the role of VASP or Formins in determining Transition Phase initiation. (Figure reproduced from Yang and Svitkina 2011.) (C) Example of Microtubule (green) debundling near protrusion and invasion into filopodium. (Actin -Red) (Figure reproduced from Ketschek et al. 2015.) (D) Describes the role of Septin and Drebrin the maturation stage of axonal branching. (Figure reproduced from Pacheco and Gallo 2016)

The preceding explanation is a superficial summary of what is known about the basic molecular mechanism underlying axon collateral branching. Which actin patches transition into filopodium? Is convergent elongation model perfect and what are proteins that play a major role in this model? Which are the proteins that involve in microtubule invasion and stabilization of filopodium? What are the defining characteristics of a mature branch? Collateral branching is still a hot topic for research and this project is focusing on one such question. Here we are trying to unravel the role of FMN2 (Formin2) in filopodium initiation and maturation. (FMN2 is a protein from Formin family.)

1.4 The Formin family

Formins are a family of proteins with highly conserved FH domains (FH1, FH2 & FH3). Most of the proteins in this family have FH1 &FH2 domain but their exceptions in this. FH1 domain is absent in ForC and Nir2 have only FH2 like domain (Fig 1.5A). FH2 is the most highly conserved domain in Formins and this is a dimeric domain that involved in altering the dynamics of actin polymerization (Higgs 2005). FH2 domain aids in accelerating *de-novo* actin polymerization (Fig 1.5B) and elongation rate (Fig 1.5C). It also prevents capping protein from capping the barbed end of the actin filament. Formin based actin nucleation and elongation are regulated by Profilin. Profilin is an actin-binding protein which facilitates cross-linking between FH1 and G-actin (Fig 1.5C). Through this FH1 domain boost polymerization rate by increasing the chance of G-actin interaction with FH2. Some proteins in this family have the ability to bundle actin (eg: INF2, FMN2) (Fig 1.5C) and interact with microtubules (eg: mDIA1, FMN2). Involvement of Formins with microtubule is still poorly understood. Since Formins interact with and regulate both actin and microtubule dynamics, directly and indirectly, these proteins are essential for major cellular activities like cell mitosis, endosome mobility etc (Higgs 2005; Wallar and Alberts 2003; Zigmond 2004; Goode and Eck 2007). From these proteins, Formin2 (FMN2) stood apart because of its importance in neurodevelopment (Fig 1.6).

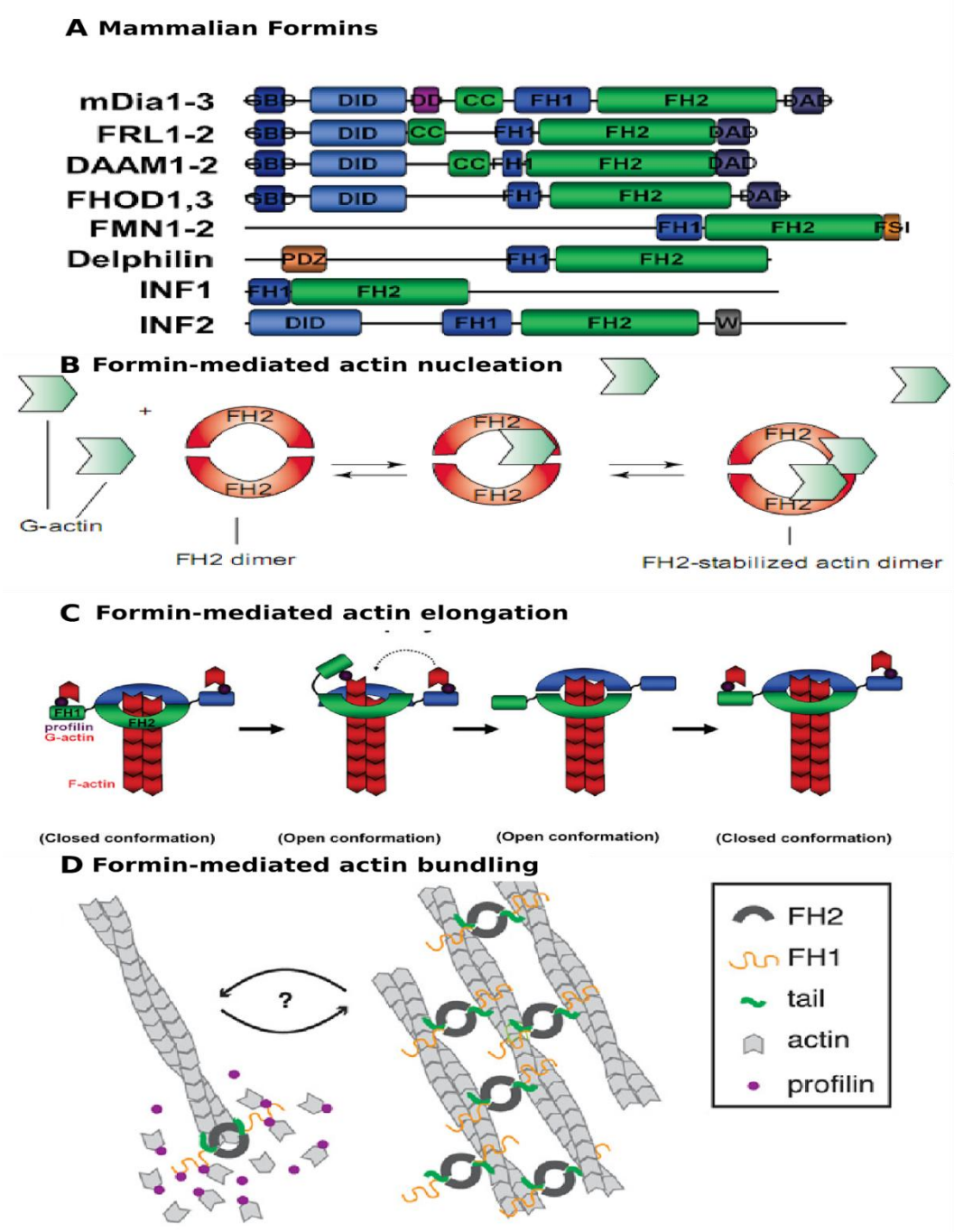
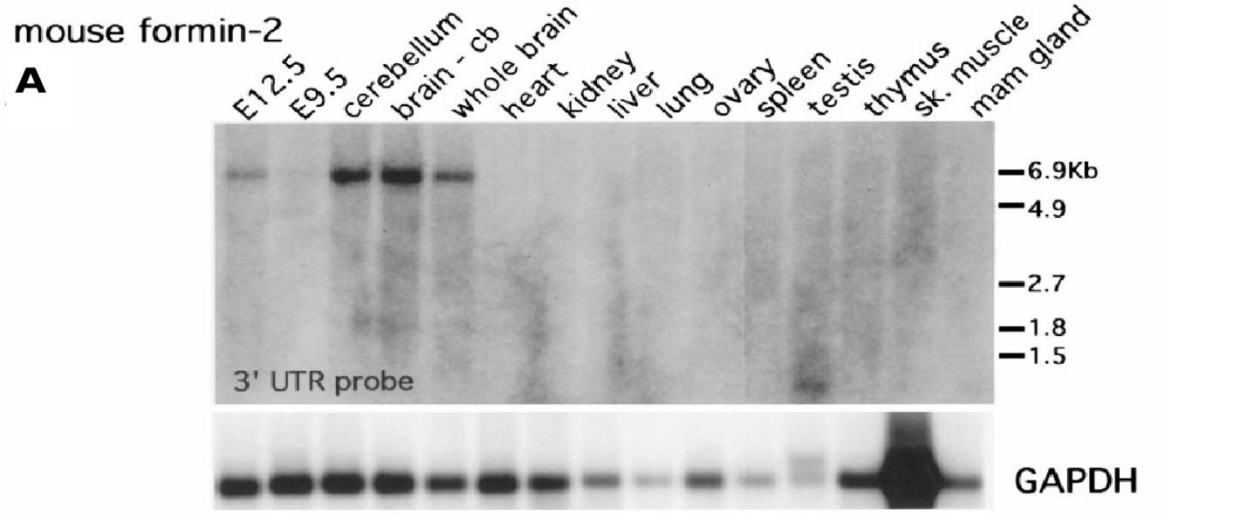


Fig 1.5: **Formin Domains and Activity Over Actin**. (A) Formins have highly conserved FH1 and FH2 domain that regulate actin polymerization. Formin family have other varied regulatory domains. (Figure reproduced from Campellone and Welch 2010) (B) Formins facilitate in forming FH2 stabilized actin dimers from G-actin. (Figure reproduced from Zigmond 2004. (C) Formins promote actin elongation with the aid of profilin. reproduced from Campellone and Welch 2010) (D) C-terminal Tail region of Formin aid in actin bundling. (Figure reproduced from Vizcarra et al. 2014.)

1.5 FMN2 Structure and Neurodevelopment:

In the C-terminal of FMN2 there is conserved FH1 and FH2 domain but in addition to that, there is the FSI (Formin Spire Interaction) domain at the tail end.FSI domain binds to Spire protein and aid in the displacement Spire from the barbed end of the actin filament. Then FH2 get replaced on to the barbed end of actin filament subsequently. This increases actin polymerization rate. Recent discoveries also indicate that FSI domain has a role in actin bundling and microtubule interaction (Montaville et al. 2016; Roth-Johnson et al. 2014, Dr. Priyanka Dutta, unpublished data.). Hence apart from regulating actin nucleation and elongation, FMN2 also bundle actin filaments and interact with microtubules. Compared to the whole body FMN2 have high expression level in both developing and adult CNS (Central Nervous System) of mammals (Fig 1.6). Also, FMN2 is highly expressed in both human and mouse hippocampal neurons (Leader and Leder 2000). Recent studies have implicated that total loss of FMN2 gene function can cause non-syndromic intellectual disability (Law et al. 2014). Also reduction in FMN2 expression level has a link with the rapid increase in age-related memory loss (Agís Balboa et al. 2017). Since FMN2 have high implication with neurodevelopmental disorders I hypothesized that FMN2 might have a role in axonal branching process.



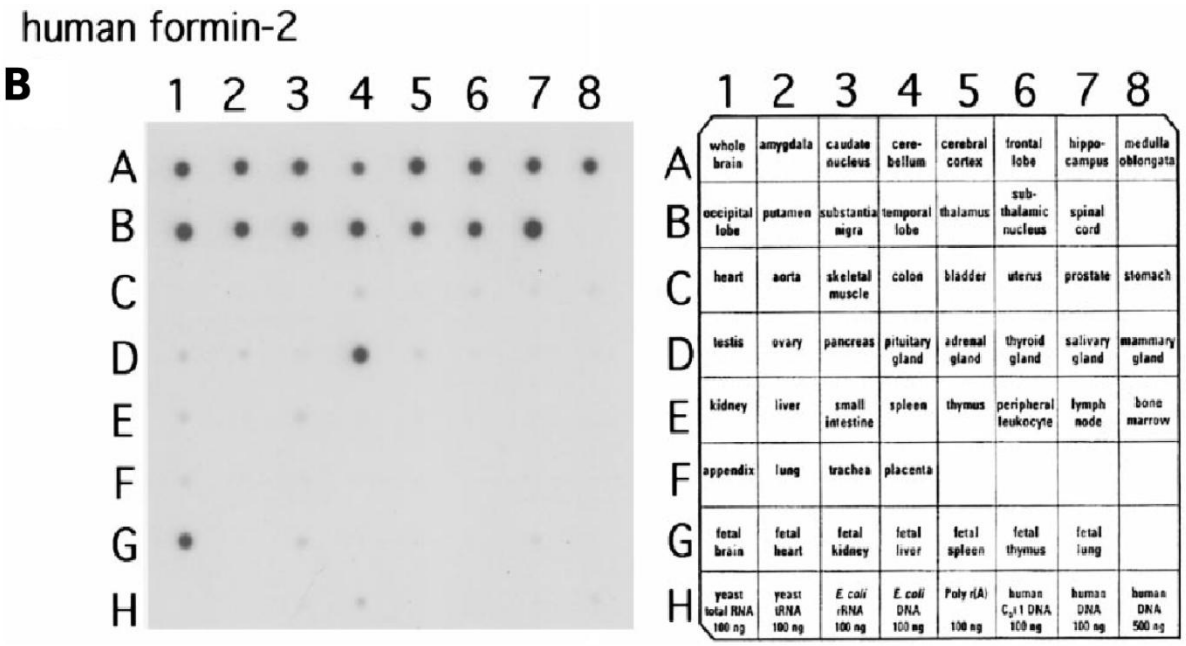


Fig 1.6: **FMN2 expression in Mammals**. (A) Northern blot analysis of FMN2 protein from mouse exhibit unique high expression in neural tissue. (B) Dot blot of FMN2 protein from human tissue show significantly high expression level in all neural tissue (A1-8, B1-7, and D4). Also, tissue from fetal brain (G1) shows similar expression level. (Figures reproduced from Leader and Leder 2000.)

1.6 Objectives:

On the basis of recent studies, I can come to a hypothesis that FMN2 might be involved in multiple stages of an axonal branching process. Our primary objective was to understand whether changing FMN2 expression level in neurons affect the axonal branching in any way. I quantified the phenotypic change in axonal protrusions to understand the effect of FMN2 on axonal branching. As mentioned earlier FMN2 is primarily involved in actin nucleation and elongation. So the FMN2 is highly likely to have a role in actin patch formation or in its maintenance. So the other part of the project is focused on the actin patch dynamics with respect to FMN2 expression level. This project explores the role of FMN2 in filopodial maturation into a branch. Effort has also been devoted to dissecting the role of different domains of FMN2 in different stages of branch formation. Further understanding of the mechanism of axonal branching through this will benefit in neurodevelopment studies.

