The Capping Activity of Eps8 Regulates Axonal F-Actin Patch Dynamics and the Formation of Collateral Branch

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by

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From May 2022 to Mar 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled

"The Capping Activity of Eps8 Regulates Axonal F-Actin Patch Dynamics and the Formation of Collateral Branch"

towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sakshi Jayvant Pagrut at Indian Institute of Science Education and Research under the supervision of Prof. Aurnab Ghose Department of Biology, during the academic year 2022-2023.

Prof. Aurnab Ghose
Date: April 10, 2023

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Prof. Aurnab Ghose

Prof. Nagaraj Balasubramanian

Dedicated to

" To My Parents & Sister "

Declaration

I hereby declare that the matter embodied in the report entitled

**"The Capping Activity of Eps8 Regulates Axonal F-Actin Patch Dynamics
and the Formation of Collateral Branch"**

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 Prof. Aurnab Ghose and the same has not been submitted elsewhere for any
other degree

Sakshi Jayvant Pagrut

Date: 01-04-2023

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Abstract

Axon collateral branching is a critical process that enables neurons to form complex neural circuits and exhibit neural plasticity. Collateral branching is a highly cytoskeletal-dependent process that is initiated by the formation of dynamic F-actin patches. However, the regulatory mechanisms governing these actin patches' dynamics are poorly understood. Eps8, a capping protein implicated in several neurodevelopmental disorders. We find that Eps8 plays a role in regulating actin patches and the formation of protrusions in chick spinal neurons. This study reveals that Eps8 colocalizes with actin patches. The capping activity of Eps8 regulates the lifetime and stability of actin patches but not initiation. We further investigated the counterbalancing activities of Eps8 and Fmn2 in regulating actin patches and protrusions. Spatiotemporal regulation of Eps8 and Fmn2 during protrusion formation was also studied using live imaging. Neurodevelopmental defects due to Eps8 may be associated with improper regulation of axon branching.

Acknowledgments

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Contributions

Contributor name	Contributor role
SP and AG	Conceptualization Ideas
SP	Methodology
SP	Software
SP	Validation
SP	Formal analysis
SP	Investigation
AG	Resources
-	Data Curation
SP	Writing - original draft preparation
SP and AG	Writing - review and editing
SP	Visualization
AG	Supervision
AG	Project administration
AG	Funding acquisition

SP - Sakshi Pagrut

AG - Prof. Aurnab Ghose

Chapter 1 Introduction

1. 1 The development of neurons

The human brain is a remarkably intricate organ comprised of more than 100 billion individual neurons that interact with each other through trillions of synaptic connections. This connectivity forms the basis of neural circuits that regulate higher-order brain functions such as learning, memory, sensory perception, cognition, and motor behavior. Despite the vast number of neurons and connections, the brain can adapt and reorganize itself in response to environmental experiences and changes. This capacity for plasticity allows the brain to develop and refine its abilities over time, enabling us to learn, grow, and adapt to new challenges.

Neural development starts with the proliferation and differentiation of neural progenitor cells . As this process continues, these progenitor cells differentiate into specific neuronal or glial cell types (Sanes et al., 2005). The initial step consists of forming protrusions known as lamellopodia and filopodia, followed by their consolidation and engorgement. In immature neurons, these protrusions are referred to as neurites, and this process is known as neuritogenesis. Subsequently, one of the neurites polarizes and elongates to form the axon, acquiring axonal markers, while the other neurites become dendrites and acquire dendritic markers. The axon is identified by the presence of a highly dynamic and motile structure called the growth cone, which navigates the environment by responding to attractive or repulsive cues to reach its target. As axonal development proceeds, dendritic, axonal branching, and arborization occur in parallel. Throughout the development of the nervous system, the final stage is synaptogenesis, where the axon ultimately reaches its desired target and forms synapses. The entire process heavily depends on cytoskeletal remodeling to enable the necessary structural and dynamic changes to the cell (Lowery and Van Vactor, 2009; Kessels et al., 2011; Flynn, 2013; Lewis et al., 2013; Kerstein et al., 2015; Menon and Gupton, 2018; Flynn and Bradke, 2020).

Defects in developmental genes can result in the occurrence of mislocalized trajectories in the brain regions, which can lead to aberrant neural connections and subsequent neurodevelopmental disorders (NDDs). Furthermore, circuitry abnormalities may arise after successful neuronal targeting, particularly during synapse formation.

1.2 Axonal branching

Neural cells undergo a morphological transformation into peculiar-shaped neurons, which enables the establishment of synaptic connections with adjacent neurons. However, establishing multiple connections between a single neuron and various targets, resulting in trillions of connections, poses a significant challenge. This "connectivity issue" is particularly daunting in more extensive and more complex organisms with a centralized nervous system. In order to achieve this intricate neuronal circuitry, nature has devised an efficient strategy that involves the connection of one neuron with multiple targets, known as axonal branching. Axonal branching allows single neurons to communicate with multiple targets, which is crucial for establishing multiple connections with other neurons that are not in their direct trajectory (Gallo, 2011; Armijo-Weingart and Gallo, 2017). During neural development, it is crucial to form functional maps and regeneration and plasticity in the adult brain nervous system (Sofroniew, 2018; Tuszynski and Steward, 2012). As a result, studying mechanisms that regulate the branching aspects of neurons is critical for understanding how complex neuronal circuitry functions.

1.3 Types of Axonal branching

Based on their morphology, complexity, and function, axonal branching processes are typically categorized into three main types: Arborization, Bifurcation, and collateral branching(Figure 1.1A). Arborization refers to developing and forming higher-order branches at the axon shaft's terminal region that have successfully reached their intended target site. Growth cone bifurcation occurs because the leading and lateral edges of the growth cone have different growth rates, resulting in the formation of Y or T-shaped branches. Consequently, two branches emerge from

the lateral edge (Wessells and Nuttall, 1978). Axon collateral branching is the denovo branch formation from the axonal shaft. This is the major mechanism governing branching during nervous system development (Gallo, 2011). The emergence of filopodium from the quiescent axonal shaft initiates this process. The filopodia maturation into branches is facilitated by their stabilization and prevention of retraction back into the axon shaft. The branches that form then extend and arborize in the target field and form synapses. Notably, the mechanisms underlying the growth cone-mediated extension of the primary axon and collateral branch formation exhibit marked differences. Axonal branching is tightly regulated by a variety of factors, including guidance cues, growth factors, and neural activity (Kalil et al., 2011).Guidance cues like netrins (Dent et al., 2004) and Slit1a (Wang et al., 1999) promote the formation of protrusions, while cues like ephrin (Castellani et al., 1998) and semaphorin (Dent et al., 2004) are repulsive and inhibit protrusion formation.
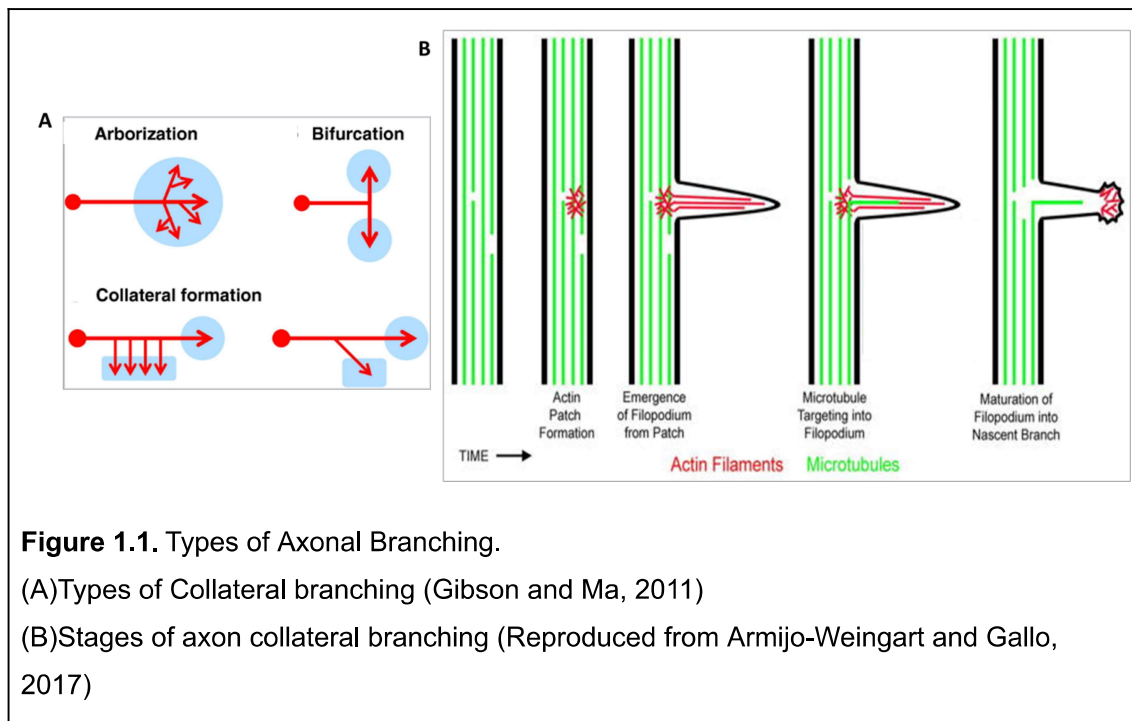
Figure 1.1. Types of Axonal Branching.

(A)Types of Collateral branching (Gibson and Ma, 2011)

(B)Stages of axon collateral branching (Reproduced from Armijo-Weingart and Gallo, 2017)

1.4 Cytoskeletal regulation during axon collateral branch formation

The process of collateral branching can be categorized into four distinct stages, each of which is a continuous process and actively demands cytoskeleton remodeling via various proteins (Armijo-Weingart and Gallo, 2017) (Figure1.1B).

1. Actin-patch Formation in the axon shaft
2. Filopodia formation from patch
3. Engorgement of Microtubule filaments into the filopodia
4. Filopodia maturation into a branch

1.4.1 Actin patch Formation

The F-actin patch is a network of F-actin filaments that are generated de novo within the axon shaft and are precursors to filopodia formation. They are highly dynamic and have a short lifespan. The nerve Growth factor (NGF) is a branch-promoting signal that increases the rate of formation of actin patches and filopodia in the axon through the PI3K pathway (Ketschek and Gallo, 2010).

The lifespan of an actin patch can be classified into three different stages: formation, elaboration, and dissipation. The Arp2/3 complex is a crucial player during the initial steps of actin patch formation. The Arp2/3 complex functions by catalyzing the nucleation of actin filaments from the sides of pre-existing actin structures, resulting in the formation of a network of actin filaments (Spillane et al., 2012). This meshwork subsequently bundles together to form an actin filament bundle, which serves as the core of the developing filopodium. WAVE1 (Sweeney et al., 2015) activates the Arp2/3 complex, and cortactin stabilizes the Arp2/3-mediated branched filaments. Formin and Cordon blue cooperate with the ARP2/3 complex to facilitate actin patch initiation (Ahuja et al., 2007; Yang and Svitkina, 2011).