Chapter 2

Materials and Methods

2.1 Materials

22mm circular coverslips, 35mm culture plates, Silicon sealant (Dow Corning), Poly-L-Lysine solution (10mg/ml), 1x Trypsin EDTA solution, Laminin in Tris-buffered saline(1mg/ml), 10x PBS, L-15, 100x Penstrep, Hi-FBS(Heat Inactivated), Methocel Powder, 0.2um Syringe Filter, 50ml Syringe, 16% Paraformaldehyde (PFA), 25% Glutaraldehyde, 4x PHEM, 1N HCl, Triton-X-100, (98%) Albumin from Bovine Serum lyophilized powder, Alexa Fluro 568 goat anti-mouse IgG (1:1000), Alexa Fluro 633 Phalloidin, 1x Opti-MEM,mouse DM1 anti-α-tubulin antibody,100mM sodium pyruvate.

2.2 Plasmids and Morpholinos

pCAG-GFP (Addgene Plasmid #11150), pCAG-gFMN2-GFP (gFMN2 - Gene ID: 775973), pCAG-gFMN2-mCherry, pCAG-mFMN2-GFP (mFMN2 - Gene ID: 54418), pCAG-mFMN2-I1226A-GFP (1226th codon replaced from Isoleucine to alanine), pCAG-mFMN2-ΔTail-GFP (Deletion of FSI domain from the mFMN2), pCAG-Tractin-GFP (Tractin- addgene #58473), gFMN2 specific morpholino (CACGGTTTTTATTGCCT GCATTGCA), Standard Control Morpholino (CCTCTTACCTCAGTTACAATTTATA).

Note: mFMN2 FL and mutants were used for the rescue experiments. This is because mRNA of mFMN2 doesn't have any matching region with gFMN2 MO.

2.3 Methods:

2.3.1 Preparation of 10X PBS:

80g NaCl, 2g KCl, 14.4g Na2HPO4.2H2O, and 2.4 g KH2PO4 were added to 800ml of distilled water, and topped up to 1 L. The pH of 10 x PBS should be 6.8, so it was adjusted using NaOH and HCl. For the experiments, this stock solution was diluted to 1x PBS concentration with 7.4pH.

2.3.2 Preparation of 4x PHEM:

73.56 g of PIPES (Piperazine-N, N′-bis(2-Ethanesulfonic Acid)) was added to 800 ml of distilled water. The pH of this solution was raised to 7 for proper dilution of PIPES. Then 23.84 g of HEPES (4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid), 15.2 g of EGTA, and 2.94 g MgSO4.7H2O were added to the solution. The total volume of the solution was topped up to 1L with distilled water and a pH of 7 was adjusted using HCl and NaOH. For the experiment, this stock solution of 4x PHEM was diluted to 1X PHEM with a pH of 6.9. The final concentration of PHEM should be 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO4·7H20.

2.3.3 Sterilization of 22mm Circular Coverslip:

The coverslips were separated from one another and were placed into the 500ml beaker. 200 ml of 1N HCl was poured into this beaker and it was heated at 60^{oC} for 6 hrs in a water bath. After this, HCl was removed, and the coverslips were washed with distilled water twice. These coverslips were then stored in 70% ethanol at 4^{oC}.

2.3.4 Preparation of Culture Plates:

20 mm hole was drilled in the 35 mm culture plates, and the edge of the hole was smoothened. It is important that the edge of the hole should be smooth for the coverslip to stick to the plate with a watertight seal. An adequate amount of Silicon glue was added over the edge of the hole, and a coverslip was placed on top of it. These culture plates were then kept for drying for 2-3 hrs in room temperature. Culture plates were washed with 70% ethanol and UV sterilized inside the culturing hood (Laminar Flow Hood) for 1hr. 500 µl of PLL (1 mg/ml) was added in each plate after removing residual ethanol from the plates. Then plates were incubated for 2-3 hrs at 37^{oC}. After the incubation, the plates were washed with distilled water three times. 20µg/ml laminin in 1x PBS was prepared from the stock concentration of 1mg/ml (1x PBS should be filter sterilized using a .2 µm syringe filter). An adequate amount of this laminin solution should be added to these plates so that the glass coverslips were covered by them. These plates were then incubated for least for 12 hrs at 4^{oC} before using.

2.3.5 Keeping Eggs for Incubation

Eggs were stored in the 12^{oC} incubator for inhibiting the embryo development. These eggs were kept in the 37^{oC} incubator 5-6 days before the day of culturing. Before keeping these eggs in the 37^{oC}, it was kept at room temperature for 2-3 hrs. This is to give the egg a transition temperature so that it won't experience a rapid change in temperature and disrupt development.

2.3.6 Preparation of Electroporation Cuvettes

1.5 ml of HCl was added into the cuvette and washed vigorously using the dropper. This was kept in HCl for 5mins and then washed with 70% ethanol. This cuvette was UV sterilized for 1hr inside the culturing hood. It was made sure that this cuvette doesn't contain any ethanol residue.

2.3.7 Dissection and Culturing of Chick Spinal Neurons

2.3.7.1 Media Prepared

(i)BCM (Basic Culture Medium): L-15 (Hi-Media) containing 10% Hi-FBS,1x Penstrep, 1x Sodium Pyruvate ($CH_3COCOONa$).

(ii)BCM with Methocel: L-15 (Hi-media) containing 10% Hi-FBS,1x Penstrep, 1x Sodium Pyruvate ($CH_3COCOONa$) and 0.6% Methocel. (This for live imaging)

(iii)EM (Embryonic Medium) for dissection: L-15 (Hi-Media) containing 1x Penstrep.

2.3.7.2 Procedure

1.5 ml of BCM or BCM-Methocel was added to the plates after removing the laminin solution from the culture plate. BCM, EM, 1x Trypsin, Opti-MEM and culture plates were kept for incubation at 37^{oC} before dissection. 5-6 days old Chicken (*Gallus gallus*) embryo was taken out and dissected in EM. The spinal cord was dissected out from the embryo and collected in a 1.5 ml centrifugation tube containing EM. Then the tube was centrifuged at 3000 rpm for 3 mins and the supernatant was drained out. 500ul of 1x trypsin was added into this tube and gently dislodged the spinal cord tissue using a pipette. The tube was kept for trypsinization at 37^{oC} for 15 mins and then centrifuged it at 3000 rpm for 3 mins. The supernatant was drained out from the tube.

2.3.7.3 For Non-Transfected Cultures

Preheated BCM was added into the dissociated tissue and using a pipette the tissue was gently homogenized. This was plated into the culture plates containing BCM. (Make sure that you add the solution on the glass coverslip and nowhere else.) The culture plates were kept for incubation at 37^{oC}.

2.3.7.4 For Transfected Cultures

After removing the supernatant, opti-MEM and the necessary amount of plasmid or morpholino was added into it. This mixture was poured into the electroporation cuvette using a 1 ml dropper. For transfection, a NEPA21 type II square wave electroporator (Nepagene) was used. After transfection by electroporation, immediately 500 µl of preheated BCM was added into the cuvette. Gently homogenized the tissue using a 1ml dropper and plated them on to the preheated culture plates. The culture plates were kept for incubation at 37^{oC}.

2.3.7.5 Electroporation Parameters

There were 2 stages of electroporation in the procedure. The initial set of pulses with unipolarity were let to cruise through the cuvette to create pores in the cell membrane (Poring pulse). Here two 150 V pulses with a pulse length of 5 msec and interval of 50 msec were let to cruise through the cuvette (decay rate 10%). Then a set of pulses with alternating polarity at each interval were let to cruise through to transfect DNA into the cell (Transfer pulse). Here five 20 V pulses with a pulse length of 50 msec and interval of 50 msec were let to cruise through the cuvette (decay rate 40%).

2.3.8 Fixing and Staining of Primary Neuron Culture:

24 hr or 48 hr post transfection the cultures are fixed using fixative solution (4% PFA, 1X PHEM, 0.25% Glutaraldehyde in distilled water) by replacing BCM solution in the culture. (Make sure that fixative solution is preheated to $37^{oC.}$ Then it was kept at 37^{oC} for 20 mins. After fixing the culture, the plates were washed using 1x PHEM

solution three times. Cells were permeabilized using PHEMT (0.1% Triton-X-100 in 1x PHEM) for 30 mins. Then the plates were washed with 1x PHEM for three times. Cells were blocked with 3% BSA in 1x PHEM for 1 hr. For staining microtubules, primary antibody (anti-α-tubulin antibody) solution (1:3000 in 3% BSA 1xPHEM) was added to the plate and kept in 4^{oC} refrigerator for 16 hrs. After primary antibody staining, the plates were washed with 1x PHEMT for three times. Then plates were stained with secondary antibody (Alexa Fluro 568 anti-mouse) solution (1:1000 in 3% BSA in 1x PHEM) for 1hr and washed the plates with 1x PHEMT for three times. For staining actin structure, phalloidin (633 nm) solution (1:100 in 3% BSA in 1x PHEM) was added to the plates and kept in it for 1hr at room temperature. Then the plates were again washed with 1x PHEMT for two times and after staining plates were stored and imaged in 1x PHEM at 4^{oC}.

2.3.9 Imaging

For fixed cultures, images were captured using 63x/1.4 objective on an LSM 710 confocal system using appropriate laser lines and Z-stack set up. For live imaging, images were captured using Olympus IX81 system (ApoN/TIRF 100x/1.49) equipped with a Hamamatsu ORCA-R2 CCD camera. During imaging, culture plates were kept inside temperature controlled setup maintained at 37^{oC} without CO_2. It also has a focus drift correction mechanism. Each time-lapse images were captured for 5 mins with a 2-sec frame interval. For FMN2 and F-actin colocalization experiment, time-lapse images were captured using 100x/1.4 objective on an ONI (Oxford Nanoimager) with HILO (High Inclination and Laminated Optical sheet) imaging setup. Multi-color imaging was captured simultaneously with an exposure time of 500 ms. This system also has a temperature controlled imaging stage maintained at 37^{oC}.

2.3.10 Quantification parameter and methods

2.3.10.1 Protrusion analysis

Here the protrusions from the axon are counted and the length of these protrusions was measured using ImageJ. A structure rising from the axon shaft showing a length of less than 0.5μm is not considered as a protrusion. Only images containing a full axon were accepted for analysis. Length of the protrusion, protrusion density in the axon and distribution of protrusions along the axon was measured from the data. For normalizing the distribution of protrusion along the axon, axon was divided into 10 equal segments and the protrusion that comes under each segment was count to portray the distribution.

2.3.10.2 Patch Area Analysis

The first frame of a time-lapse image of tractin-GFP over-expressed neurons was used for the patch area quantification. The auto threshold method in ImageJ was used to isolating high-intensity actin blob structures in the axon. There were 16 sets of thresholding algorithms (Huangs, Intermodes, IsoData, etc.) and a suitable algorithm is selected for image depending upon the intensity profile that it gives. Boundaries of the high-intensity actin blobs were manually drawn, and these set of ROI (Region of Interest) were saved. Using this set of ROI, the area of the actin patches was measured. (Credits to Divya S for the analysis)

2.3.10.3 Patch Dynamics Analysis

For calculating speed and lifetime of an actin patch, the time-lapse images were bleach and drift corrected using ImageJ plugins. **Bleachcorreaction** plugin manages intensity decay due to photobleaching using three methods. Here exponential fitting method was used for bleach correction. For correcting XY drift in the time-lapse image, **Stagreg** plugin was used. There are four types of drift correction methods (Scaled rotation, Affine, Translation, and Rigid body). A suitable drift correction method was adapted for each set depending upon which method provide the best drift correction. After preparing the image for analysis, kymographs are produced from the axon region for the whole 5 min using **Kymoclear 2.0** plugin. From these kymographs, regions representing actin patches and trails were marked manually using lines, and these sets

of ROI were used to calculate the speed and lifetime of an actin structure using the **velocity-measurement -tool** plugin.

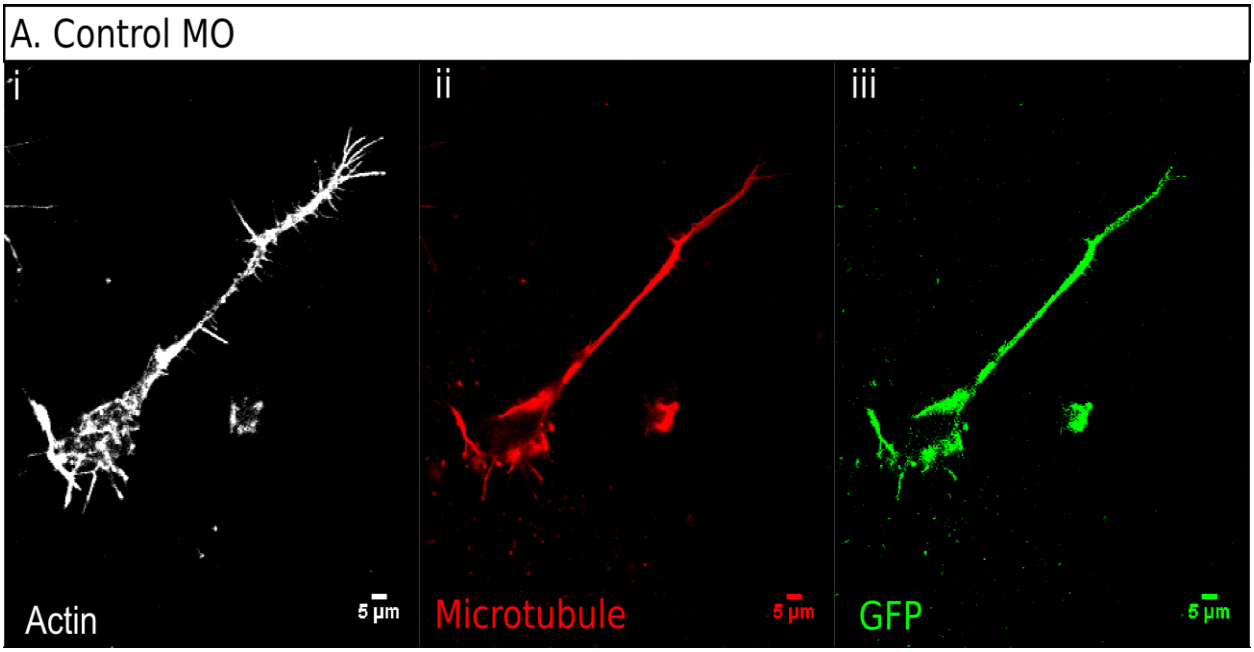
2.3.11 Statistical Analysis:

All statistical comparisons for the column plots were made using the Mann-Whitney test in Prism 5 software. All statistical comparison for the axonal branch distribution plots were made using regular two-way ANOVA test with repeated measuring.

Chapter 3
Results

3.1 Reduction in Protrusion Activity in Axon Shaft upon Knockdown of FMN2

To investigate the role of FMN2 in axonal branching, the FMN2 expression level was depleted in primary cultures of spinal neurons from chick embryo. Translation blocking gFMN2 specific morpholino (Gallus gallus FMN2; gFMN2) was used to knock down the protein expression level. As a control standard nonspecific morpholino was used for these experiments. The pCAG-GFP plasmid was cotransfected with both morpholinos. Neurons expressing pCAG-GFP was considered as an indicator for successful transfection. This was on the basic assumption that neurons exhibiting successful transfection of pCAG-GFP plasmid have a high probability of success in taking in morpholino as well. Using western blotting technique our lab previously quantified FMN2 expression level in FMN2 depleted neurons in comparison to control. Neurons treated with FMN2 specific morpholino exhibited 70% reduction FMN2 expression level and this quantified.



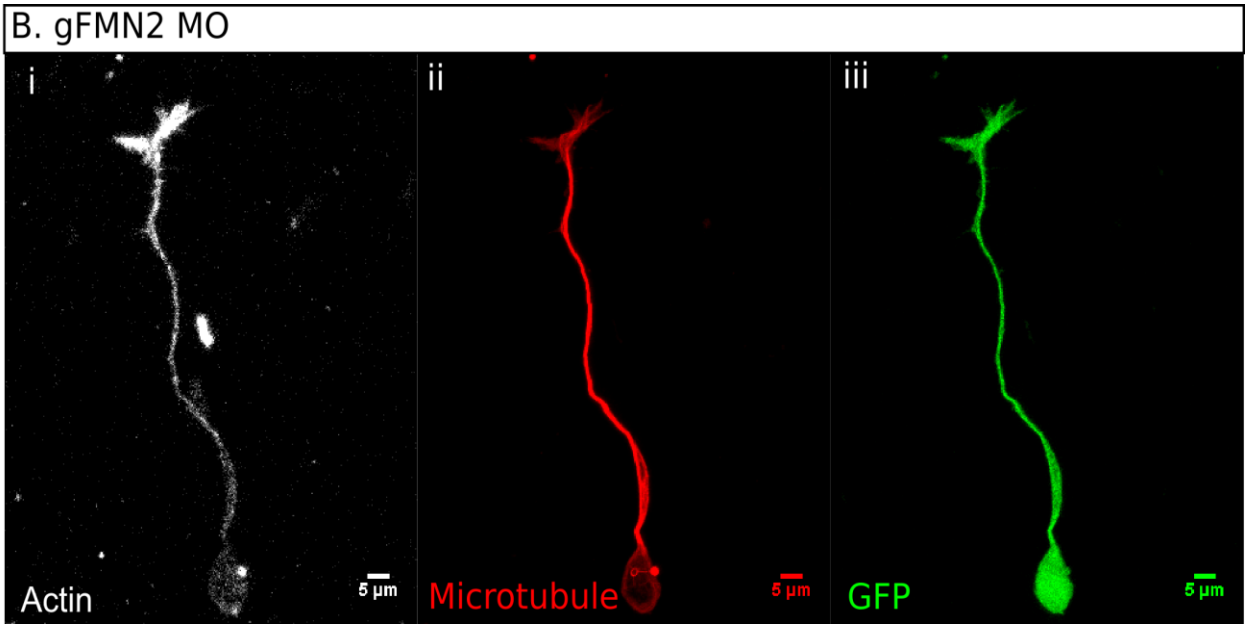


Fig 3.1 **Observable Reduction in Protrusions in Axon in FMN2 Depleted Neurons**. Spinal neuronal tissue was extracted from E5-E6 chick embryo. Neurons were fixed and stained for Microtubule (Red) and Actin (White). Zeiss LSM 710 confocal microscope was used to image using a 63x objective. (A) Neurons were cotransfected 0.1 mM of standard control morpholino and 5 µg of pCAG-GFP Plasmid. (B) Neurons were cotransfected with 0.1 mM of gFMN2 morpholino and 5 µg of pCAG-GFP Plasmid.

There was an observable (Fig 3.1) and quantifiable decrease in protrusion density along the axon shaft in FMN2 depleted neurons in comparison to control (Fig 3.2A). There was around 40% reduction in protrusion density. The length of the protrusions also showed a significant decrease (Fig 3.2B). In control neurons, the axonal protrusions were distributed in a proximo-distal gradient with the lowest density closest to the soma. This trend was disrupted in FMN2 depleted neurons. The distribution of protrusions along the axon was more or less uniform in FMN2 depleted neurons (Fig 3.2C).

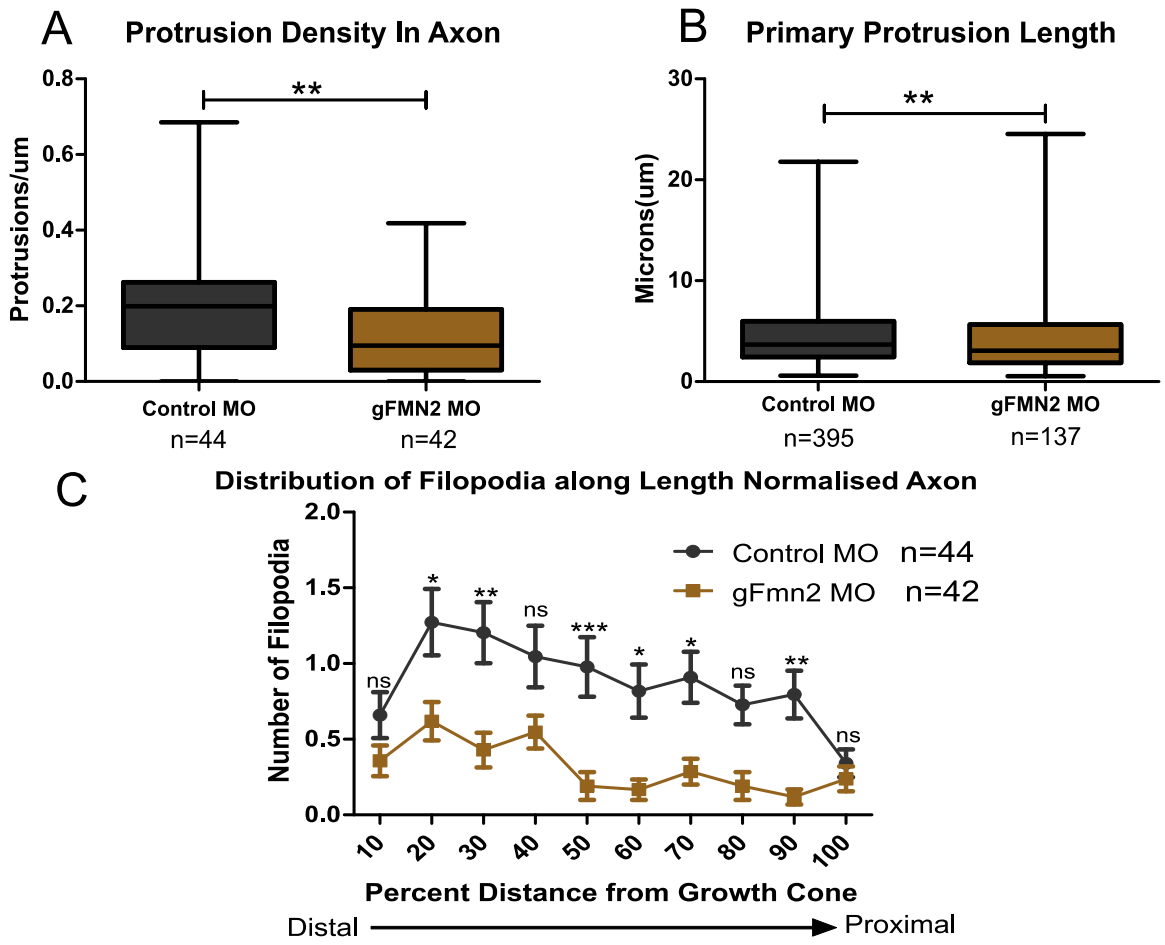
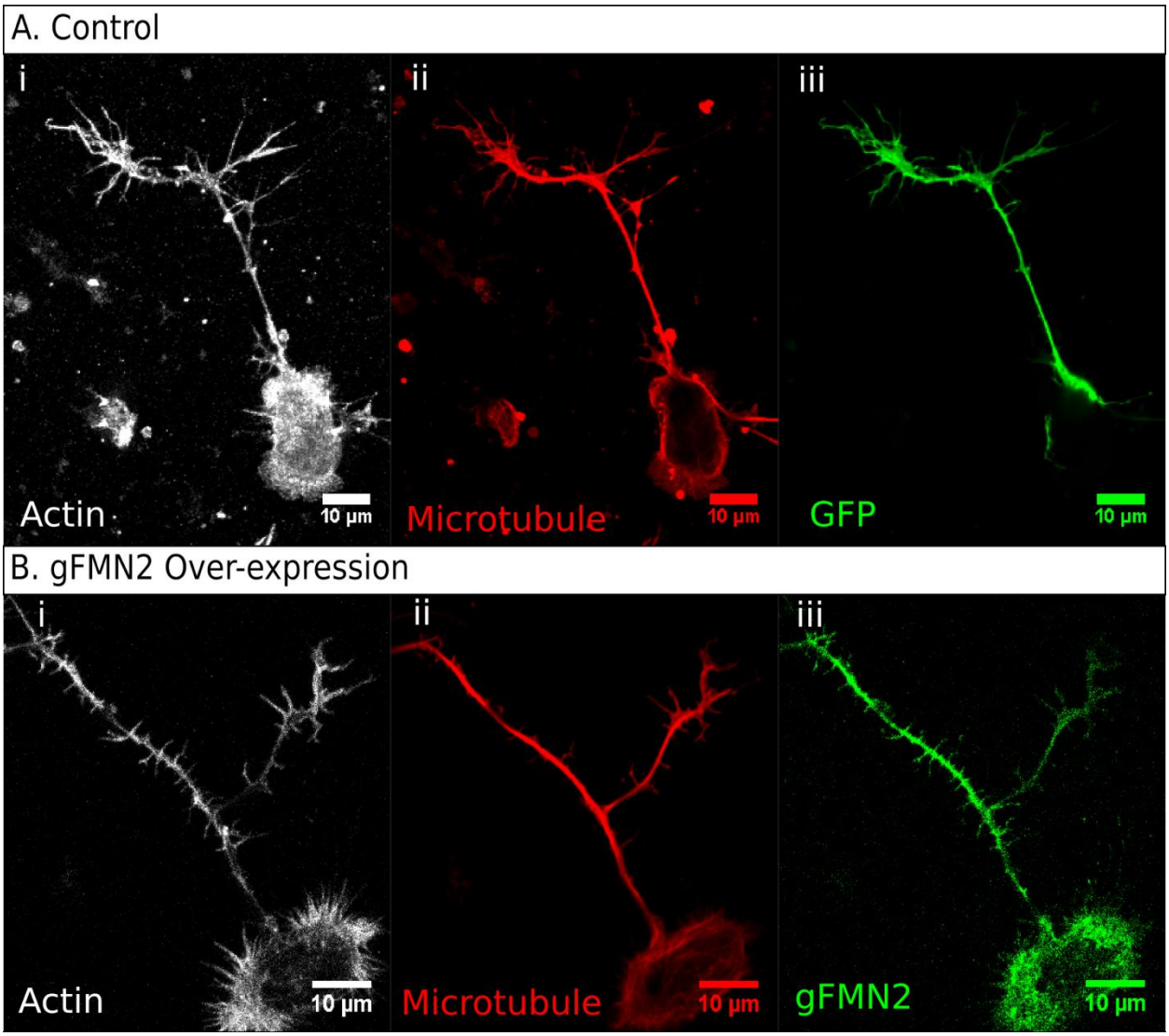


Fig 3.2 **Knockdown of FMN2 Decreases Protrusion Activity** (A) gFMN2 -transfected neurons exhibit a significant decrease in protrusion density **(P-value=0.0032)**. (B) gFMN2 -transfected neurons exhibit a significant decrease in protrusion length **(P-value=0.0096)**. (Mann-Whitney 2-tailed test for A & B) (C) The graph exhibits the distribution of protrusions along the axon and there is a significant decrease in protrusion numbers in most of the section of FMN2 MO axons. (2-Way ANOVA test)(N - biological replicates, n- numerical replicates and total number of axon analyzed control MO = 44, gFMN2 MO = 42)

3.2 Increased Protrusion Activity upon Overexpression of FMN2

Since FMN2 depleted neurons showed decreased protrusive activity in the axon, FMN2 overexpression studies were undertaken. Neurons overexpressing gFMN2-GFP exhibited a significant increase in protrusion density and reduction in primary protrusion length in comparison to control GFP expressing neurons. (Fig 3.3A-B; Fig 3.4A-B). Further, Fmn2 was found to be enriched at the base of protrusions (Fig 3.3C).