The next subsequent step is patch elaboration. A higher polymerization rate than depolymerization in the patch characterizes this stage. Consequently, there is an increase in the lifetime and stability of the patch (Figure 1.2A). The ENA/VASP and formin family proteins are actin-binding proteins. They bind to the F-actin barbed end and enhance the actin elongation rate, thus contributing positively to filopodia formation (Lebrand et al., 2004; Dwivedy et al., 2007). Fmn2 increases the actin patch lifetime and stability (Kundu et al., 2022). The majority of patches undergo an

elongation phase, while only few of them give rise to filopodia (Armijo-Weingart and Gallo, 2017; Ketschek and Gallo, 2010). The trigger for branch formation, however, remains unknown.
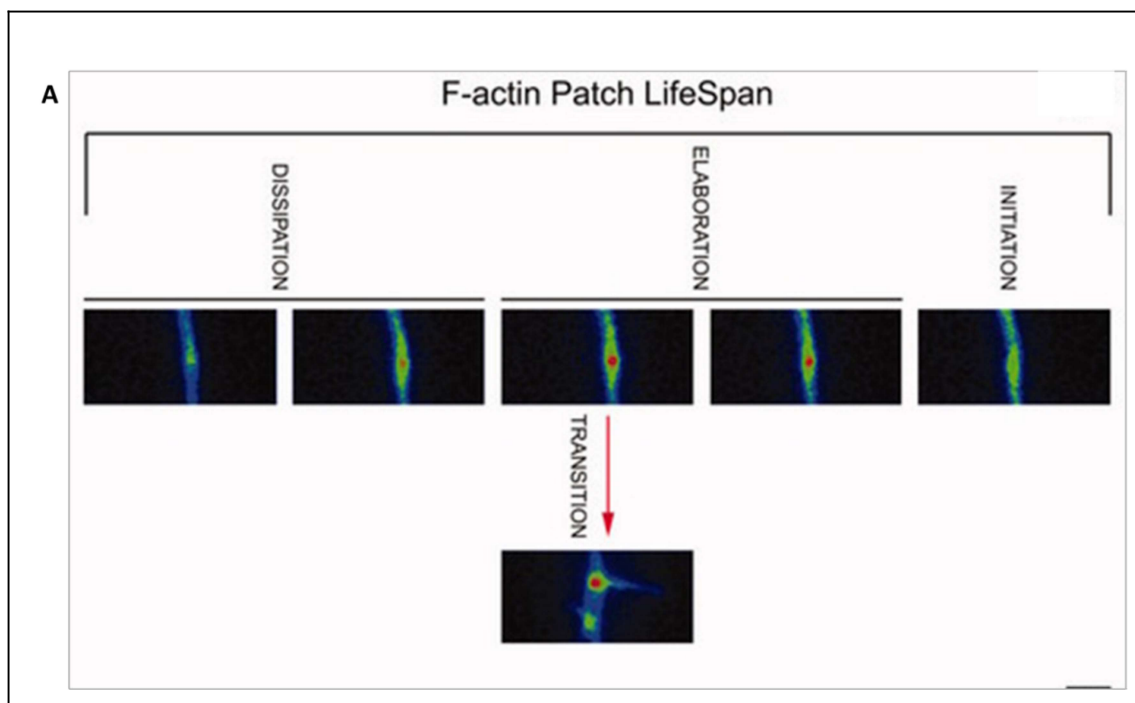
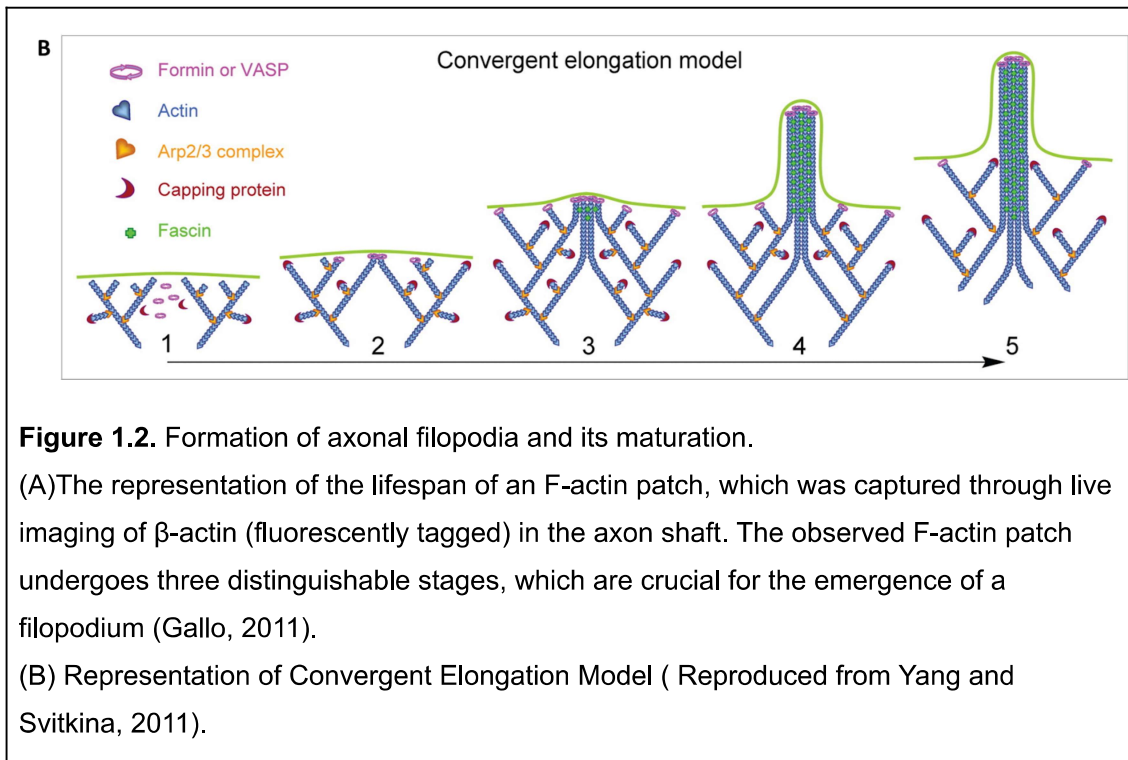### 1.4.2 Filopodia formation and maturation

According to the convergent elongation hypothesis, the actin patch cannot produce a protrusion on the cell membrane alone. The actin patch is a network of actin filaments that cannot exert a targeted force on the cell membrane (Yang and Svitkina, 2011). In order to generate such a force, it is necessary for the actin filaments to be bundled—proteins like Debrin and Fascin bundle parallel elongating actin filaments. There is a complex interaction of actin bundling, elongation, depolymerization, and capping proteins to form a convergent cluster of actin filaments at a specific site in the axon, leading to the formation of the protrusion (Figure 1.2B). Following the elaboration of the patch and subsequent formation of the filopodium, the patch dissolves, leaving behind the pointed ends of the bundled actin filaments present within the filopodium.

Filopodia are highly dynamic structures consisting of actin bundles that undergo multiple cycles of growth and collapse on the cellular membrane throughout their lifespan. The engorgement of microtubules is an essential event of filopodium maturation. The arrangement of microtubules in an axon typically involves parallel bundles of filaments that are tightly packed. To enable the entry of microtubules into the filopodium, these bundles undergo local debundling and fragmentation. Severing proteins, such as spastin and katanin, mediate this fragmentation, which promotes the formation of the mature collateral branch. Although the process of fragmentation and debundling is not well studied, recent investigations have revealed that nerve growth factor (NGF) in local debundling and fragmentation of axonal microtubules prior to branch formation (Ketschek et al., 2015; Yu et al., 1994). Furthermore, the effects of NGF on microtubule debundling are triggered by the depolymerization of actin filaments and inhibition of myosin ||. MAP Tau exerts an inhibitory effect on collateral branch formation by safeguarding microtubules from the severing action of katanin (Qiang et al., 2006). Septin7 (displaces MAP tau from microtubules) and Drebrin (inhibits myosin II activity) promotes microtubule localization and

organization in axonal filopodia (Ketschek et al., 2016; Hu et al., 2012). The entry of microtubules provides filopodia with the proper length and girth required for maturation into a branch. The energy needed to maintain a branch's actin tubule network structure is substantial. To meet this energy demand, mitochondria function as power stations during the branch maturation process (Spillane et al., 2013). This branch continues to expand in order to acquire its target region and form a synapse at some point.

Collateral branching is still a hot topic for research. This project aims to investigate the function capping activity of Eps8, a member of the actin-binding protein family, in the dynamics of F actin patches and its role in the formation of collateral branching. We also studied the role of the capping activity of Eps8 in growth cone regulation. Additionally, this study aims to examine the interaction between Eps8, capping protein, and Fmn2, a formin family member, at the actin patch and protrusion level.

Figure 1.2. Formation of axonal filopodia and its maturation.

(A)The representation of the lifespan of an F-actin patch, which was captured through live imaging of β-actin (fluorescently tagged) in the axon shaft. The observed F-actin patch undergoes three distinguishable stages, which are crucial for the emergence of a filopodium (Gallo, 2011).

(B) Representation of Convergent Elongation Model (Reproduced from Yang and Svitkina, 2011).

1.5 Eps8 : Eps8L-family member

Eps8 is a capping protein that belongs to the Eps8L family. In general, capping proteins bind to the barbed end of an actin filament and prevents further monomer addition. Eps8 possesses a modular domain organization consisting of PTB and SH3 domains, along with a C-terminal actin-binding region (Narita et al., 2006). This modular organization is more commonly observed in signaling adaptors. The SH3 domain typically plays a crucial role in transducing signals from Ras to Rac, which, in turn, leads to actin remodeling (Fiore and Scita, 2002). The conserved C-terminal region of Eps8 is responsible for its core biological activity of capping filament barbed ends, thereby regulating motility processes based on actin (Croce et al., 2004, Disanza et al., 2004). Notably, this region does not share any sequence conservation with other known cappers, such as gelsolin, Twinfilin, or Capping proteins (McGough et al., 2004; Littlefield et al., 1998).

Eps8 is a multifunctional protein that performs several actin-based activities, including capping and bundling, which are tightly regulated by its binding partners, Abi1 and IRSp53, respectively (Disanza et al., 2006). The C-terminal domain of

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Eps8L comprises five alpha helices that are connected by linkers of varying lengths. The amphipathic helix (H1) in this domain is mainly responsible for the capping activity of Eps8, as it blocks the further addition of monomer to the barbed end of the actin filament (Figure 1.3A, B). The globular domain (H2-H5) binds to the sides of filaments and promotes bundling (Hertzog et al., 2010). The Eps8 capping activity plays a critical role in the regulation of endomembrane cellular trafficking and actin-based motility, acting as a negative contributor to filopodia formation. In contrast, its bundling activity is essential for the regulation of intestinal morphogenesis during the development of Caenorhabditis elegans and shows a positive contribution to filopodia formation (Croce et al., 2004; Disanza et al., 2004).

In this study, The selection of Eps8 to the collateral branch as the preferred capping protein over others, such as CapZ, was based on compelling evidence that supports its regulatory role in filopodial protrusions in primary hippocampal neurons. Specifically, genetic removal of Eps8 significantly increases the density of filopodial protrusions, and BDNF was found to inhibit the capping activity of Eps8 through MAPK-dependent phosphorylation of specific Eps8 residues (S624 and T628) (Menna et al., 2009). Additionally, the contribution of CapZ and other capping proteins to collateral branching has yet to be explored (Davis et al., 2009). Furthermore, research using knock-out mice has demonstrated that Eps8 deficiency results in immature spines and an inability to respond to enhanced stimuli, thereby impairing cognitive function (Menna et al., 2013). In particular, individuals with autism have been found to exhibit lower levels of Eps8 in the brain as compared to the general population. The dynamic regulation of the capping activity of Eps8 may play a crucial role during both brain development and in the adult brain.

Figure 1.3. Capping activity of Eps8

(A)Representation of   Eps8 actin-binding region(Red) and a single actin monomer(Gray).
(B)The barbed ends of the actin filament have an accessible H1 binding site that allows
the H1 to bind in the hydrophobic pocket, which stops the addition of more G-actin
monomers to the filament (Reproduced from Hertzog et al., 2010)
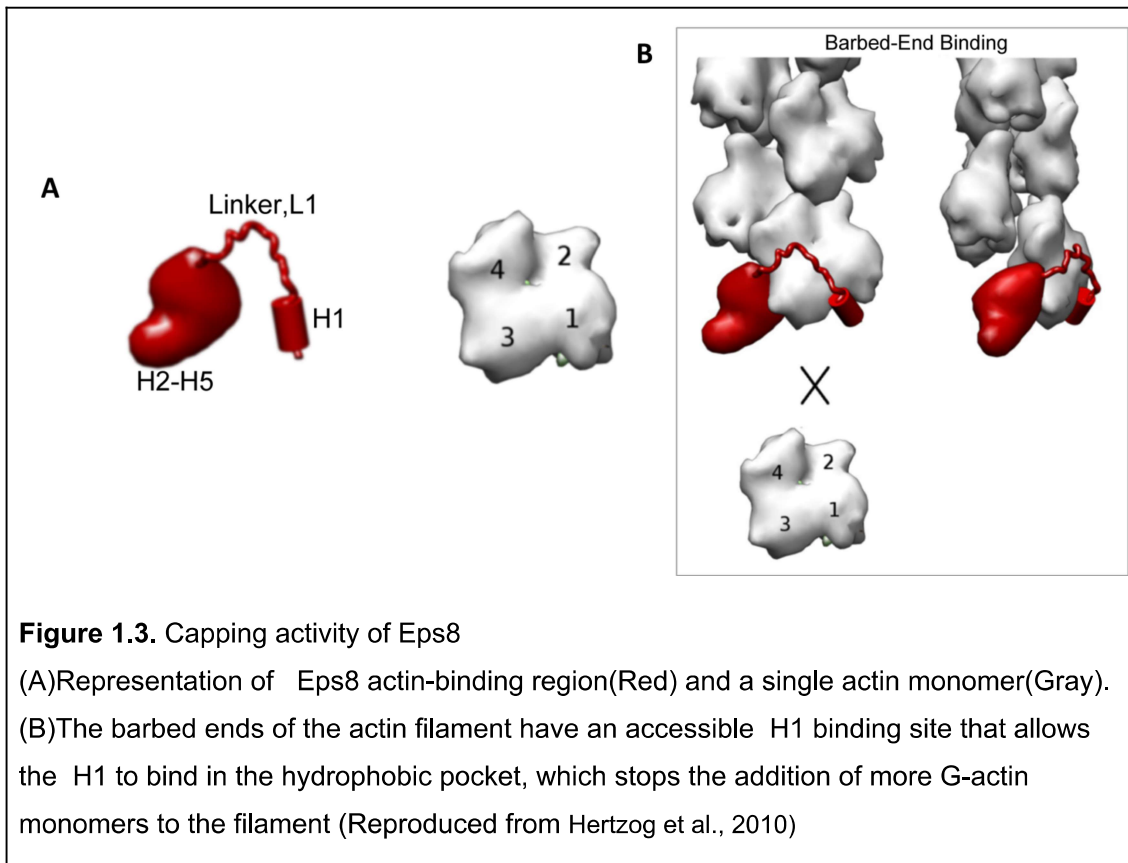
1.6 Fmn2: Formin family member

Formins are a family of proteins with a high molecular weight (120- 220kDa) and
conserved formin homology domains FH1 and FH2 (Castrillon and Wasserman,
1994; Higgs, 2005; Grunt et al., 2008). Based on differences in FH2 domains, the
formin family is divided into seven classes, and now 15 formins are known (Higgs
and Peterson, 2006; Campellone and Welch, 2010).