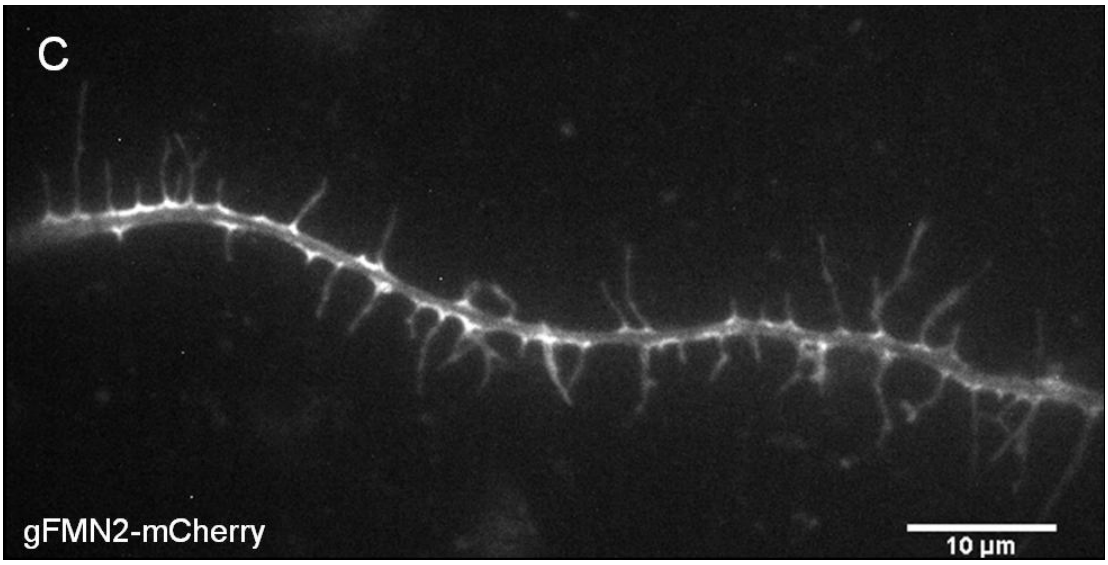


Fig 3.3 **Observable Increase Protrusion Activity in FMN2 Overexpressed Neuron.** The neuronal cultures were fixed and stained for microtubule (red) and actin (white). (A) Neurons were transfected with 5 µg of the pCAG-GFP plasmid. (B) Neurons were transfected with 15-20 µg of the pCAG-gFMN2-GFP plasmid. (C) A representative image showing the localization of the FMN2 in the axon. Zeiss LSM 710 confocal microscope was used to image using a 63x objective.

Distribution of protrusions along the axon in control-GFP transfected neurons showed a similar distribution pattern as in the control MO transfected neurons described earlier. However, the gFMN2 overexpressing neurons had the highest protrusion number in the mid-region of the axon and a subsequent decrease in protrusion number towards both distal and proximal ends of the axon. Most of the sections exhibit increased protrusion number in comparison to control (Fig 3.4C).

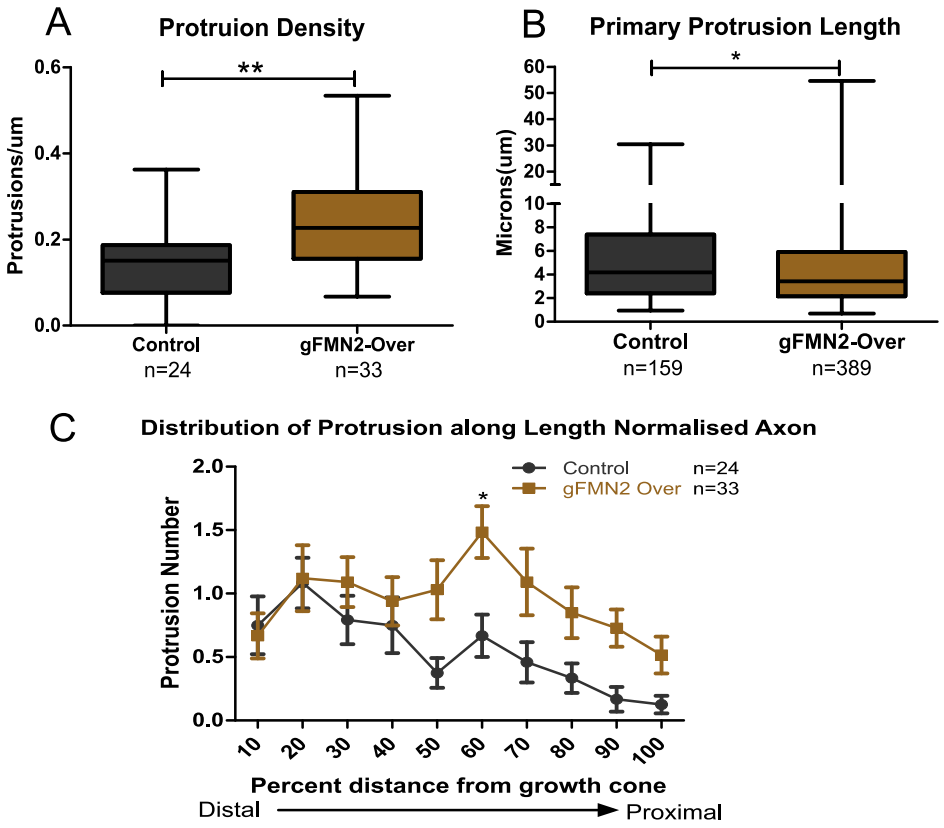


Fig 3.4 **Overexpression of FMN2 Increases Protrusive Activity**. (A) gFMN2 overexpressing (gFMN2-Over) neurons show a significant increase in protrusion density (P-value = 0.0012). (B) gFMN2 overexpressing neurons exhibit significant reduction in protrusion length (P-value = 0.0211). (Mann-Whitney test for A & B) (C) The graph exhibits the distribution of protrusions along the axon and there is a significant boost in protrusion numbers in most of the section of gFMN2-Over axons. Overall distribution trend seen in control MO neurons is shown to be disrupted in FMN2 MO neurons. (Two Way ANOVA test) (N - Biological replicates, n- Numerical replicates, and number of neurons analyzed control = 24, gFMN2 Over = 33.)

3.3 Protrusion Activity is rescued by Morpholino Resistant full length FMN2

Expression of morpholino resistant mouse FMN2 full length (FL Rescue) in neurons depleted of endogenous FMN2 by gFMN2 MO rescued the decrease in protrusion density back to control MO levels (Fig 3.5A and Fig 3.6A). Interestingly the length of the protrusions was not rescued by FL Rescue expression. On the contrary, mean protrusion length was even lower than the gFMN2 MO-transfected neurons (Fig 3.6B). The distribution of protrusions along the axons of FL Rescue neurons showed an

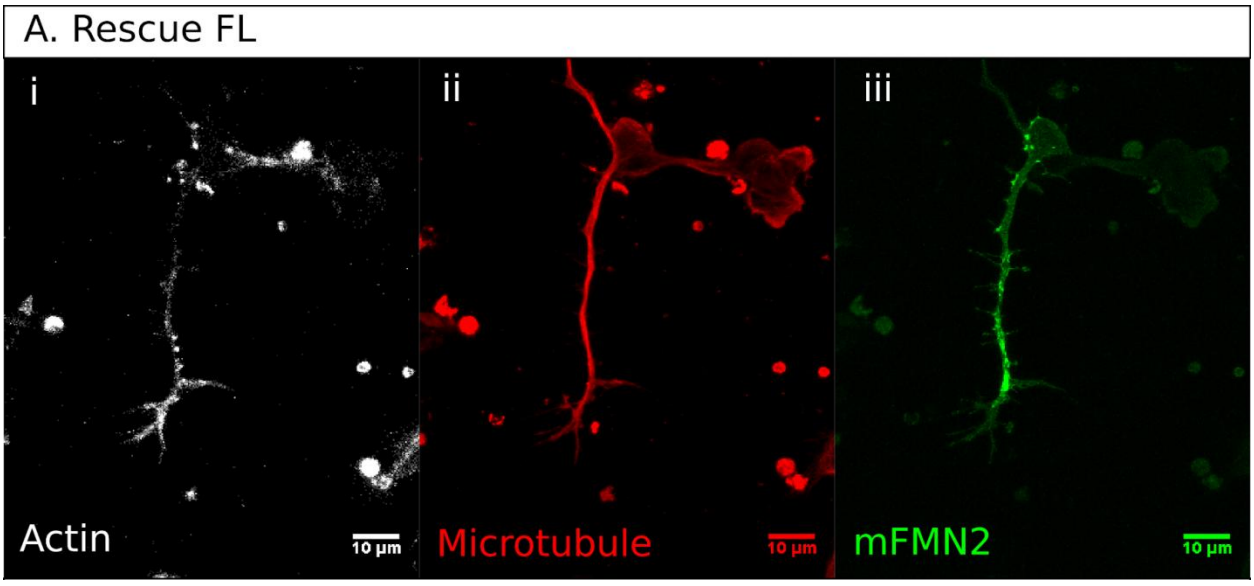
increase in the protrusion numbers across most to the axonal length with an overall there is a uniform distribution (Fig 3.6C).

3.4 Protrusion Activity is not getting Rescued by Morpholino Resistant FMN2 I2A

Mouse FMN2 I2A (I1226A) is a FMN2 mutant lacking actin nucleation and elongation activity but is resistant to the gFMN2 MO. Expression of the I2A mutant (FMN2 I2A Rescue) did not rescue the protrusion density or protrusion length compared to gFMN2 MO -transfected neurons. (Fig 3.5B & Fig 3.6A-B). However, the mean protrusion length of rescue I2A neurons was higher than gFMN2 MO -transfected and rescue FL neurons but lower than Control MO -transfected neurons (Fig 3.6B). Distribution of protrusions along the axon in rescue I2A neuron shows a similar distribution pattern to gFMN2 -transfected neurons (Fig 3.6C).

3.5 Protrusion Activity may be Rescued by Mutant Lacking the Tail Region of FMN2

FMN2 Δ-FSI is a mouse FMN2 mutant lacking the C-terminal FSI domain but is resistant to the gFMN2 MO. As mentioned in the introduction, recent studies have implicated the FSI domain in actin bundling and interaction with microtubules. In rescue Δ-FSI experiments, there was an observable recovery protrusion density in axons compared to the gFMN2 MO (Fig 3.5C). This preliminary observation needs to be quantified with more numbers.



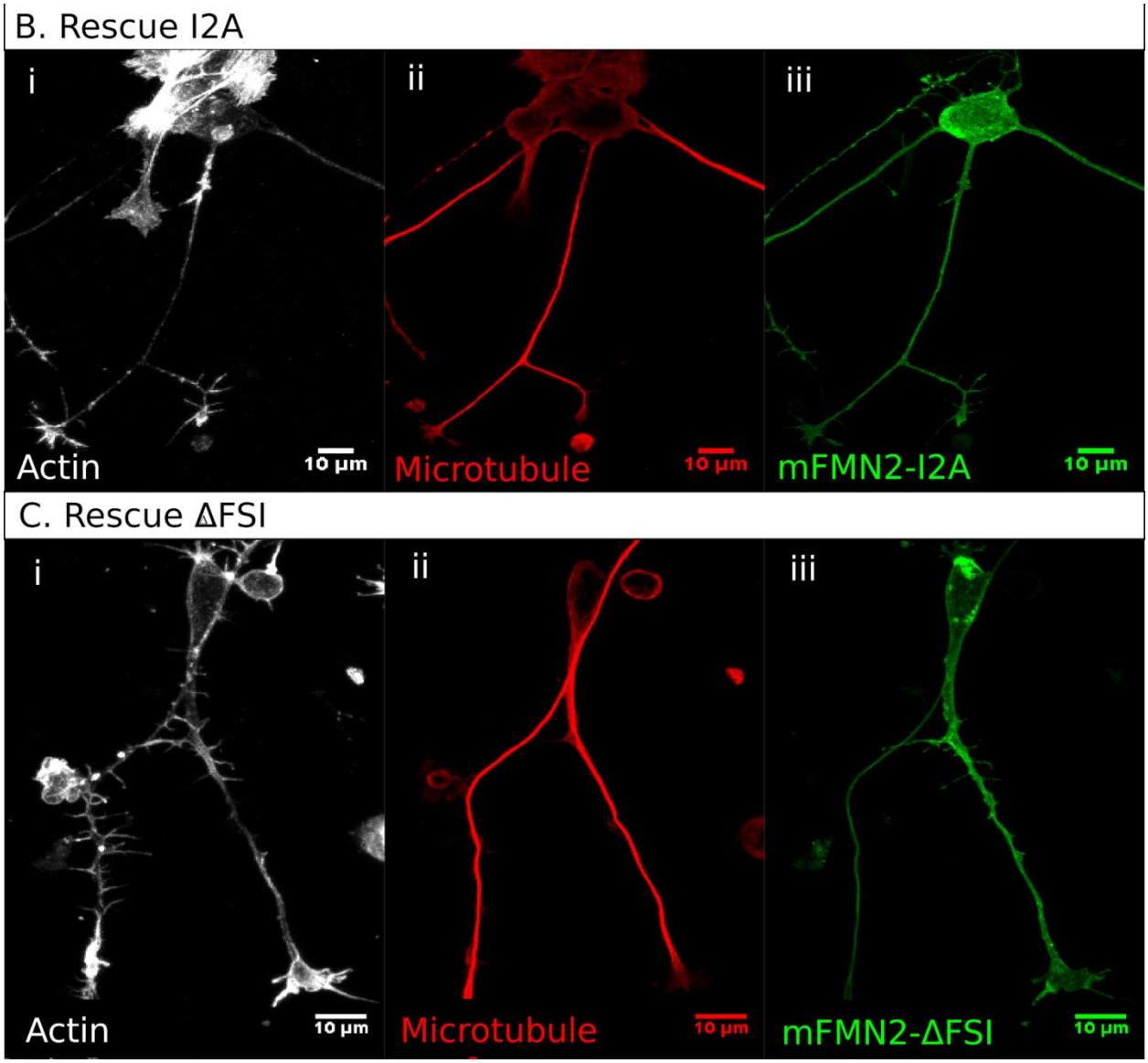


Fig 3.5 **Observable Recovery in Protrusion Density due to Nucleation and Elongation Activity of FMN2:** Spinal neuronal tissue was extracted from E5-E6 chick embryo. The cultured neurons were fixed and stained after 24 hr culturing for Actin (White) and Microtubule (Red). Zeiss LSM 710 confocal microscope was used to image using a 63x objective. (A) Neurons were cotransfected with gFMN2 MO (0.1 mM) and pCAG-mFMN2-FL-GFP plasmid (20 µg-30 µg). (B) Neurons were cotransfected with gFMN2 MO (0.1 mM) and pCAG-mFMN2-I1226A-GFP plasmid (20 µg-30 µg). (C) Neurons were cotransfected with gFMN2 MO (0.1 mM) and pCAG-mFMN2-ΔFSI- GFP plasmid (20 µg-30 µg)**.**