Formins facilitate the nucleation and elongation of actin filaments. The FH1 domain
contacts with profilin-actin monomers, whereas FH2 binds to the barbed end of the
polarized actin filament by forming a doughnut-shaped dimer. Profilin is an
actin-binding protein that mediates cross-linking between FH1 and G-actin, boosting
the polymerization rate by increasing the chance of G-actin interaction with FH2.
During actin filament elongation, formin switches its conformation from close to open.
In close conformation, the FH2 dimer binds to the terminal actin subunit, thus

blocking the monomer addition. One of the FH2 dimers steps towards the barbed end in an open conformation, thus facilitating monomer addition (Figure 1.4 A) (Dong et al., 2003; Brandt et al., 2007; Paul and Pollard, 2009). Fmn2 has a highly conserved FH2 domain, and it is expressed in both developing and adult nervous systems. Its involvement has been implicated in a variety of neurodevelopmental disorders. Fmn2 regulates actin patch lifetime and protrusion formation in the collateral branching in chick spinal neurons and zebrafish motor neurons (Kundu et al., 2022).

Figure 1.4. Formin mediated actin polymerization

(A) Representation of formin-mediated actin polymerization through stair-stepping mechanism (Reproduced from Campellone and Welch, 2010).
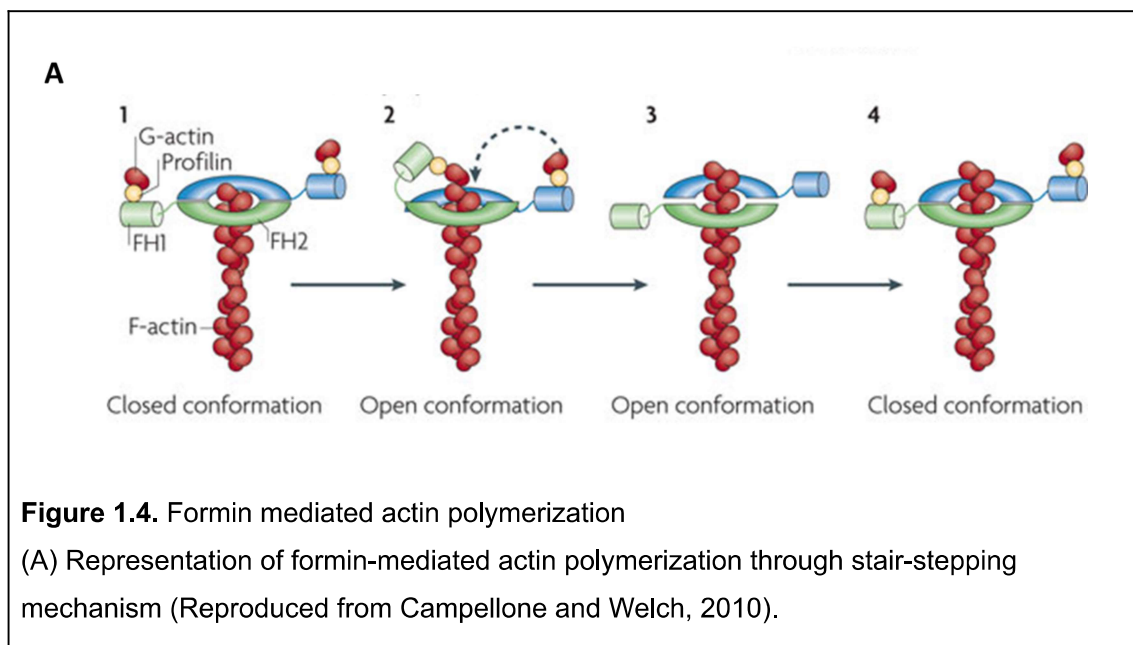
1.7 Regulation of formins and capping proteins to bind F-actin barbed end.

Actin filaments exhibit polarity with distinct barbed and pointed ends, with monomer addition occurring primarily at the barbed end and disassembly occurring mainly at the pointed end. The polarized growth of actin filaments is precisely regulated by various barbed-end binding proteins that either cap or elongate them in a processive manner. This regulation is achieved through a delicate balance of active barbed-end regulators controlled by inhibition (sequestration) or local activation. While the contribution of individual barbed end-binding proteins during collateral branching in

forming protrusion is well studied, how these proteins work together spatiotemporally to maintain the dynamic balance of the F-actin patch remains unclear.

The other objective of my project is to investigate whether any interplay exists between Fmn2-mediated F-actin assembly and Eps8-induced capping activity, ultimately leading to a dynamic equilibrium during actin patch formation. This study sheds light on the genetic interaction between Fmn2 and Eps8 to regulate collateral branch formation.

Capping proteins are regulated by capping interacting proteins through two mechanisms. Firstly, proteins such as myotrophin sequester free capping proteins by binding to their actin-binding sites with high affinity, reducing the pool of available capping proteins for barbed end binding (Bhattacharya et al., 2006; Zwolak et al., 2010). Secondly, the capping protein interaction (CPI)-motif-containing family. These proteins bind to both free and barbed end-bound capping protein through CPI motifs and reduce capping protein affinity for actin sites (Fujiwara et al., 2010). This promotes uncapping by enhancing capping protein dissociation from barbed ends (Figure 1.5 A).

Similarly, formins are also subject to regulation by several factors that limit their activity. Profilin is essential for the efficient and fast processive actin assembly mediated by formin. However, it has been observed that excessive concentrations of profilin can lead to a reduction in formin-based elongation rates (Cao et al., 1992; Joy et al., 2014). Furthermore, Bud14, in cooperation with Kelch proteins in yeast, binds and inhibits the FH2 domain of formin, resulting in increased formin dissociation from barbed ends (Chesarone et al., 2009; Gould et al., 2014).

Invitro studies have shown that formins and capping protein form ternary complexes at the barbed end of actin instead of mutually exclusive binding (Shekhar et al., 2016). Due to the weak affinity of both proteins in the ternary complex, one can easily displace the other(Figure 1.5 B, C). According to biochemical experiments and studies conducted in fission yeast cells, Capping protein SpCP and formin Cdc12p compete for actin filament barbed ends, indicating genetic antagonism (Kovar et al.,

2005). Studies suggest that CP and formins counterbalance each other's activities in vivo (Sinnar et al., 2013).

Figure 1.5. Regulation of Actin barbed filament by Formins and capping proteins (Reproduced from Shekhar et al., 2016).

1.8 Objectives

1. To study the subcellular localization of Eps8 protein by ectopically expressing fluorescently labeled Eps8 in cultured chick spinal neurons

2. To study the subcellular localization of mutant Eps8 protein lacking capping activity (Eps8$^{\Delta cap}$) by ectopically expressing fluorescently labeled mutant construct.

3. To study the morphological aspects of the neurons, such as growth cone area, number, and length of growth cone filopodia upon ectopic expression of both Eps8 and Eps8$^{\Delta cap}$ protein.

4. To study the effect on axonal filopodia, size, and lifetime of F-actin patch upon Eps8 and Eps8$^{\Delta cap}$ protein overexpression

5. To study genetic interaction between Fmn2 and Eps8 by coexpressing them in the chick spinal neurons.

Chapter 2 **Materials and Methods**

2.1 Plasmids

1. pCAG-GFP (Addgene Plasmid #11150), GeneID: 74950
2. pCAG-mEps8-GFP - GeneID: 74950 <u>(Credits to Lisas Sewatkar for subcloning of mEps8 into pCAG GFP)</u>
3. pCAG mΔCapEps8 - GFP - Gene ID : 74786 <u>(Credits to Lisas Sewatkar for subcloning of mEps8$^{\Delta cap}$ into pCAG GFP)</u>
4. pCAG-gFMN2-GFP (gFMN2 - Gene ID: 775973) <u>(Credits to Sooraj das for subcloning of Fmn2 into pCAG GFP)</u>
5. pCAG-gFMN2-mCherry
6. pCAG-mFMN2-GFP (mFMN2 - Gene ID: 54418)
7. pCAG-Tractin-mCherry (Tractin- Add gene: 58473)

2.2 Materials

22mm circular coverslips, 35mm culture plates, Silicone sealant (Dow Corning), Poly L lysine (10 mg/ml; PLL), 1x Trypsin EDTA solution, Laminin in Tris-buffered saline (1mg/ml), 10X PBS, L-15, 100x Penstrep, 1x Opti-MEM, 0.2um syringe filter, 50ml syringe, 16% Paraformaldehyde, 25% Glutaraldehyde, 4x PHEM, 1N HCl, Triton-X-100, Albumin from Bovine serum lyophilized powder, 100mm Sodium pyruvate, Alexa Fluro 568 Phallodin, Alexa Fluro 647 Phalloidin.

2.3 Methods

2.3.1 Primary neurons culturing and transfection

The spinal tissue from Day 6 chick embryos were dissected in Embryonic media (EM), which consists of L-15 supplemented with 1x Penstrep. The tissue was then collected in EM and pelleted at 3000 rpm. The EM was discarded, and the tissue was resuspended in 1x Trypsin-EDTA, which had been preheated to 37°C. Trypsinisation was performed for a duration of 20 minutes.

For untransfected cultures, the spinal tissue was resuspended in BCM (L-15 medium supplemented with 10% FBS, 1mM Sodium Pyruvate, 1x Penstrep, and 0.6% Methylcellulose). The cells were then plated on glass-bottomed 35mm plastic dishes and incubated at 37°C for 36 hours. For transfected cultures, spinal tissue was trypsinized and resuspended in Optimem. Plasmids were added to the solution in appropriate amounts (according to the plasmid size), and the entire mixture was transferred to a cuvette. Electroporation was performed using the NEPA21 type II square wave electroporator under the specified conditions. Following transfection, the cuvette was supplemented with BCM, and the cells were plated on PLL and laminin-coated plates and grown for 36 hours. Poly-L-lysine (PLL) at a concentration of 1 mg/ml, followed by laminin at a concentration of 20 ug/ml coating was done prior to plating the dishes.

2.3.2 Transfection parameters

The NEPA21 type II square wave electroporator was utilized to perform transfections, employing two distinct electrical pulses. The initial pulse type involved a high voltage short interval poring pulse, characterized by a pulse length of 5ms, a pulse interval of 50 ms, a decay rate of 10%, and a voltage of 125 V. This facilitated the formation of pores on the membrane of cells. The second pulse type comprised a low-intensity alternating polarity transfer pulse with a pulse length of 50ms, a pulse interval of 50 ms, a decay rate of 40%, and a voltage of 20 V. This pulse resulted in the minor movement of charged particles (DNA), leading to a higher likelihood of transferring plasmids into the cell.

2.3.3 Fixing and Staining of Primary Neuron Culture

The neural cultures were subjected to fixation using a solution of paraformaldehyde(4%) and glutaraldehyde (0.25%) in PHEM buffer, which consisted of 25 mM HEPES, 60 mM PIPES, 10 mM EGTA, and 4 mM $MgSO_4 \cdot 7H_2O$, at a temperature of 37°C for a duration of 20 minutes. Subsequently, the cells were permeabilized using 0.1% Triton in PHEM buffer for 30 minutes at room temperature. 3% BSA in PHEM buffer was used to block the cells (to prevent non-specific binding) for one hour at room temperature. Phalloidin 568 at a dilution of 1:150 was used to

label F-actin, and Phalloidin 647 at a dilution of 1:400 was used before mounting in 1x PHEM buffer.