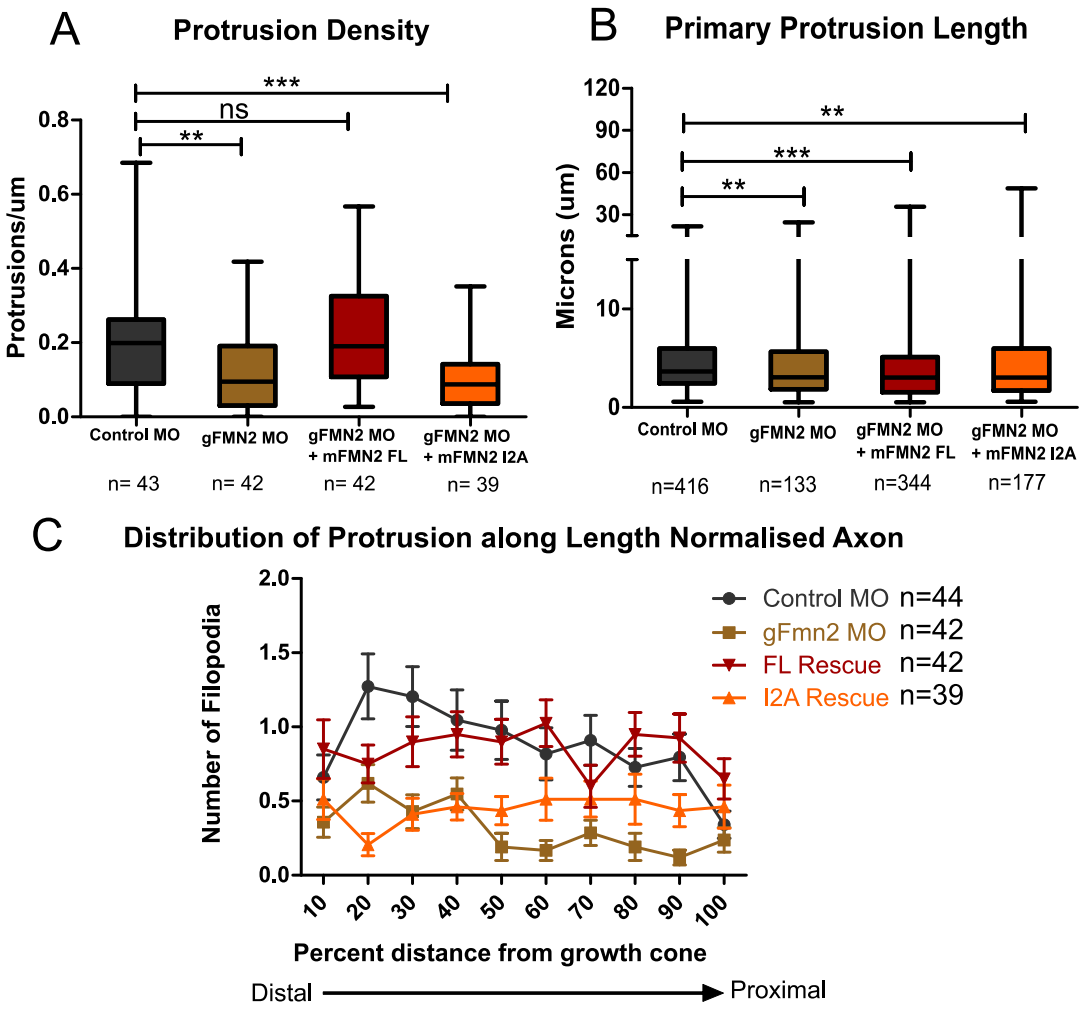


Fig 3.6 **Nucleation and Elongation activity of FMN2 Rescues Protrusive Activity in Axon Shaft** (A) Rescue FL neurons show a significant recovery in protrusion density from gFMN2 MO and have protrusion density levels comparable to control MO **(P-value = 0.0007)**. However, Rescue I2A neurons do not show significant recovery in protrusion density in comparison to gFMN2-MO transfected neurons **(P-value= 0.8572)**. (B) Rescue FL and Rescue I2A show significant decrease in protrusion length in comparison to control MO transfected neurons **($P_{Con\text{-}FL}$-value < 0.0001 and $P_{Con\text{-}I2A}$-value = 0.0035)**. (Mann-Whitney two Tail Test for A & B). (C) Distribution of protrusion along the axon in Rescue FL show recovery in protrusion number from gFMN2 MO. Distribution was almost uniform in nature for Rescue FL. In Rescue I2A there was no significant change in comparison to control MO. (Two-way ANOVA test) (N= biological replicate, n= numerical replicate, number of neurons used Rescue FL - 42 & Rescue I1226A - 39.)

3.6 Neurons have Increased Proportion of Long Protrusions in 48 hr Incubation Period.

In 24 hr incubation period, the protrusions exhibit average length of around 5μm and only 10% of neurons have least 1 protrusion longer than 15μm in length. So, to study maturation stage of axonal branching neuronal culture after 24hr incubation is not suitable. Long protrusions are indicative of stability and maturity and we assumed that proportion of protrusions in a neuron would be longer in 48hr incubation period. To check whether there were drastic increase in longer branches (long and mostly invaded by microtubule) primary cultures were kept for 48 hr incubation. This is a standardization experiment to decide the time point for understanding branch maturation mechanism. 48 hr culture showed an observable increase in long branches than 24 hr culture (Fig 3.7A-B). Here we quantified the total number of neurons observed with one or more long branches (15μm) in its axon relative to the total neurons in each set. Over 50% of all neurons from the 48hr primary neuronal culture have long protrusion (Fig 3.7). This observation indicated that 48 hr culture was the most appropriate time window to study the role of FMN2 in the maturation of an axonal branch.

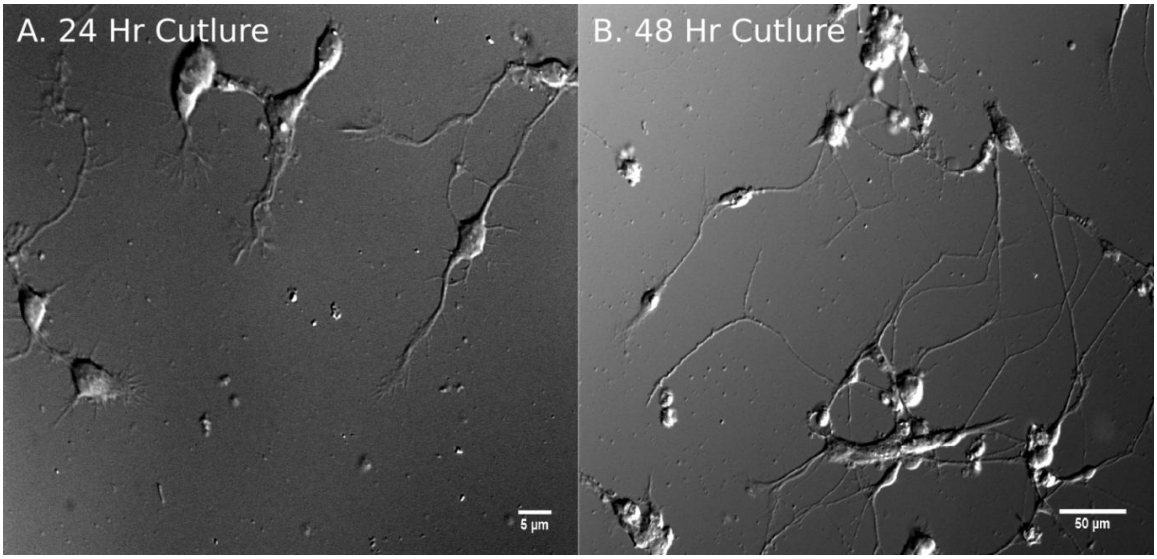


Fig 3.7 **Neurons have Increased Proportion of Long Protrusions in 48 hr Incubation Period.** (A) Olympus IX81 inverted microscope was used to image using a 100x objective (Scale Bar 5 μm). (B) Olympus IX81 inverted microscope was used to image using a 20x objective (Scale Bar 50 μm).

3.7 Observable Decrease in Longer Branches in Neurons Grown in vitro for 48 hrs following FMN2 Knockdown.

Preliminary experiments showed a decrease in the number, length, and degree of branching in FMN2 depleted neurons in comparison to control. It remains to be seen whether this trend is significant over multiple biological replicates. However there was observable reduction in protrusions along the axon of FMN2 depleted neurons. Most of protrusions from the axons of control neurons show microtubule invasion as well. As mentioned earlier, microtubule invasion into the protrusion is an indicator for the maturation of a protrusion.

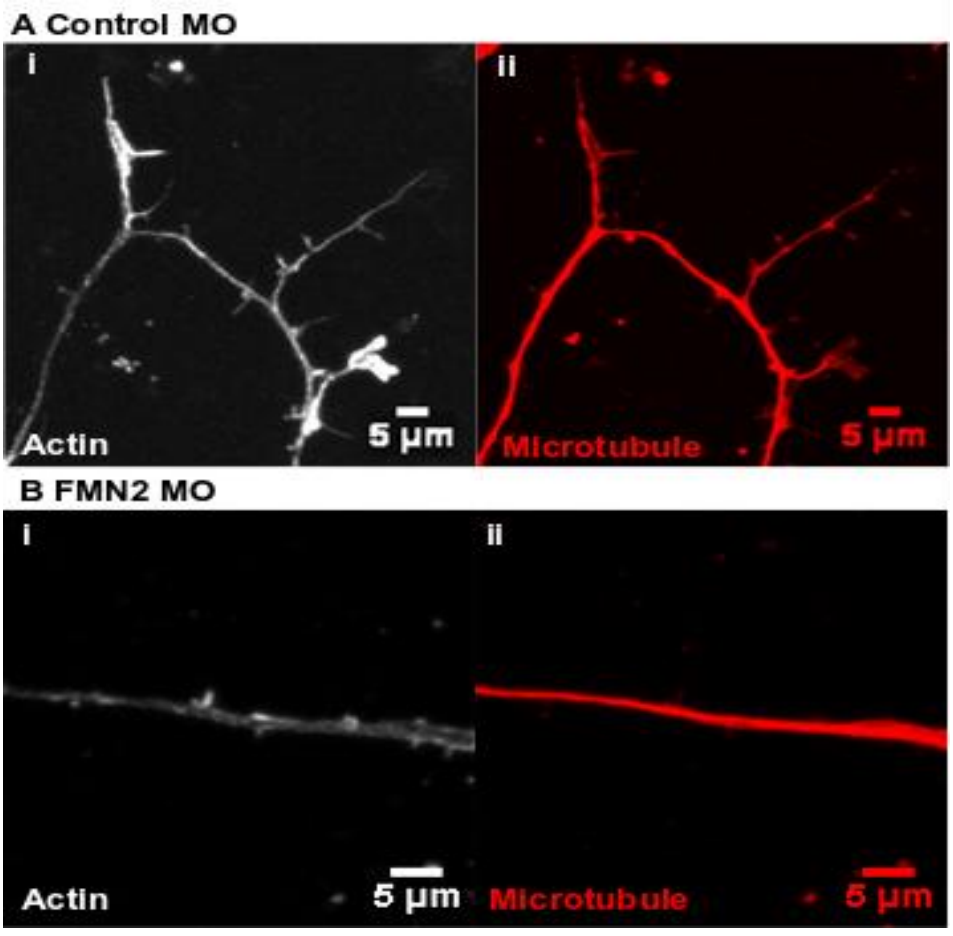


Fig 3.8 **Decrease in Protrusion Initiation and Maturation Activity in FMN2 depleted neurons cultured in vitro for 48 hrs**. Spinal neuronal tissue was extracted from E5-6 chick embryos. These cultures were fixed and stained for Microtubule (Red) and Actin (White). (A) Neurons were cotransfected with control morpholino (0.1mM) and pCAG-GFP Plasmid (5 µg). Zeiss LSM 710 confocal microscope was used to image using a

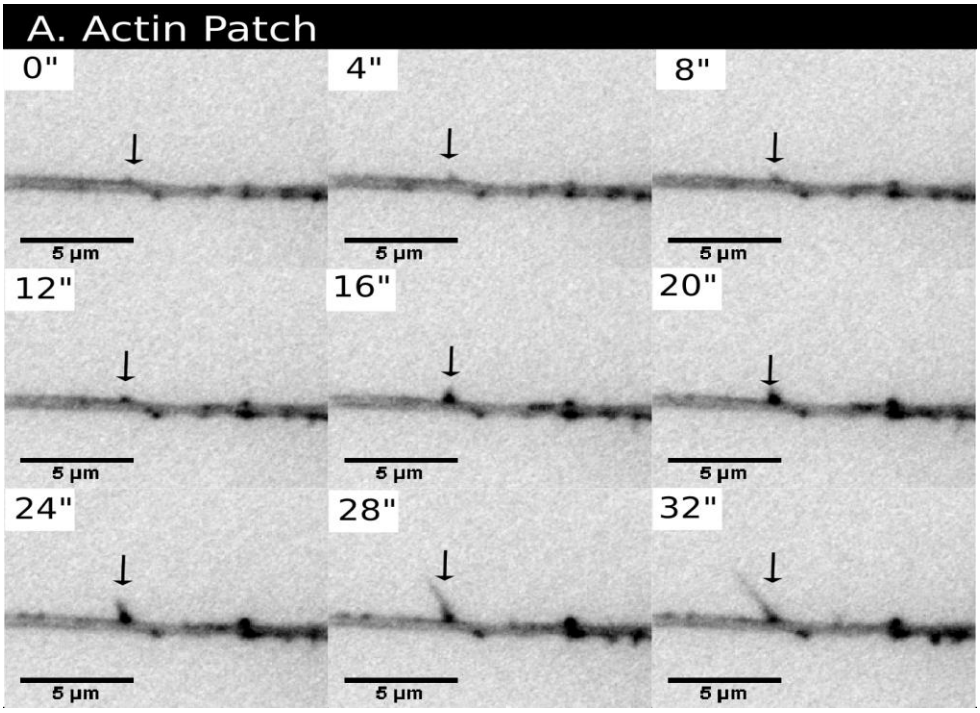
40x objective. (White arrow in A(ii) is pointing out few branches with microtubule innervation) (B) Neurons were cotransfected with gFMN2 morpholino (0.1mM) and pCAG-GFP Plasmid (5 µg). Zeiss LSM 710 confocal microscope was used to image using a 63x objective.

3.8 Recognizing the Actin Structures that Facilitate Collateral Branching

To study the actin structures that lead to collateral branching, pCAG-Tractin-GFP was transfected in neuronal cultures. Tractin is an F-actin binding protein used to visualize F-actin dynamics. Tractin is one of the best actin probes available due to its minimal effect in altering the axonal and dendritic morphology (Patel et al. 2017). Live imaging of neurons revealed two distinct actin structures involved in the initiation of collateral branching.

They are:

- Actin patch, a blob of F-actin (Fig 3.9A).
- Actin trail, a highly mobile, elongated blob of F-actin. Sometimes, it originates from an actin patch. (Fig 3.9B)



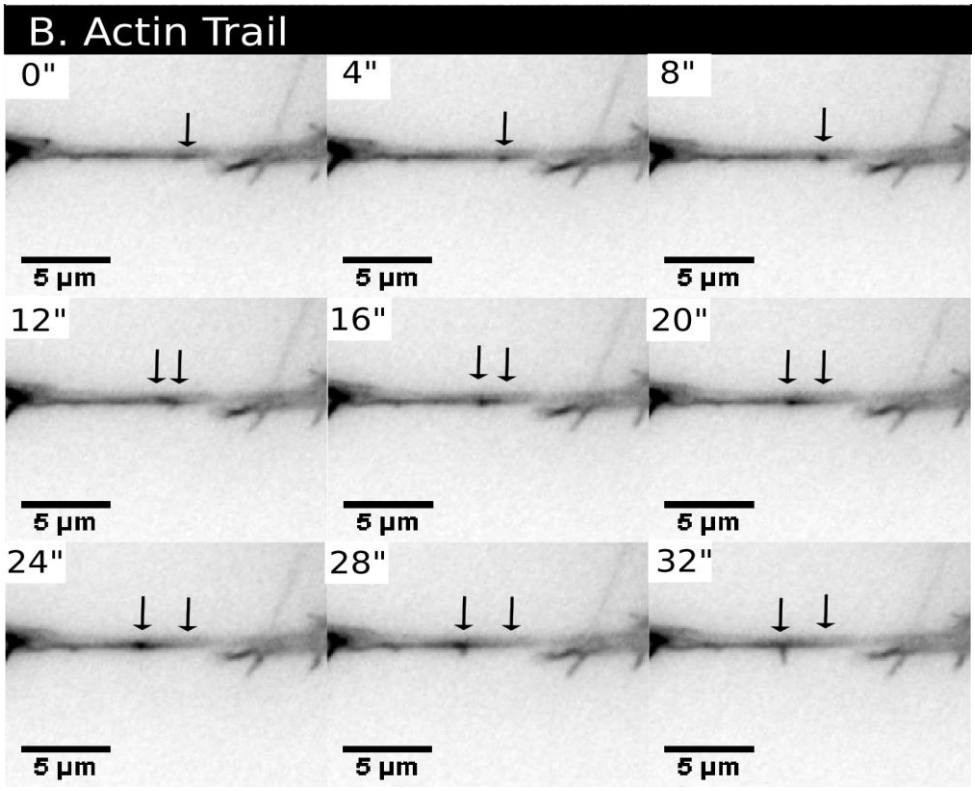
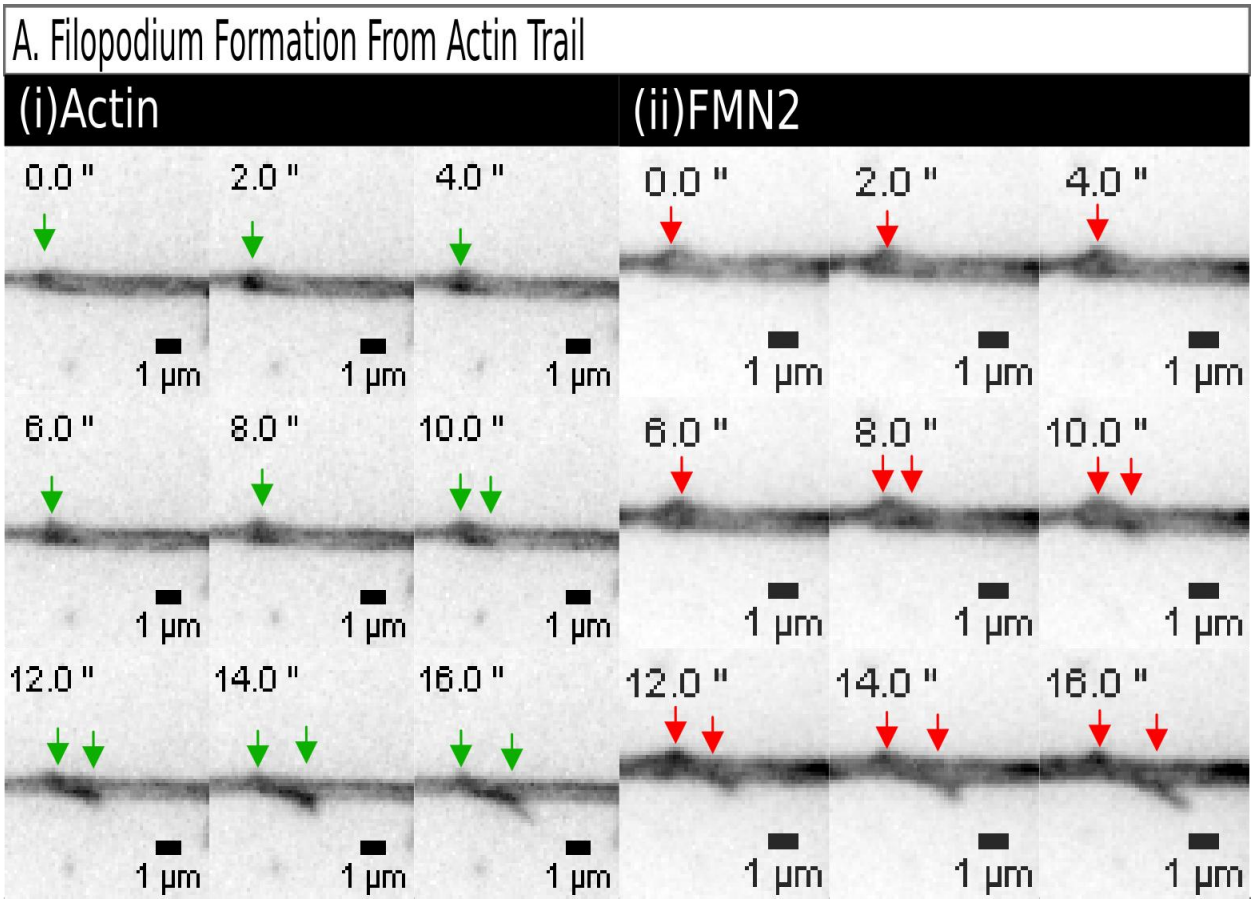


Fig 3.9 **2 Types of Actin Structures that Facilitate in Axonal Branching**. Neuronal cultures were transfected with pCAG-Tractin-GFP (10µg) and imaged after 36 hr incubation. (A) At time 0, the marked region is devoid of actin patch. At 8th sec patch initiated in the axon from a pool of F-actin and started to elaborate in size rapidly after the 12th sec. This patch then gives rise to a filopodium at the 24th sec. (B) At 4th sec an actin patch starts to aggregate. Then at the 12th sec from the actin patch, an actin trail moves out towards the distal side (proximal- left side). At 28th sec the trail halts and initiates the formation of filopodium. Olympus IX81 inverted microscope was used to image, using a 100x objective. Frame interval for the imaging was 2 sec. (proximal- left side)

Since FMN2 actin nucleation and elongation activity regulates protrusion formation in axons, we hypothesized that FMN2 might have a role in regulating actin structures that facilitate in axonal branching process. To investigate this hypothesis, gFMN2-mCherry and Tractin-GFP were cotransfected in spinal neuronal culture. If FMN2 have a direct involvement in formation or regulation in actin patch or trail, then FMN2 would co-localize with these actin structures.

3.9 FMN2 Co-Localizes with the Actin Patch/Trail that Facilitates Collateral Branching

FMN2 colocalization with actin patch and trail was observed in time-lapse images of neurons that were cotransfected with tractin-GFP and gFMN2-mCherry. Whenever an actin patch or an actin trail gives rise to a filopodium, it was observed that the FMN2 protein invaded the protrusion simultaneously with the actin (Fig 3.10A-B). It was inconclusive that among F-actin and FMN2 which localize initially but it is evident that FMN2 colocalize with actin patch and trail. Also not all patches and trail have FMN2 in it.