2.3.4 Imaging and Image analysis

Zeiss LSM 710 system with a 63x 1.4 NA oil objective using appropriate laser lines and Z-stack setup was used to image fixed samples. For the co-expression study, the Leica SP8 system with a 63x 1.4 NA oil objective was used. Live imaging was conducted using culture plates placed in a temperature-controlled setup maintained at 37°C without CO_2, and images were captured with the Leica SP8 system and 63x 1.4 NA objective. Actin patch lifetime analysis was conducted by capturing time-lapse images at 6-minute intervals with 6-second frame intervals, and the Image J software was used to analyze the acquired images. Single neurons with visible cell bodies and whole axons were selected for analysis. Moderate to low-intensity fluorescence neurons were selected for analysis.

2.3.5 Statistical analysis

GraphPad Prism 8 software was used to generate graphs and represented as violin plots, which display the median and interquartile range, as well as the total spread and density of data in each category. For comparisons between two groups, Welch's t-test was employed if the data distribution was normal, while the Mann-Whitney U test was performed if the distribution was not normal.

2.3.6 Quantification Parameters and methods

2.3.6.1 Protrusion density

Whole axon length was considered for protrusion density measurement without soma and growth cone. Protrusion density (providing a measurement of protrusion density per unit length (micron) of the axon) is defined as the number of protrusions in the axon divided by axon length. ROI for axonal filopodia and axon length was marked using a segmented line tool from the ImageJ software.

2.3.6.2 Actin patch size

Statistical region merging (Segmentation) plugin from ImageJ software was used to determine the size of actin patches. Q value was set between 100-300, which is a rough estimate of the number of regions in the image. Actin patches in the segmented version were verified by comparing them with the original image, and the ROI of the patch area was marked by the freehand selection tool.

2.3.6.3 Patch Dynamics Analysis

For calculating the speed and lifetime of an actin patch, the time-lapse images were drift-corrected using ImageJ plugins. For correcting XY drift in the time-lapse image, the Stackreg plugin was used. Kymographs were then produced from the axon region for the entire six-minute period using Kymoclear 2.0 plugin. From these kymographs, regions representing actin patches and trails were marked using a segmented line tool from the ImageJ software.
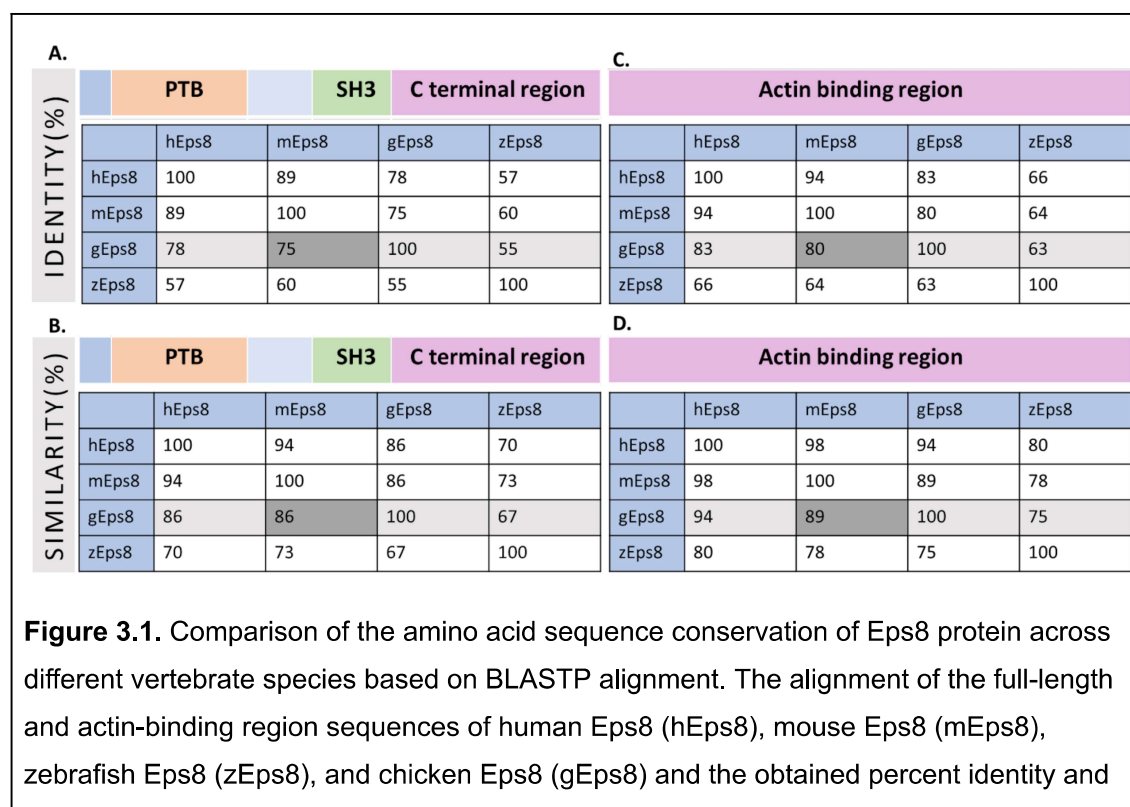
2.3.6.4 Growth cone analysis

The area of the growth cone was marked by the free hand selection tool from the Image J software, and the length of the growth cone filopodia was marked by the segmented line tool. After marking the ROI in the ROI manager toolbox, a measure option was selected to obtain the area and length values.

Chapter 3 **Results**

3.1 The Actin binding region of Eps8 is conserved across vertebrate species.

The Chick Eps8 protein (gEps8) (A0A8V0YFV0; UNIPROT) shares a high degree of sequence identity (SI) and sequence similarity (SS) with both human Eps8 (SI: 78%, SS: 86%) and mouse Eps8 (SI: 75%, SS: 86%) (Figure 3.1 A, B). The actin-binding region of gEps8 shares 80% identity and 89% similarity with mouse Eps8 (mEps8). Overall, we observed a high degree of identity and similarity between both Full-length Eps8 and only the actin-binding region of Eps8 in humans (Q12929), mice (Q08509), chick (A0A8V0YFV0), and zebrafish (A0A0R4IID9) orthologs (Figure 3.1C, D).

In summary, Eps8 is highly conserved across vertebrates, especially the actin-binding domain. We used the mEps8 plasmid construct for overexpression studies to explore Eps8 function in subsequent studies of chick spinal neurons.

Figure 3.1. Comparison of the amino acid sequence conservation of Eps8 protein across different vertebrate species based on BLASTP alignment. The alignment of the full-length and actin-binding region sequences of human Eps8 (hEps8), mouse Eps8 (mEps8), zebrafish Eps8 (zEps8), and chicken Eps8 (gEps8) and the obtained percent identity and

similarity are shown. (A)Full-length gEps8 has a sequence identity of 78%, 75%, and 55% with human, mouse, and zebrafish Eps8, respectively. (B) The full-length gEps8 has a sequence similarity of 86%, 86%, and 67% with human, mouse, and zebrafish Eps8, respectively. (C) Further, only the actin-binding region of Eps8 (648-841 for mEps8) was compared across species, and the percent identity matrices were obtained. The actin-binding region of gEps8 has a sequence identity of 83%, 80%, and 63% with human, mouse, and zebrafish Eps8, respectively. (D)The actin-binding region of gEps8 has a sequence similarity of 94%, 89%, and 75% with human, mouse, and zebrafish Eps8, respectively.
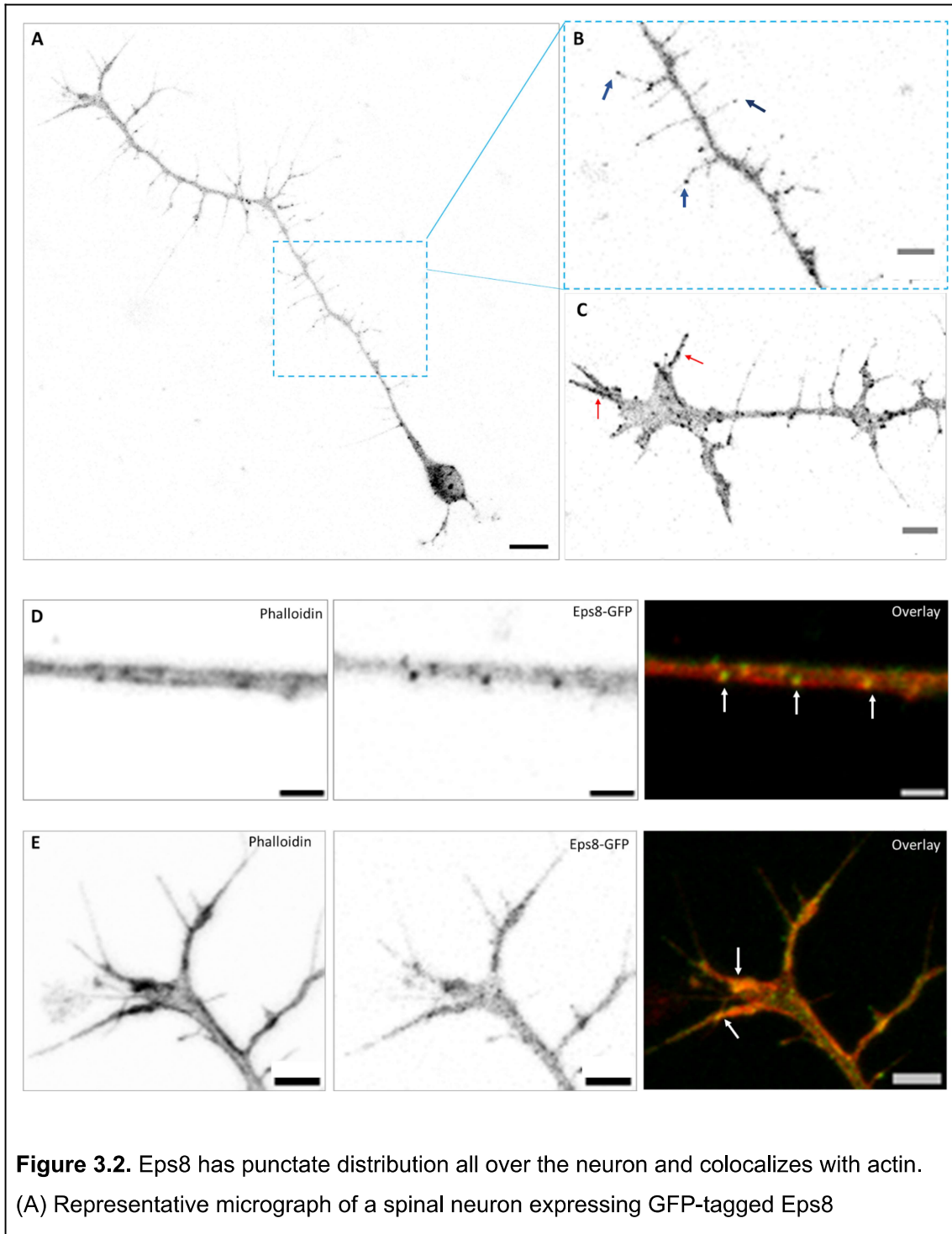
3.2   Eps8 has punctate localization throughout the neuron and colocalizes with actin.

To investigate the role of Eps8 in chick spinal neurons, we evaluated its subcellular localization. We used GFP-tagged Eps8 (Eps8-GFP) to visualize the protein's distribution. Eps8 exhibited punctate distribution throughout the neuron, with punctae present in the cell body, axon shaft, and prominently at the growth cone and filopodial tips of the axon (Figure 3.2A).

Axonal filopodia are composed of bundles of polarized actin filaments, where the barbed end of the filaments is oriented towards the tip of the filopodium. The barbed end of the actin filament continuously grows with the aid of elongation factors. The activity of capping protein is essential to regulate the filopodial dynamics at the tip. The presence of punctate expression at the tips of filopodia implies that its location is conducive to its intended function.

Next, we examined the colocalization between Eps8 and F-actin structures present in the axon shaft and growth cone. We overexpressed spinal neurons with Eps8-GFP and stained them with Phalloidin to visualize the actin structures. The protein Eps8 exhibited colocalization with F-actin patches present in the axon shaft (Figure 3.2D). Further, It colocalizes with actin structures present in the central domain as well as the peripheral domain of the growth cone of chick spinal neurons

(Figure 3.2E). In summary, Eps8 localizes to actin-rich structures in the chick spinal neurons. Eps8 is localized at the tips of the axonal filopodia, where a capping protein is likely to present.