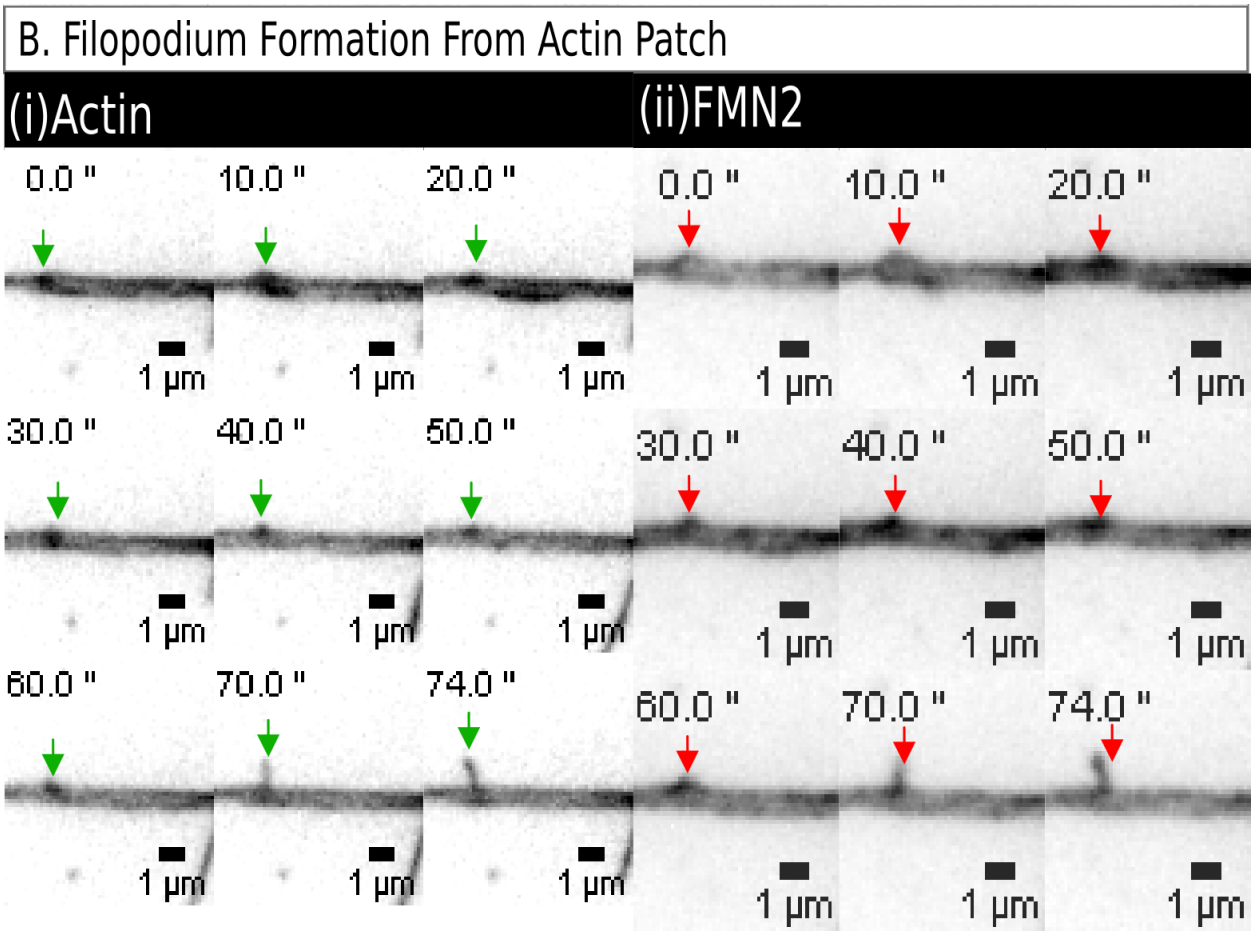
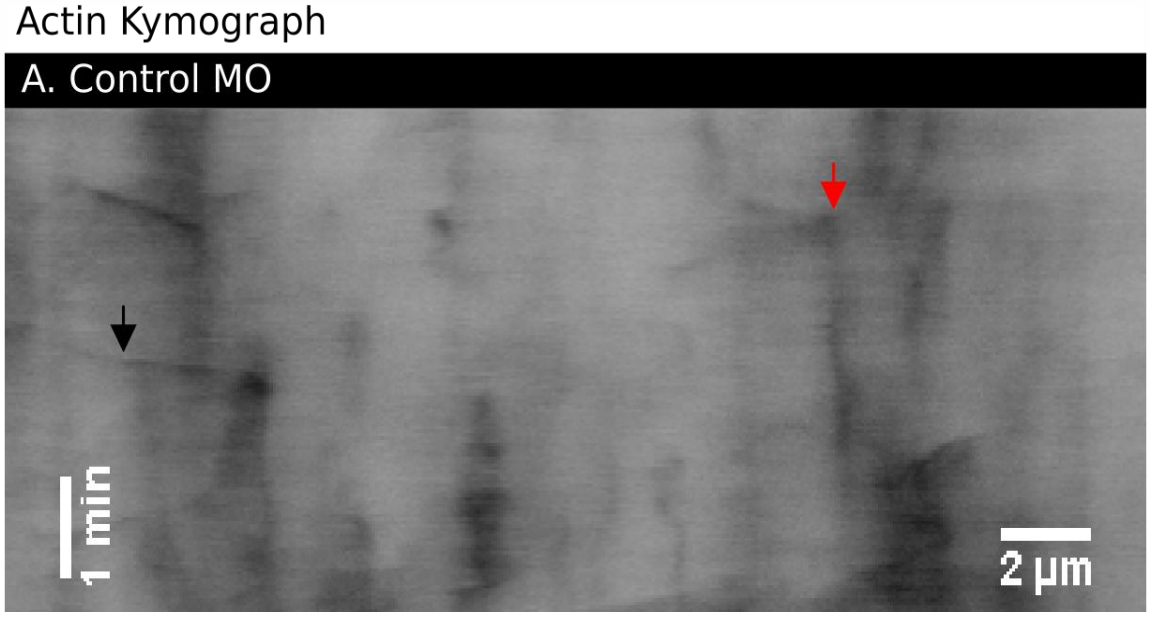


Fig 3.10 **FMN2 Colocalize with Actin Patch and Trail.** For the experiment neurons was transfected with pCAG-Tractin-GFP plasmid (3.5-4 µg) and pCAG-gFMN2-mCherry (20-30 µg). These images were captured after 36 hr incubation, using 100x ONI widefield microscope. F-actin and gFMN2 dynamics were captured simultaneously at 500 ms exposure time. (A) In the initial stage of an actin trail, F-actin aggregate to form an actin blob which then in a later stage a part of it or the whole rapidly move along the axon. In this case, the actin trail resulted in the formation of a filopodium. Here in the initial stage, the FMN2 doesn't seem to have a conclusive localization with actin patch but from 8th sec onwards FMN2 started localizing along the trail and at the site of the protrusion. Then FMN2 invaded the filopodium simultaneously with F-actin. (B) Here the FMN2 increase in colocalization with actin patch in a later time point and invades the filopodium along with the F-actin. (Proximal - left side)

3.10 Fmn2 Influences Actin Patch and Trail Dynamics

From the time-lapse images of Tractin-GFP (F-actin probe) over-expressed neurons, kymographs were made using Kymoclear 2.0. Kymograph is a graphical representation of spatial variations along a time period. (X-axis space and Y - axis time). In these kymographs, long inclined lines represent actin trail and vertical line represent actin patch. Using Velocity Measurement Tool plugin from ImageJ lifetime and velocity of actin patch/trail was measured.

Change in FMN2 expression level in neurons shows changes in actin patch lifespan, size, speed and density in the axon. When FMN2 expression was depleted in neurons using gFMN2 morpholino, actin patch showed an observable decrease in lifespan (Fig 3.11A-B). While FMN2 was over-expressed in neurons, there was distinguishable an increase in patches with a longer lifespan (Fig 3.11C). These distinguishable changes neurons with varying FMN2 expression level were observed using kymographs formed from time-lapse images.



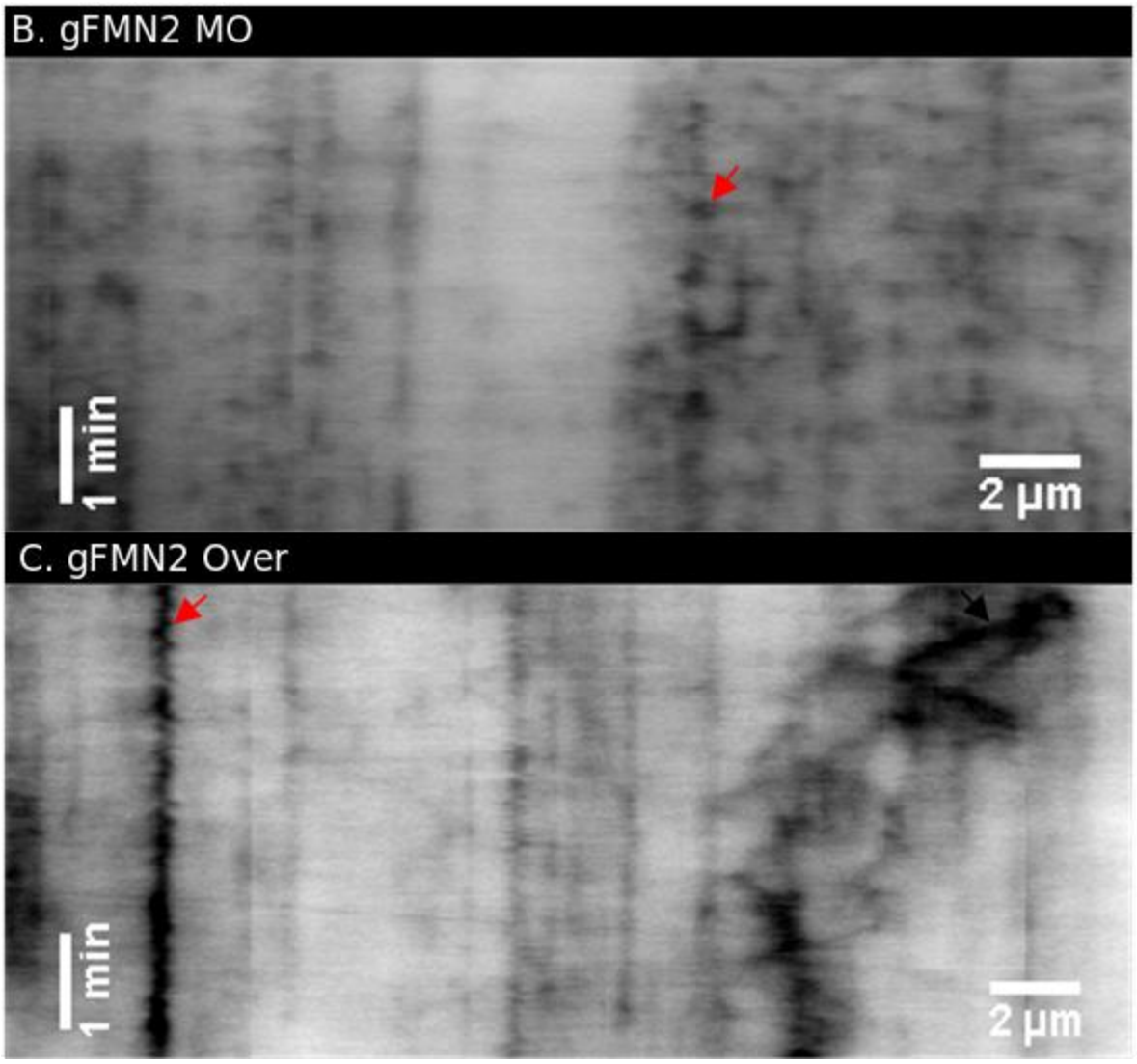


Fig 3.11 **Lifetime of Actin Patch is Directly Proportional to FMN2 Expression Level**. Spinal neuronal cultures were prepared from E5-E6 chick embryos. Time-lapse images were taken after 36 hrs of incubation for all sets. These kymographs were generated using Kymoclear 2.0 plugin in ImageJ. (Distal - left side) (A) Kymograph was generated from the axon shaft of a neuron that was co-transfected with standard non-specific morpholino(0.1mM) and pCAG-Tractin-GFP (3.5µg). An immobile actin patch is seen as a straight line in kymograph (Red arrow) and an actin trail as an inclined line in kymograph (White arrow). (B) Kymograph was generated from the axon shaft of a neuron that was co-transfected with gFMN2 specific morpholino (0.1mM) and pCAG-Tractin-GFP (3.5µg). This kymograph has a high number of spots in comparison to control MO kymograph. (C) Kymograph was generated from the axon shaft of a neuron that was co-transfected with the pCAG-gFMN2-GFP plasmid (20-30µg) and pCAG-

Tractin-GFP (3.5µg). (All kymographs are formed from a 5 min long time-lapse image with 2sec frame interval.)

Kymographs of control MO and gFMN2 MO transfected neurons were quantified and analyzed to evaluate actin patch dynamics. In FMN2 depleted neurons there was a significant decrease in actin patch size and lifetime in comparison to control MO transfected neurons. Interestingly there was a significant increase in patch mobility upon depleting the FMN2. (Fig 3.12C). There is around 30% decrease in actin patch size and lifetime in FMN2 depleted neurons (Fig 3.12A-B). There is a no difference in actin patch density in FMN2 depleted neurons compared to controls, though there is a trend towards increased density upon Fmn2 depletion. However, the number of data points is too low at this point to reach any conclusion. (Fig 3.12D)

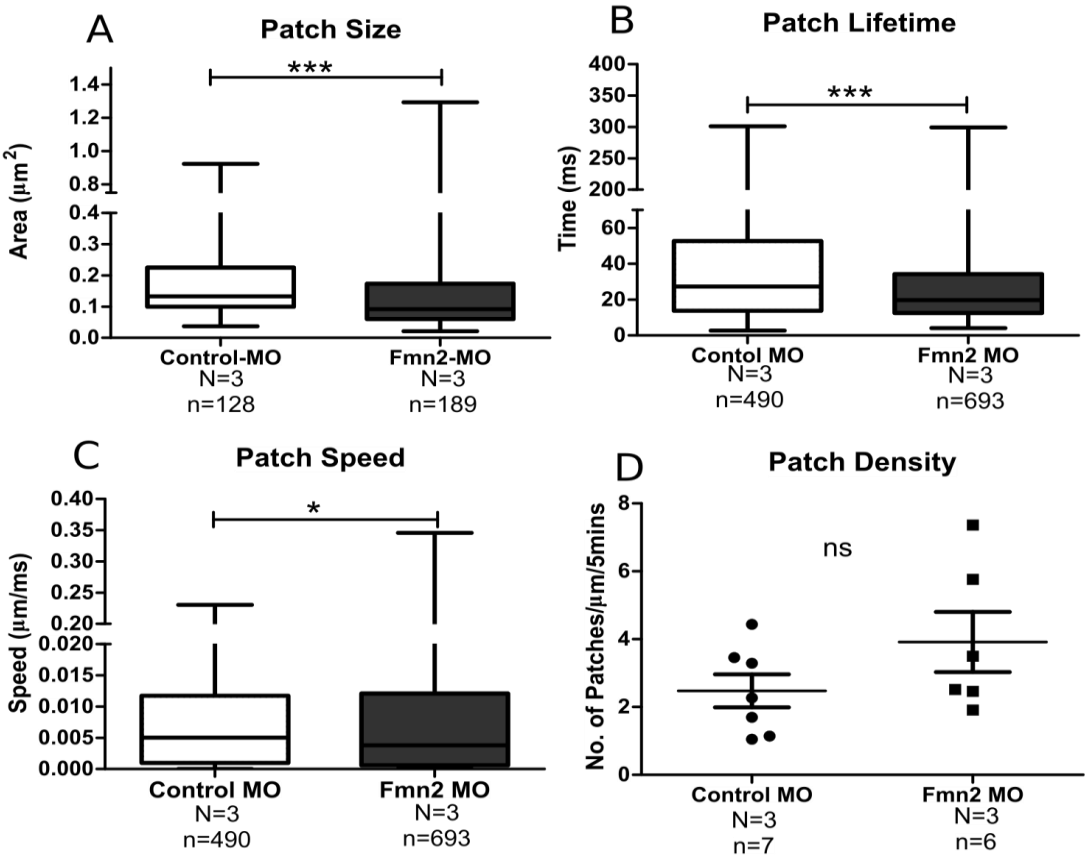


Fig 3.12 **Depletion of FMN2 Disrupted Actin Patch Dynamics.** (A) Knockdown of gFMN2 expression in neurons, exhibit a significant decrease in actin patch size in comparison to control MO transfected neurons **(P-value < 0.0001)** (Credit - Divya S for the analysis). (B) There is a significant decrease in actin patch lifetime in FMN2

depleted neurons in comparison to control MO transfected neurons **(P-value < 0.0001)** (C) There is a significant increase in actin patch speed in FMN2 depleted neurons in comparison to control MO transfected neurons **(P-value = 0.0267)**. (D) The graph shows an increase in patch density in FMN2 depleted neurons but it is non-significant **(P-value = 0.1807)**. For quantification of patch lifetime and speed from the kymograph, Velocity Measurement Tool plugin in ImageJ was used. (Mann-Whitney Test) (N= biological replicate, n= numerical replicate, number of neurons used control MO- 7 gFMN2 MO- 6.)

On the other hand, actin trails did not exhibit any significant changes in gFMN2 depleted neurons. The probability of an actin trail event is quite low, so the data acquired was not enough to show any trend in actin trail dynamics. So results are inconclusive. (Fig 3.13). The analysis of actin patch/trail dynamics was undertaken together with Tanushree Kundu and Divya S. (IISER Pune).

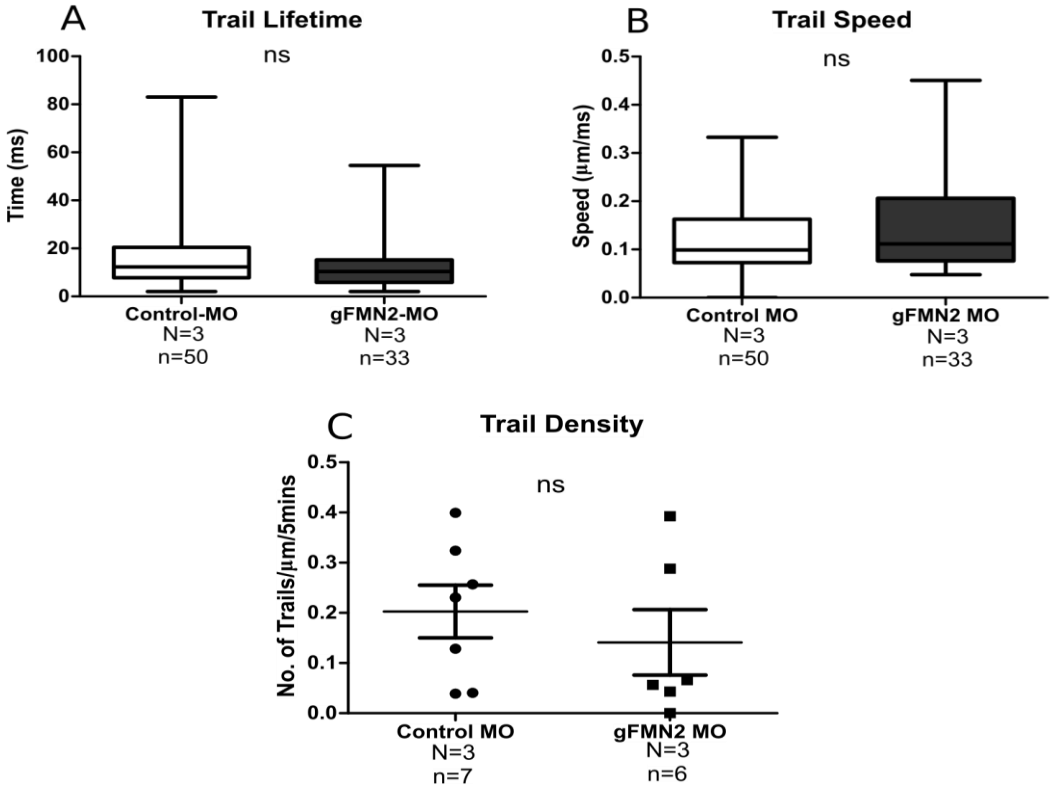


Fig 3.13: **No Significant Changes for Actin Trail in gFMN2 Depleted Neurons.** For quantification of trail lifetime and speed from the kymograph, Velocity Measurement Tool plugin in ImageJ was used. (Mann-Whitney Test) (N= biological replicate, n= numerical replicate, number of neurons used control MO- 7 gFMN2 MO- 6.)

Chapter 4
Conclusion and Discussion

4.1 Conclusion:

In this study, I investigated the role of Fmn2 in axonal branching process. During branch initiation, the activity of FMN2 in regulating nucleation and elongation of actin was found to be important for protrusion in axon shaft. Initiation of a protrusion is majorly governed by the actin patch and FMN2 was found to be influencing actin patch dynamics. Depletion of FMN2 in neurons significantly reduced the lifetime and area of actin patch in the axon shaft and by overexpressing the FMN2 in neurons displayed a distinguishable increase in actin patch lifetime.

4.2 FMN2 have a Role in Maintaining the Actin Patch

For an actin patch to maintain its size and to increase lifetime, actin polymerizing proteins have to constantly work against the depolymerizing proteins (eg: cofilin). Also, some proteins (eg: Ena/VASP) have to constantly inhibit F-actin barbed end capping for maintaining the polymerization rate. If the polymerization rate of the actin patch is greater than the depolymerization rate, then the actin patch elaborates in size. The actin patch lifetime and size was significantly affected by FMN2 depletion in neurons. There was around 30% reduction in both actin patch size and lifetime in FMN2 depleted neurons. Reduction in size and lifetime implicate that the depolymerization rate is gaining in actin patches. This suggests that FMN2 have a role in maintaining the actin structure. The kymographs of FMN2 overexpressing neurons exhibit a distinguishable increase in actin patch lifetime. This would suggest that the polymerization rate of actin patch equalized or gained over depolymerization rate due to increased FMN2 activity. Consistent co-localisation of actin patch and FMN2 was also backing evidence suggesting FMN2 is essential in maintaining the actin patch. However, the question of being the actin patch formed at the site, before or after FMN2 protein aggregation at the location was still not put to bed.

4.3 Influence of FMN2 in Transition Stage of Filopodia Initiation

Protrusion density in axons is significantly affected by the disruption of the actin nucleation and elongation activity of FMN2. This doesn't mean that the ability of transition is not necessarily compromised. However, with reduced lifetime the probability of transitioning is compromised. What if FMN2 had a direct role in the transition stage? On the basis of convergent elongation model, FMN2 might have a direct role in the transition stage by force generation on to the membrane through actin bundling and elongation. In this stage of the project, there is no strong evidence to suggest this. However, it was observed in time-lapse images of FMN2 and F-actin, that at the site of filopodium initiation, FMN2 increased in localization just before the formation of a filopodium and simultaneously with F-actin invaded the filopodium. This indicated that FMN2 might have a direct role in the transition mechanism.

4.4 FMN2 in the Maturation of a Filopodia

Maturation of filopodia starts with the invasion of microtubule and subsequent elongation of the filopodia. FMN2 depleted 48 hrs primary culture of spinal neurons exhibited short filopodia and only a few of these had microtubule innervation. On the contrary, the 48 hr primary culture of spinal neurons transfected with control-MO exhibited distinguishably longer filopodia and most these had microtubule invasion. Even though the analysis of this data is incomplete, there is an observable reduction in mature branches. This observable reduction in microtubule invasion could be an indicator for an additional role of FMN2 in filopodia maturation. Also, the localization pattern underneath the filopodia exhibited by the FMN2 is similar to Septin 7. Septin 7 is a protein that has implicated in having a role in filopodia maturation (Hu et al. 2012). Here Septin 7 speculated to be acting as a guiding track for the debundled microtubule into the filopodia. This leads to the speculation that FMN2 may also have a similar role in filopodia maturation as FMN2 is known to interact with microtubules via its FSI domain (Tanushree Kundu and Priyanka Dutta, unpublished data). Future studies probing the role of FMN2 in the maturation of filopodia will mostly focus on this domain. However, the FSI domain also has a role in actin bundling *in vitro* (Priyanka Dutta,

unpublished data). Hence, disrupting this domain may affect filopodia initiation and maturation. However, in 24 hr primary cultures of spinal neurons, FMN2 depletion was rescued using morpholino resistant FMN2-Δ-FSI (mutant lacking FSI domain) and these neurons exhibited observable recovery in protrusion density compared to FMN2 depleted neurons. Though the number of data points is low at this point, this trend suggests that in neurons the FSI domain does not contribute to filopodial initiation but may be involved in recruiting microtubules to stabilize the protrusions.

4.5 A model for FMN2 Functions in Axonal Branching:

It appears that other actin nucleators like Cobl, other formins and ARP2/3 may seed the actin patches. FMN2 appears to be involved in the dynamic stability of actin patches and by positively influencing the lifetime of the patch it contributes to the frequency of actin patches that transition to make filopodia. In addition, in the later stage of elaboration of an actin patch, FMN2 may facilitate assembly actin bundles and their elongation in order to deform the membrane and initiate a protrusion (Convergent Elongation Model). Further, in the later stages filopodia maturation, FMN2 localized at the base of the filopodia may aid innervation of filopodia by microtubules and influence protrusion persistence and stability.

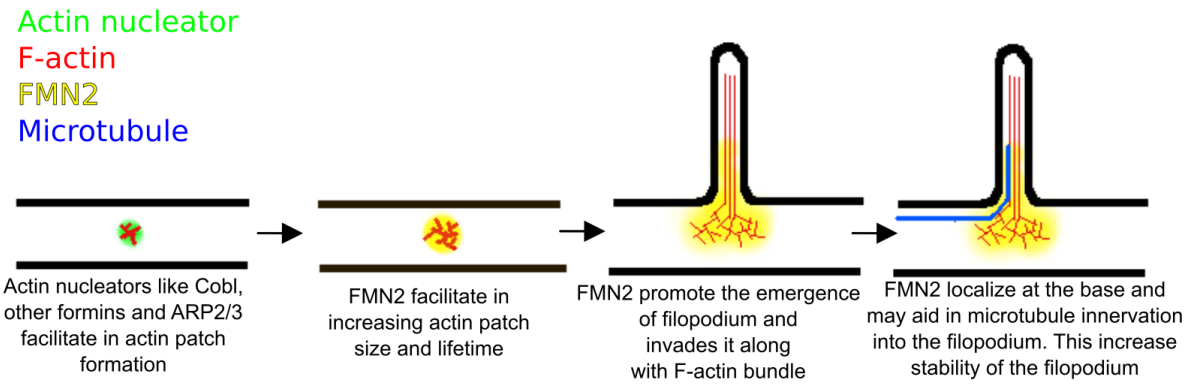


Fig 4.1 **A model for FMN2 function in Axonal Branching**

4.6 Distribution of Protrusion along Axon

There are nerve growth factors like NGF, BDNF etc induce axonal branching and recent studies have found that these factors induce axonal branching in different regions of axon. NGF prominently induces branching in the distal region and BDNF induces branching in the proximal region (Danzer et al. 2002). Region specific induction of branches in axon suggests that there are different signaling pathways at specific regions of the axon that induce the formation of an axonal branch. On the basis of this, FMN2 could be a factor in the axonal branching process acting at a specific region in the axon. To investigate the FMN2 activity in axonal branching process along the axon, distribution of protrusions along the axon were analyzed. From the control, it is already understood that protrusion density is higher over the distal region and decline sequentially towards the proximal. Knockdown and Rescue experiments had effect on protrusion density all along the axon. These changes were also almost uniform along the axon. This implicate that FMN2 is essential for axonal branching process all along the axon. In contrary to this argument, over-expression of FMN2 exhibited increase in protrusion density in the mid and proximal region of the axon. This is interesting because over-expression of FMN2 itself facilitated changes in FMN2 activity on axonal branching process along the axon. We do not have a definite explanation for this phenomenon and further investigation is necessary.

4.7 Protrusion Length and FMN2

Protrusion length analysis was performed to understand the role of FMN2 in maturation process of a protrusion. The length of a protrusion was used as a proxy for quantifying maturation. FMN2 depleted neurons exhibited significant reduction protrusion length. This was considered as an initial implication that FMN2 might have a role in maturation process. In contrary to this, over-expression of FMN2 in neurons was showing significant reduction in protrusion length as well. This might be due to the 75% increase in protrusion density causing deficiency in other factors, which involve in axonal branching, to maintain these protrusions stable. Surprisingly, rescue experiments have failed in recovering protrusion length in both FL and I2A sets. This is interesting because the growth cone filopodial length get rescued in both sets.

(Unpublished data by Tanushree Kundu (AG Lab)). We do not have a conclusive explanation for this phenomenon. Also the protrusion length comes under maturation stage of axonal branching, so the upcoming experiments focusing on the role of FMN2 in maturation stage of axonal branching might shed some light to this puzzle.

4.8 Future Experiments

Role of FMN2 in the transition stage and filopodium maturation is still inconclusive. So the major focus will be on further understanding these stages from the perspective of FMN2. Next stage of the project will be focused on the neurons at time point where branch maturation is prominent. In the initial stage, morphological disruption on mature branch due to variation in FMN2 will be quantified similar to the previous *in vitro* experiment. To further implicate the role of FMN2 in the convergent elongation model, changes in dynamics of the filopodia initiation (eg: latency period of an actin patch/trail in filopodium initiation) upon disrupting the FMN2 expression, have to be analyzed. Also the time point of FMN2 localization with actin patch/trail is still inconclusive. Resolving this ambiguity is important, since the role of FMN2 in actin patch initiation is not well understood. This would shed some light into this matter. During rescue experiments for FMN2, it was found that length of protrusion is not recovering back to normal phenotype in both Rescue FL and I2A. This is an odd observation in comparison to the protrusion density. However, slight reduction in protrusion length in FMN2 overexpressed neurons could be due to the depletion in cytoskeleton machinery because of increased protrusion activity. Since protrusion density recovers back in rescue FL experiment. Further test has to undertake for understanding the odd behavior with protrusion length.

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