Figure 3.2. Eps8 has punctate distribution all over the neuron and colocalizes with actin. (A) Representative micrograph of a spinal neuron expressing GFP-tagged Eps8
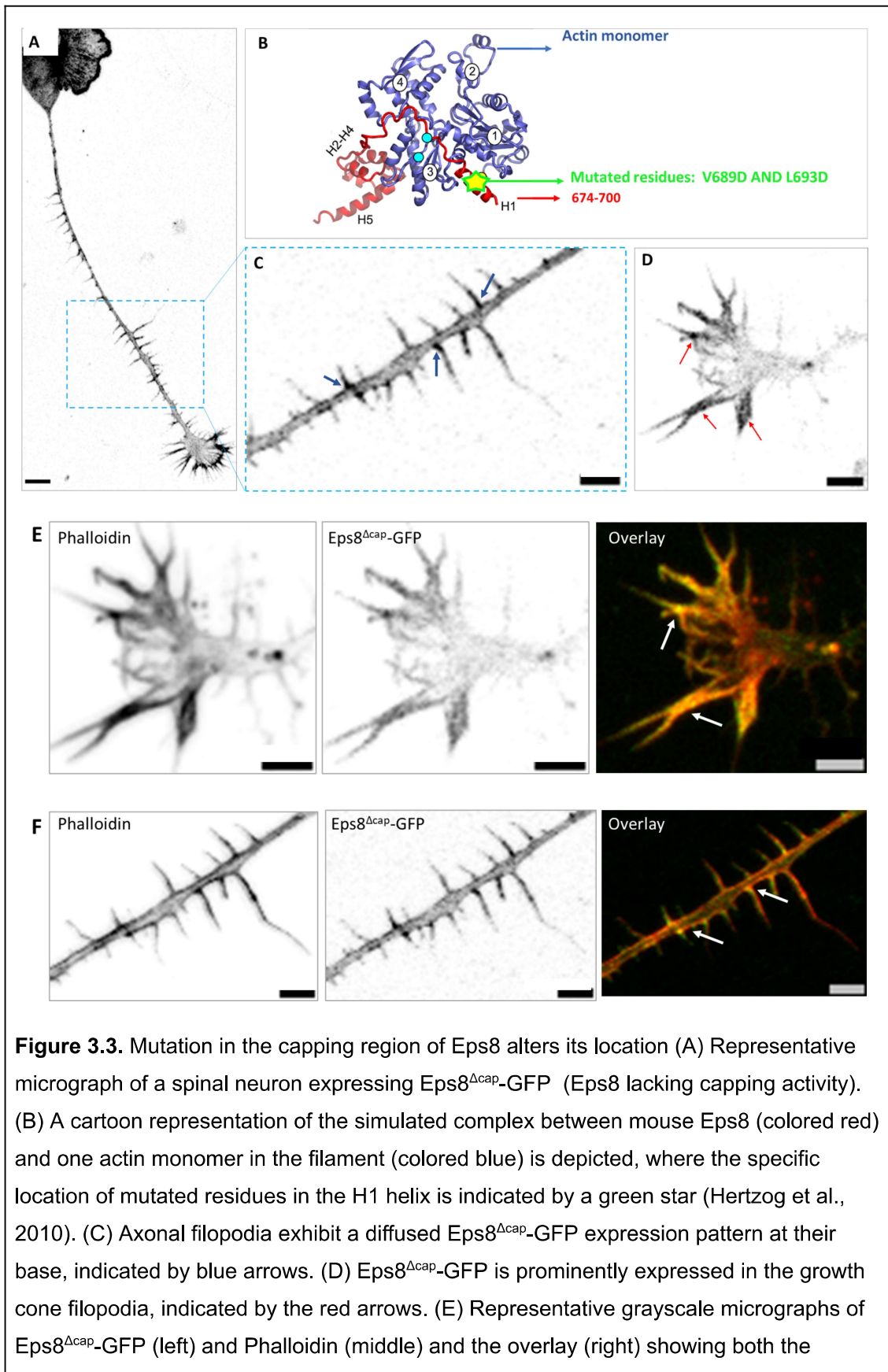
(Eps8-GFP). Eps8 has punctate expression all over the neuron. (B) Expression in the tips of axonal filopodial is indicated by the blue arrows (enlarged view of the blue dashed box). (C) Eps8 expression in the growth cone is indicated by the red arrows. (D) Representative grayscale micrographs of Eps8-GFP (left) and Phalloidin (middle) and the overlay (right) showing both the Eps8 (green) and Phalloidin (red) signals in the axonal shaft. Eps8 colocalizes with the axonal actin patches. (E) Representative grayscale micrographs of Eps8-GFP (left) and Phalloidin (middle) and the overlay (right) showing both the Eps8 (green) and Phalloidin (red) signals in the growth cone. Eps8 colocalizes with the F- actin structures in the growth cone. Scale bar: 10 µm (A), 5 µm (B,C,D,E).

3.3 Mutation in the Eps8 capping domain alters its full-length localization.

A GFP-tagged mutant Eps8 protein lacking capping activity (hereafter referred to as Eps8$^{\Delta cap}$-GFP) was transfected into cultured chick spinal neurons. This mutation involves two point mutations (V689D AND L693D) in the H1 helix region that form hydrophobic bonds with the cleft between actin barbed ends (Figure 3.3B), keeping the bundling and side-binding properties of protein unaltered. The localization of the capping mutant protein differed from that of the full-length Eps8 protein (Figure 3.3 A). In full-length protein, there is a preponderance of distinct punctae; however, in a mutant lacking capping activity, the punctae are absent, and instead, the protein exhibits a diffuse distribution pattern. The Eps8$^{\Delta cap}$-GFP has prominent expression in the growth cone filopodia (Figure 3.3C) and the axonal filopodia (Figure 3.3D). Furthermore, we looked at the colocalization of Eps8$^{\Delta cap}$-GFP with actin structures. We observed that Eps8$^{\Delta cap}$-GFP colocalizes with actin bundles present in the axon filopodia (Figure 3.3E) and growth cone filopodia (Figure 3.3F).

In summary, As the mutant preserves side-binding and bundling, it is no longer larger punctate but is found to co-localize with F-actin structures. In axonal filopodia, the expression of the mutant is more pronounced at the base than at the tips. In the growth cone, the mutant is prominently expressed in filopodia, which resembles the localization of fascin, a bundling protein.
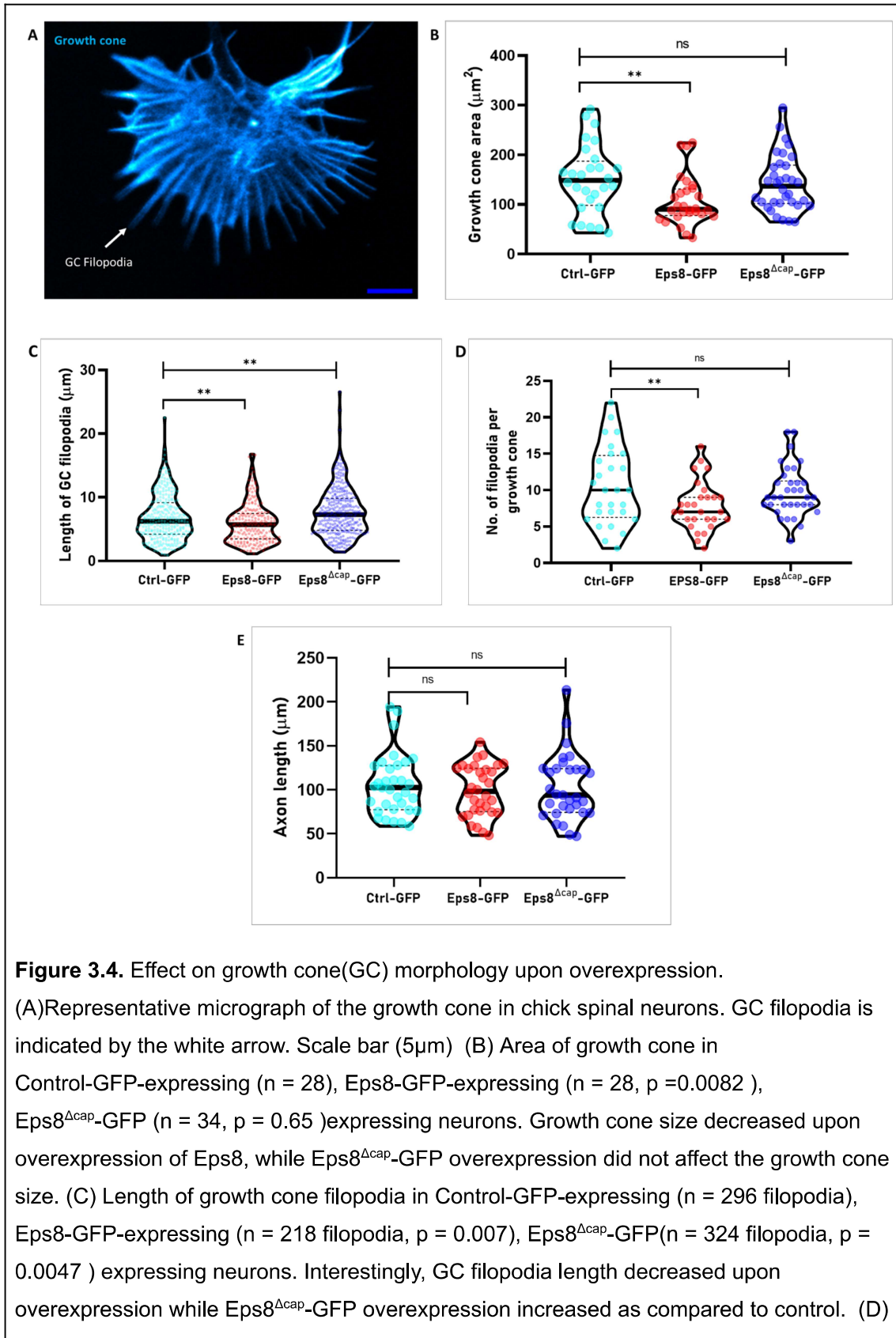
Figure 3.3. Mutation in the capping region of Eps8 alters its location (A) Representative micrograph of a spinal neuron expressing Eps8$^{\Delta cap}$-GFP (Eps8 lacking capping activity). (B) A cartoon representation of the simulated complex between mouse Eps8 (colored red) and one actin monomer in the filament (colored blue) is depicted, where the specific location of mutated residues in the H1 helix is indicated by a green star (Hertzog et al., 2010). (C) Axonal filopodia exhibit a diffused Eps8$^{\Delta cap}$-GFP expression pattern at their base, indicated by blue arrows. (D) Eps8$^{\Delta cap}$-GFP is prominently expressed in the growth cone filopodia, indicated by the red arrows. (E) Representative grayscale micrographs of Eps8$^{\Delta cap}$-GFP (left) and Phalloidin (middle) and the overlay (right) showing both the

Eps8$^{\Delta cap}$-GFP (green) and Phalloidin (red) signals in the axonal shaft. Eps8$^{\Delta cap}$-GFP colocalizes with the actin bundles present in the growth cone. (F) Representative grayscale micrographs of Eps8$^{\Delta cap}$-GFP (left) and Phalloidin (middle) and the overlay (right) showing both the Eps8$^{\Delta cap}$-GFP (green) and Phalloididn (red) signals in the axonal shaft. Eps8$^{\Delta cap}$-GFP colocalizes with the actin bundles present in the axonal filopodia. Scale bar: 10 µm (A), 5 µm (C,D,E,F).

3.4 Overexpression of Eps8 in the neurons affected overall growth cone morphology.

The regulation of growth cone dynamics requires a balance between factors promoting the elongation of the F-actin filament and those inhibiting it, namely, capping proteins. However, the specific capping proteins involved in negatively regulating filopodial dynamics in the growth cone are poorly understood, highlighting the need for further research.

We transfected chick spinal neurons with plasmid construct (Eps8 and Eps8$^{\Delta cap}$) and stained them with phalloidin to visualize actin structures. We conducted a morphometric analysis of spinal neurons overexpressing Eps8 and Eps8$^{\Delta cap}$. Our results showed that overexpression of Eps8 led to a significant reduction in the area of the growth cone (107.6 ± 9.4µm^2) compared to the control (150.8 ± 13µm^2), whereas overexpression of Eps8$^{\Delta cap}$ did not change the growth cone area compared to the control (Figure 3.4B). Interestingly both Eps8 and Eps8$^{\Delta cap}$ overexpression did not change the axon length compared to the control (Figure 3.4E). Furthermore, the overexpression of Eps8 reduced both the length of growth cone filopodia (Figure 3.4C) and the number of filopodia per growth cone (Figure 3.4D) compared to the control. Interestingly, in the case of Eps8$^{\Delta cap}$ overexpression, the length of growth cone filopodia increased (7.6 ± 0.21µm) as compared to the control (6.8 ± 0.20µm). Taken together, Eps8 is an important candidate acting as F-actin capping protein to regulate the growth cone dynamics.

Figure 3.4. Effect on growth cone(GC) morphology upon overexpression.
(A)Representative micrograph of the growth cone in chick spinal neurons. GC filopodia is indicated by the white arrow. Scale bar (5μm) (B) Area of growth cone in Control-GFP-expressing (n = 28), Eps8-GFP-expressing (n = 28, p =0.0082), Eps8$^{\Delta cap}$-GFP (n = 34, p = 0.65)expressing neurons. Growth cone size decreased upon overexpression of Eps8, while Eps8$^{\Delta cap}$-GFP overexpression did not affect the growth cone size. (C) Length of growth cone filopodia in Control-GFP-expressing (n = 296 filopodia), Eps8-GFP-expressing (n = 218 filopodia, p = 0.007), Eps8$^{\Delta cap}$-GFP(n = 324 filopodia, p = 0.0047) expressing neurons. Interestingly, GC filopodia length decreased upon overexpression while Eps8$^{\Delta cap}$-GFP overexpression increased as compared to control. (D)

Number of filopodia per growth cone in Control-GFP-expressing (n = 28),
Eps8-GFP-expressing (n = 28, p = 0.03), Eps8$^{\Delta cap}$-GFP(n = 34 filopodia, p = 0.88)
expressing neurons. (E) Axon length remains unaffected upon Eps8 and Eps8$^{\Delta cap}$
overexpression. Axonal protrusion density in Control-GFP-expressing (n = 33),
Eps8-GFP-expressing (n = 32, p = 0.63), and Eps8$^{\Delta cap}$-GFP-expressing (n = 31, p =0.68)
neurons. The Mann-Whitney test was used to compare the treatments; ns, p >0.05, **p <
0.01, ***p < 0.001, ****p <0.0001.

3.5 The F-actin-capping activity of Eps8 restricts the formation of collateral protrusions.

Next, we analyzed the effect of Eps8-GFP overexpression on axonal filopodia
(Figure 3.5 A-F). Protrusion density is the measure of the same, defined as the
number of filopodia per unit length of the axon. Overexpression of GFP-tagged Eps8
in chick spinal neurons led to a decrease in protrusion density (0.2 ± 0.02
protrusions/μm) compared to the control group of GFP-expressing neurons (0.3 ±
0.02 protrusions/μm) (Figure 3.5 G). However, the overexpression of Eps8$^{\Delta cap}$ did not
change the protrusion density compared to control GFP-expressing neurons. These
results suggest that the F-actin capping activity of Eps8 restricts the formation of
axonal filopodia and is thus crucial for regulating axon collateral branching.
Interestingly, the length of the axonal filopodia remains unaffected upon Eps8
overexpression (Figure 3.5H), suggesting that the capping activity likely regulates
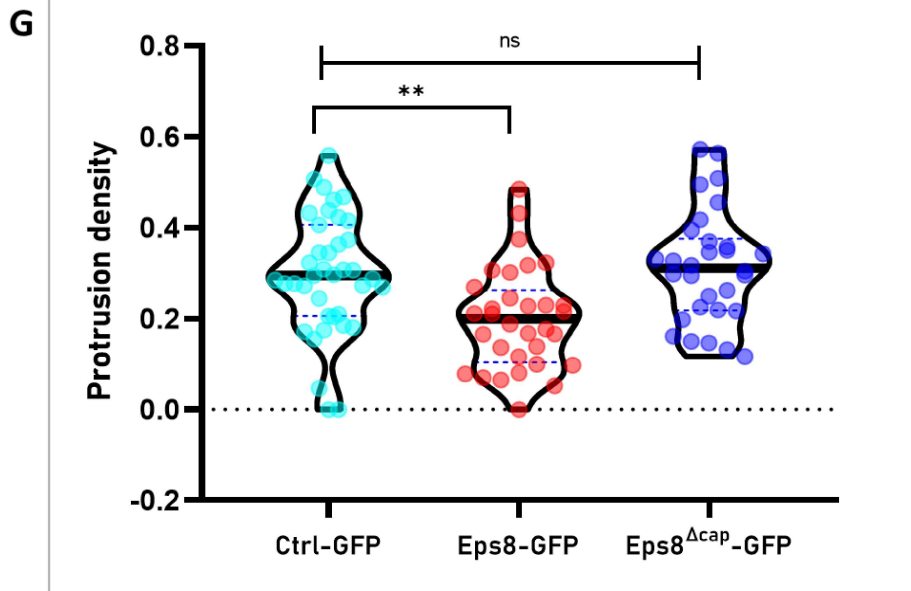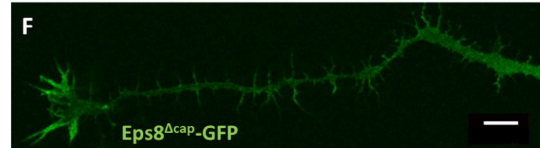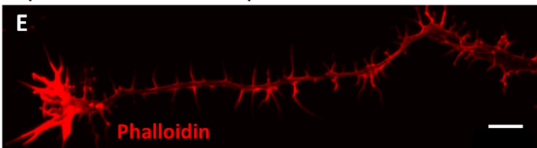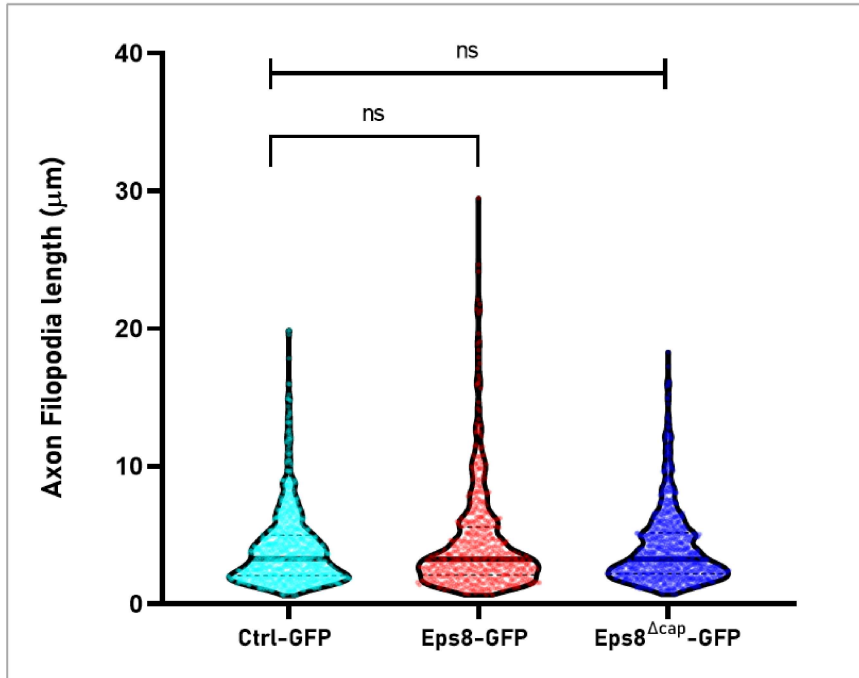initiation rather than the elongation of filopodia.

Control
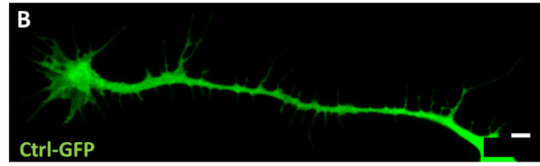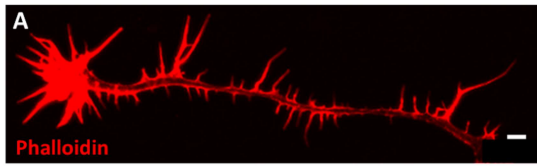
Eps8-GFP Overexpression

Eps8$^{\Delta cap}$-GFP Overexpression

G
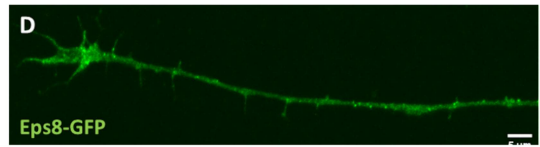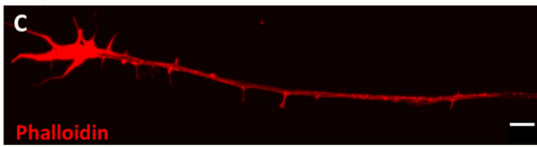
Figure 3.5. Eps8's Actin-Capping Activity regulates axonal filopodia formation

Representative images of neurons with collateral protrusions after transfection of (B) pCAG GFP (D) Eps8 GFP (F) Eps8$^{\Delta cap}$--GFP (green color).

(A, C, E)The neurons were fixed and stained with phalloidin after 36 h of incubation. Phalloidin is depicted in red color. Overexpression of Eps8 shows a reduction in protrusion density, whereas overexpression of Eps8$^{\Delta cap}$ does not affect the protrusion density.

(G)Graphical representation of the Axonal protrusion density in Control-GFP-expressing (n = 39), Eps8-GFP-expressing (n = 32, p = 0.0013), and Eps8$^{\Delta cap}$-GFP expressing (n = 30, p,0.58) neurons. Welch's t-test was used as a check for significance.

(H) Protrusion length remains unaffected upon Eps8 overexpression. Axonal protrusion length in Control-GFP-expressing (n = 1123 protrusions), Eps8-GFP expressing (n = 635 protrusions, p = 0.46), and Eps8$^{\Delta cap}$-GFP expressing (n = 948, p=0.31) neurons. The Mann-Whitney test was used as a check for significance.

ns, p >0.05, **p < 0.01, ***p < 0.001, ****p <0.0001. Three separate experiments were carried out to obtain the resulting data values. Scale bar: 5 μm (A, B, C, D) and 10 μm (E, F).

## 3.6 Eps8 regulates the dynamics of axonal F-actin patches.

F-actin patches are the precursor to form axonal filopodia. The axonal F-actin patch is a complex structure that is maintained in a dynamic equilibrium between F-actin nucleation, elongation, capping, as well as depolymerizing activities. However, only a small proportion of actin patches have the capacity to initiate a protrusion, with the largest and long-lived patches having the highest probability (Kundu et al., 2022). The precise mechanisms that regulate the dynamic equilibrium of the F-actin patch remain unclear. Given the involvement of Eps8 in generating axonal filopodia , we investigated if the capping function of Eps8 regulated the axonal F-actin patches.

We overexpressed spinal neurons with Eps8 and Eps8$^{\Delta cap}$ and stained them with phalloidin to visualize actin structures. Upon Eps8 overexpression, the number of F-actin patches per unit length axonal length was unchanged compared to control GFP transfected neurons (Figure 3.6A). However, the size of the F-actin patches was significantly reduced to almost half upon Eps8 overexpression (0.25 ± 0.02 µm^2) compared with the Ctrl-GFP neurons (0.44 ± 0.037 µm^2) (Figure 3.6B). The F-actin patch size of Eps8$^{\Delta cap}$ neurons did not change (0.43 ± 0.04 µm^2) as compared to the control.

To further investigate the role of Eps8 in regulating F-actin patch dynamics, we took time-lapse images of F-tractin mCherry (Trac-mCherry) to visualize the F-actin networks. Using time-lapse images, we generated kymographs to analyze the dynamics of F-actin patches. Our kymograph analysis revealed that the lifetime of the F-actin patches was significantly reduced upon overexpression of Eps8, from 177.5 ± 15 s in control groups to 121.5 ±13.21 s (Figure 3.6 E). This suggested that in neurons overexpressing Eps8, the newly formed actin patch was unstable, unable to elaborate, and dissipated rapidly (Figures 3.6C and 3.6D). A frequency distribution of patch numbers across 20-second bins showed that the Eps8 overexpression resulted in more patches under fell within the 20-60 s bin, while very few patches crossed the 240 s, as compared to the control group (Figure 3.6E).

In summary, results suggest that Eps8 does not play a role in the initiation of actin patch formation but rather regulates the size and lifetime of newly nucleated actin

patches. The regulation of actin patch size is dependent on the capping activity of Eps8. Overexpression of Eps8 is likely to result in excessive capping of F-actin filaments resulting in smaller and short-lived F-actin patches. In turn, smaller and more labile patches are likely to generate fewer collateral protrusions.
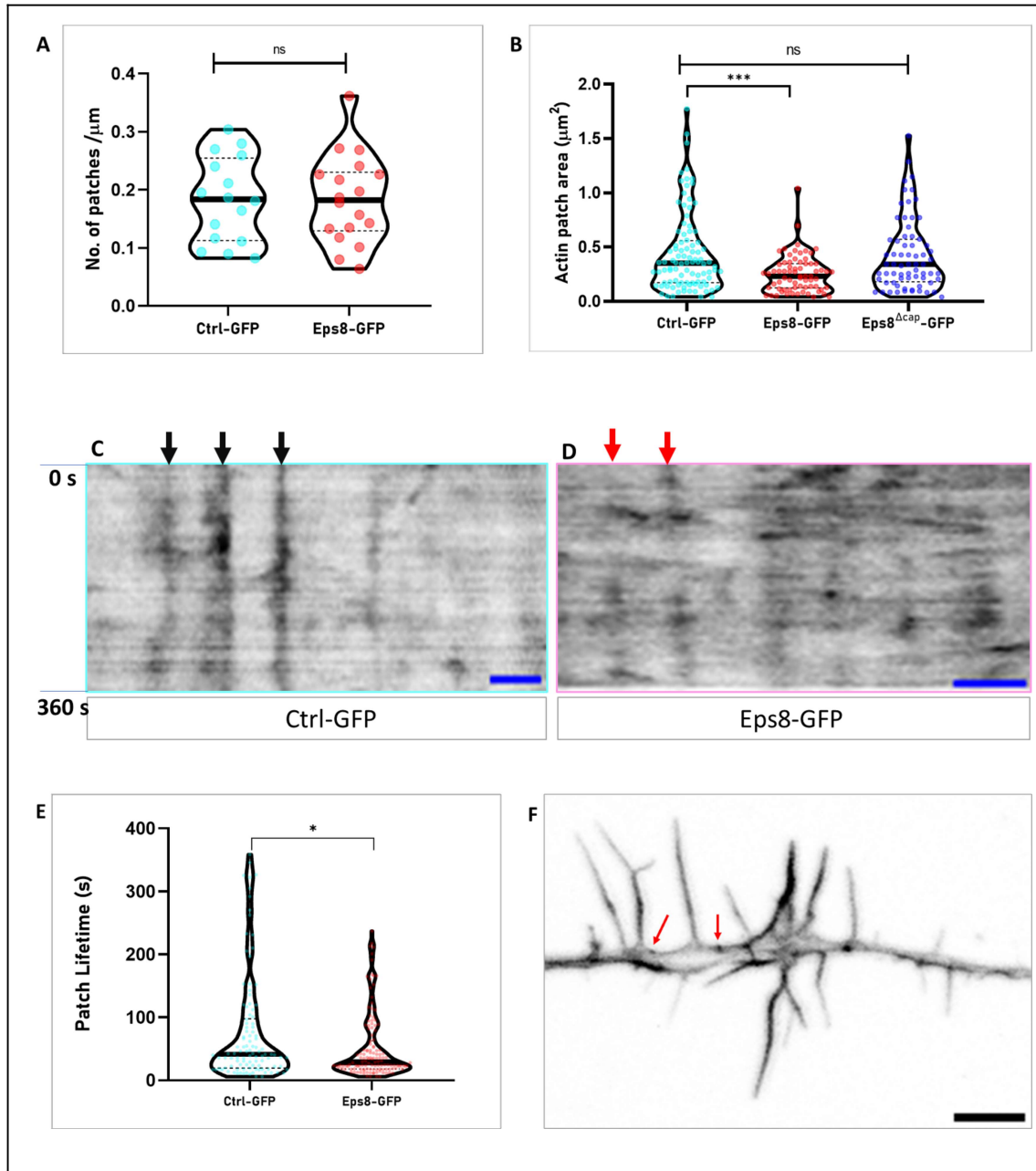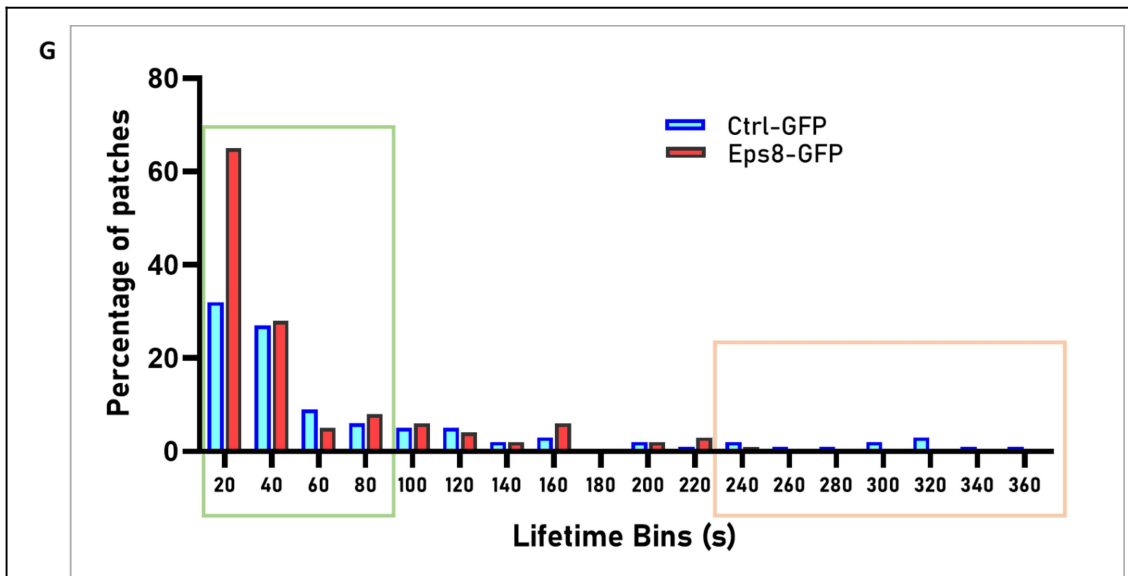
Figure 3.6. Eps8 regulates the newly formed patch.

(A)The density of F-actin patches in the entire axon is unaltered between Control-GFP (n = 16 axons) and Eps8-GFP (n = 18 axons; p = 0.9725) transfected neurons. (B) Actin patch area significantly reduces upon Eps8 over-expression. Actin patch in Control-GFP-expressing (n = 96 actin patches), Eps8-GFP-expressing (n = 70 actin patches, p = p=0.0004), and Eps8$^{\Delta cap}$-GFP expressing (n =68 actin patches, p= 0.85) neurons. (C) Representative kymograph for Ctrl-GFP (C) and (D) for Eps8-GFP transfected neurons. Arrows mark actin patches, black arrowheads in C indicate the long-lasting patches in control whereas red arrowheads in D indicate the patch cannot elaborate further after some point due to excessive capping by Eps8. (E) Graphic representation of patch lifetime in Control-GFP (n = 106 actin patches) and Eps8-GFP (n = 134 actin patches; p = 0.014) transfected neurons. (F) Representative micrograph showing actin patch in spinal neurons indicated by red arrows. (G)Frequency Distribution of all actin patch lifetimes in 20-s bins across the 6 min of time-lapse imaging in Eps8 overexpression (Ctl-GFP; n = 47and Eps8-GFP; n = 33).

Eps8 GFP overexpression increases the proportion of short-lived patches (green box) compared with control-GFP (orange box) (p =0.04; Kolmogorov–Smirnov test). Scale bar (C,D) 20um; (F) 5um. (A, B, E) The Mann-Whitney test was used as a check for significance. ns, p >0.05, **p < 0.01, ***p < 0.001, ****p <0.0001.

3.7 The anti-capping activity of formins is counterbalanced by the capping activity of Eps8 to regulate the formation of protrusion.

Recent studies in our lab show that overexpression of Fmn2 increases the axon collateral protrusion density along with the size and lifetime of the axonal F-actin patches in chick spinal neurons (Kundu et al., 2022). Conversely, the reduction of Fmn2 levels reduces collateral branching and actin patch sizes and stability. Fmn2 regulates F-actin patches, in part, by insulating the F-actin filaments from ADF-mediated disassembly and thereby maintains a dynamic balance to regulate collateral branching (Kundu et al., 2022). In addition to actin filament nucleation/elongation, formin family members are also known to compete with F-actin capping activities. Both in vivo and in vitro studies have shown that formins and capping protein counterbalance each other's activities (see Shekhar et al., 2016, for a review). Fmn2 can also compete with capping protein in vitro (unpublished data from Priyanka Dutta and Aurnab Ghose). The axonal F-actin patch regulatory role of the capping protein Eps8 identified in this study offers an excellent system to test Fmn2 and Eps8 collectively maintain F-actin patch dynamics and, consequently axonal protrusions.

First, we performed live imaging of neurons transfected with fluorophore-tagged Eps8 and Fmn2. We observe that Eps8 and Fmn2 assemble together at the same time at the base of the branch before the formation of the protrusion (Figure 3.7 F, G, H).

Further, we co-transfected Fmn2 and Eps8 in the chick spinal neurons and stained with phalloidin. Coexpression of both Fmn2 and Eps8 was able to rescue the reduction in actin patch size (Figure 3.7D) and protrusion density (Figure 3.7C) caused by Eps8 overexpression alone. Consistent with the earlier report (Kundu et al., 2022), the overexpression of Fmn2 alone increased the actin patch size and also the density of axonal protrusions as compared to the GFP control.

In summary, the localization of Eps8 and Fmn2 at the base of the branch prior to its formation and rescue of the phenotype caused by Eps8 overexpression suggest a
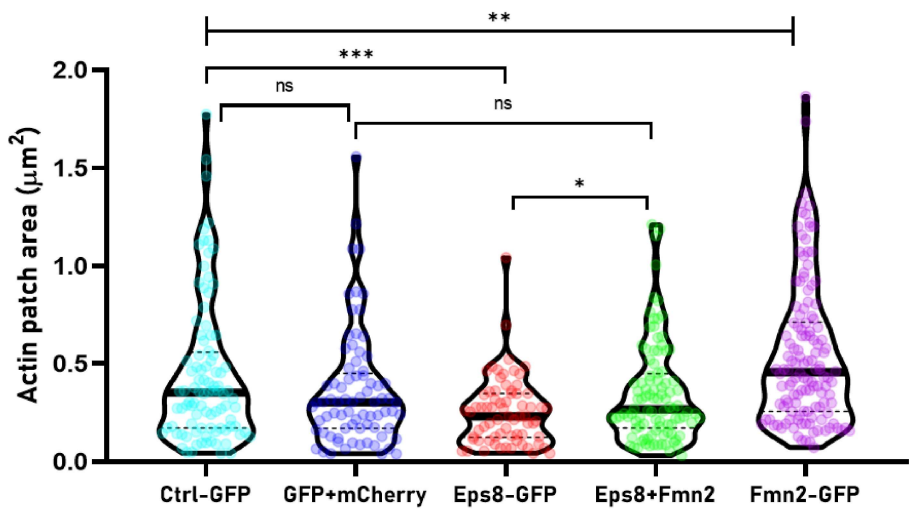
possible interaction between Fmn2 and Eps8 in regulating collateral branch formation.
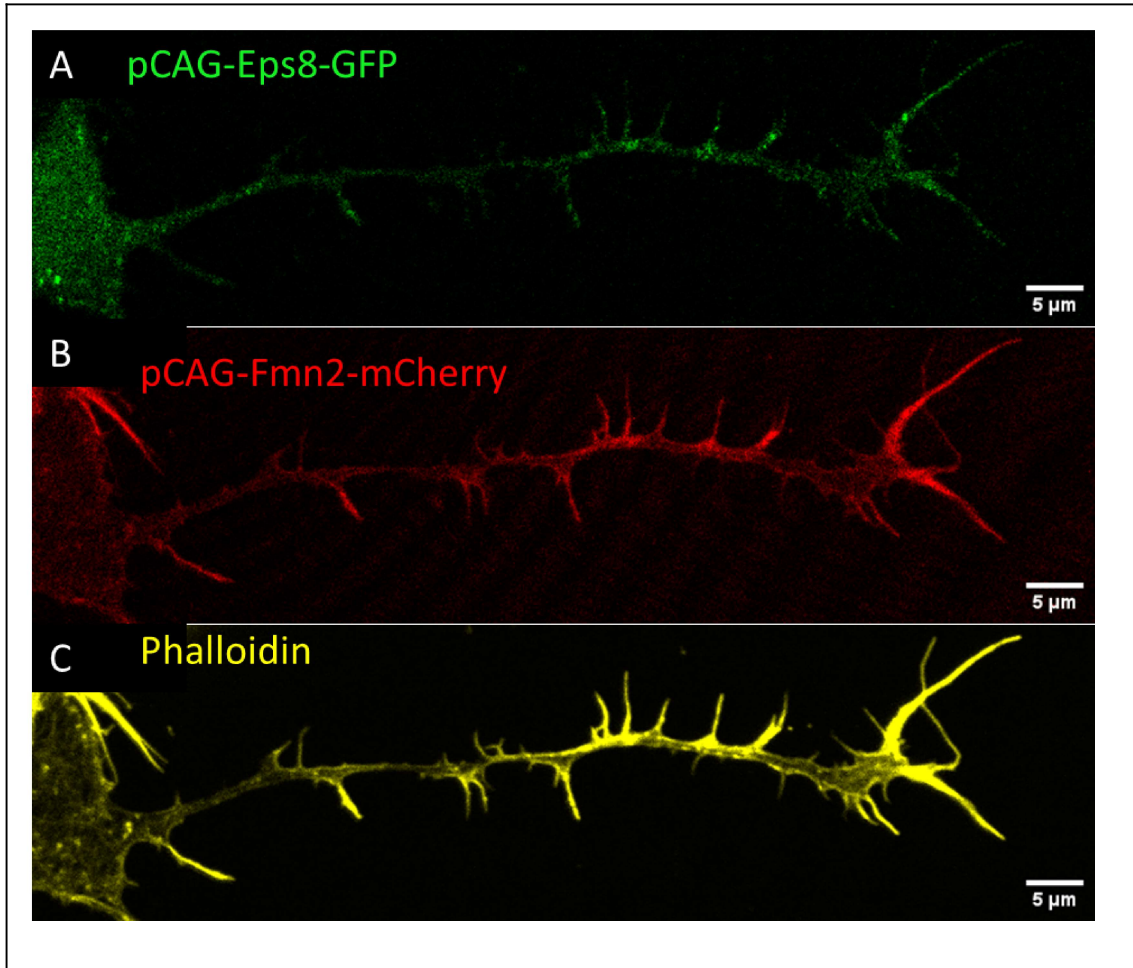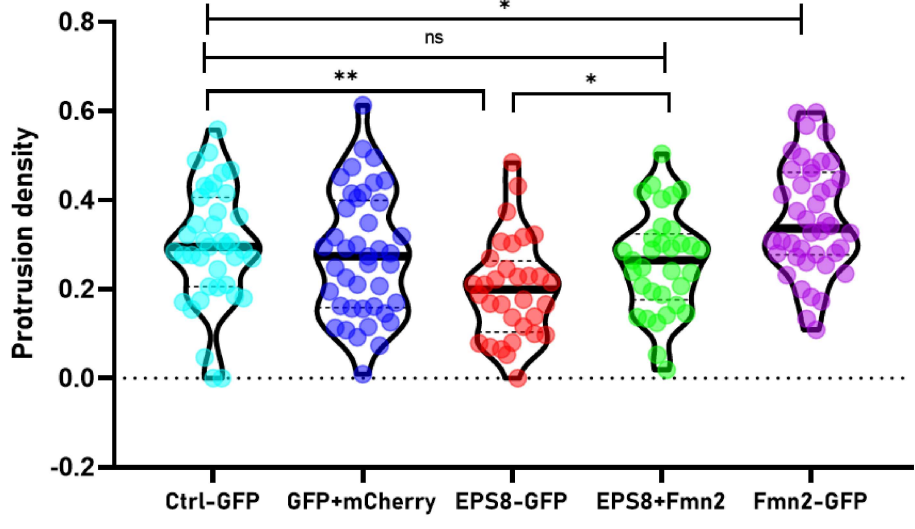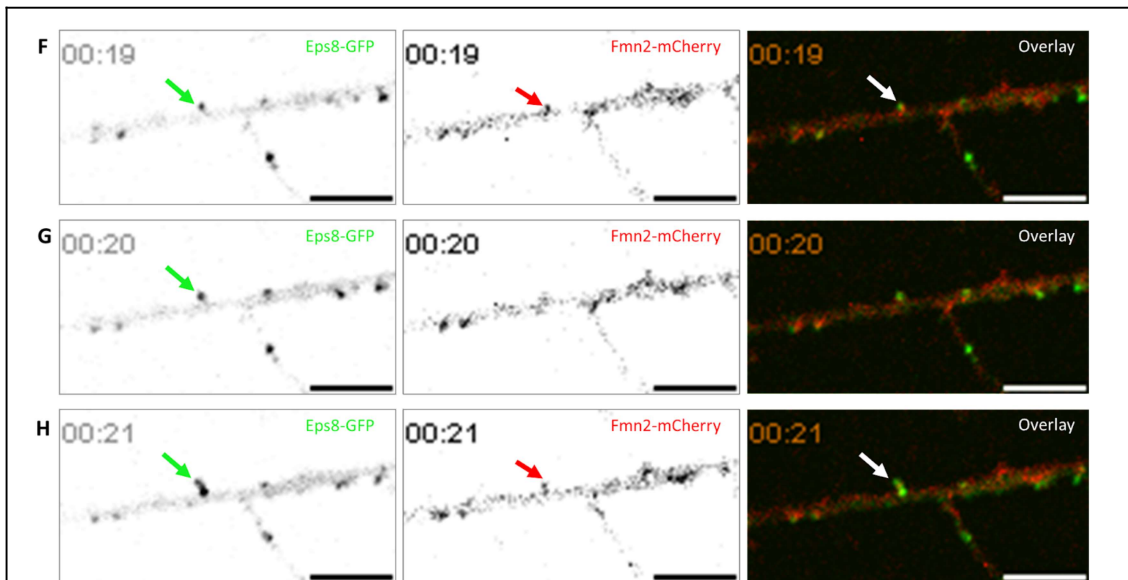
Figure 3.7. Counterbalancing activities of Eps8 and Fmn2

(A, B, C) Co-expression of both Eps8 GFP and Fmn2 mCherry, followed by phalloidin staining. (A) represents Eps8 overexpressed, (B) represents Fmn2 mCherry overexpressed in the same neuron (C) represents phalloidin 647 staining. (D) Graphic representation of axonal protrusion density in Ctl-GFP-expressing (n = 39 axons), Ctl-GFP+ mCherry expressing (n = 41axons, p =0.5), Eps8-GFP-expressing (n = 32axons , p = 0.0013), Eps8 GFP+Fmn2 mcherry-expressing (n =33 axons, p =0.2) , and Fmn2 GFP-expressing (n =42 , p =0.035) neurons. Welch's t-test was used as a check for significance. (E) Comparision of F-actin patch areas in Ctrl-GFP-expressing (n =96 actin patches), Ctrl-GFP + mCherry expressing (n =77actin patches, p=0.18), Eps8-GFP-expressing (n =70 actin patches , p = 0.0004), Eps8 GFP+Fmn2 mcherry-expressing (n =100 actin patches , p =0.12) , and Fmn2 GFP-expressing (n =130 actin patches , p =0.0044) neurons. (F, G, H) Representative grayscale micrographs of Eps8-GFP (left) and Fmn2-mCherry (middle) and the overlay (right) showing both the Eps8 (green) and Fmn2-mCherry (red) signals . (F) The snaps taken at 19 sec (G) indicate the snaps taken at 20 sec (H) indicate the snaps taken at 21 sec of 5 minutes of live imaging. The Mann-Whitney test was used to compare the treatments. Scale bar (A, B, C, F, G, H) 5um.

Chapter 4 **Discussion**

The axon shaft comprises functionally distinct cytoskeletal structures, including F-actin trails, hotspots, the membrane-associated periodic cytoskeleton (MPS), and F-actin patches that induce collateral branching. Precise regulatory mechanisms are required to maintain the unique properties of these structures and control actin assembly spatiotemporally. Capping protein is known to have a critical role in maintaining the structurally and functionally distinct actin architecture. While the initiation of the axon collateral branch with filopodia as a precursor is well studied, the regulation of the F-actin patch remains poorly understood. In this study, we present evidence demonstrating the crucial role of the capping activity of Eps8 in developing spinal neurons. Eps8 regulates collateral branching by regulating actin patch dynamics. Furthermore, we show that Eps8 and Fmn2 counterbalance their respective activities, thereby maintaining a dynamic equilibrium of the F-actin patch and regulating collateral branch formation.

We find that Eps8 is present at the axonal filopodia tips and colocalizes with the actin patch, both of which are critical sites for collateral branch regulation in the chick spinal neurons. Overexpression of Eps8 did not affect the density of actin patches in the axon, suggesting that it is not involved in initiating actin patches. The overexpression of Eps8 in the neurons decreased the actin patch size and lifetime. However, Eps8$^{\Delta cap}$ overexpression does not affect protrusion density and the size of actin patches. Lifetime analysis of the patch upon Eps8 overexpression demonstrates that the formation of the patch is impeded and subsequently dissipated, indicating that it is unable to elaborate further. Studies have shown that capping proteins caps the barbed end of the nucleated core formed by the Arp2/3 complex and protects them from formin activity in the fission yeast (Billault-Chaumartin and Martin, 2019). This supports our results as overexpression of Eps8 can rapidly cap newly nucleated actin filaments by its capping activity to prevent their elongation. In turn, excessive capping activity would lead to reduced

size and a lifetime of the actin patch. A further consequence of smaller and more labile F-actin patches is decreased axonal protrusion density. A larger patch that stays for a long time has a higher propensity to transition into a filopodium (Loudon et al., 2006). If most of the newly formed patches are strongly capped, they do not reach critical size and stable lifetimes required to initiative mebrane protrusion. This suggests that the capping activity of Eps8 is regulating F-actin patch dynamics and thus collateral branching. If most of the newly formed patches are capped in the axon, stability decreases, and the patch cannot live for a long time and thus cannot form protrusive structures.

Our analysis indicate that overexpression of Eps8 does not affect the length of axonal filopodia, despite the fact that increased capping activity is generally expected to inhibit actin polymerization and thereby shorten the length of the axonal filopodia. This can be explained by considering the availability of a limiting cellular factor, namely monomeric G-actin. Due to the fact that the majority of the newly formed actin filaments are capped within the axon, and thus, monomeric G-actin pools remain free in the axon shaft to facilitate actin polymerization in the filopodia. Moreover, capping protein (CP) is known to play a crucial role in maintaining sufficient supply of actin monomers for actin-based motility (Carlier and Pantaloni, 1997; Loisel et al., 1999; Pollard and Borisy, 2003). Computer simulations and mathematical modeling have demonstrated that molecular noise arising from capping protein binding and unbinding can result in macroscopic fluctuations in filopodial lengths. Some filopodia rapidly grow longer due to bundle thinning, requiring less actin transport, while others are capped and retract towards the filopodial base, ultimately disappearing. This observation was in contrast to system that lack capping proteins, which exhibit only minuscule fluctuations in filopodial length (Zhuravlev and Papoian, 2009).

The proper regulation of various activities such as F-actin nucleation, elongation, bundling, and capping, retrograde flow of actin is essential for achieving appropriate growth cone motility and retraction to response extracellular cues. The heterodimeric Capping Protein (CP) regulates axon elongation, and growth cone morphology and may have a role in axon guidance in Drosophila (Drees and Gertler, 2008). Of note, the Capping protein subunit, CapzB2 may modulate the morphology of growth

cones in mouse hippocampal neurons not by its well-known capping activity but rather by interacting with microtubules (Davis et al., 2009). However, the specific capping proteins involved in regulating growth cone morphology in vertebrates remain unclear. We find that elevated levels of Eps8 led to significant changes in overall growth cone morphology, and overexpression of capping mutant did not elicit any observable changes. This suggests a role for the capping activity of Eps8 in growth cone regulation. However, despite the decrease in growth cone size, the length of axons remains unaffected. According to Stamatakou et al.'s studies, Eps8 gain of function increases the growth cone area with no change in the length and number of growth cone filopodia in DRG neurons of newborn mice, which contradicts our results. It should be noted that there were differences in the model organism and developmental time point used in our respective studies. Stamatakou et al. study stated that Eps8 does not show its capping and bundling activities during the terminal remodeling of axons. Instead, Eps8 interacts with Dvl1 protein via Wnt signaling and promotes axon remodeling by changing actin dynamics in the growth cone (Stamatakou et al., 2015). Taken together, the capping activity of Eps8 regulates growth cone morphology and may have a role in axon guidance.

Recent studies in our lab suggest that Fmn2 does not participate in the initiation process of actin patch formation. Instead, its nucleation activity serves to stabilize and maintain the lifetime of the actin patch, which in turn increases the probability of generating actin-rich structures that are essential for membrane protrusion formation. This study further indicates that Fmn2 knockdown results in a "patch blinking phenomenon," where certain regions of the axon exhibit repeated cycles of actin patch formation and disappearance. The blinking phenomenon observed in a specific region of the axon can be attributed to the inability of the nucleating core to elongate further due to the absence of the elongating factor Fmn2 (Kundu et al., 2022). In our study, we did not observe repeated cycles of actin patch formation. Rather, we observed that the newly formed actin patch exhibits a limited ability to elaborate and subsequently becomes unstable, ultimately leading to its sudden dissipation. This implies that when a patch is formed and starts to expand, the presence of abundant eps8 in the axon results in the capping of the developing actin patch leading to the disassembly from the pointed end.

The aforementioned study also reveals an antagonistic relationship between Fmn2 and ADF, which regulates actin patch stability and axonal branching. ADF/cofilin, a member of the F-actin severing and disassembly factor family, plays a crucial role in the turnover of actin and regulating diverse F-actin networks, including collateral branching. Fmn2 acts as an insulation factor to protect actin filaments from disassembly by ADF and maintains dynamics equilibrium of actin assembly and disassembly in the actin patch (Kundu et al., 2022). A recent study by Mizuno et al. provides evidence that mammalian formin protein mDia1 can facilitate F-actin assembly and confer resistance to the disruptive effects of cofilin. This resistance is because of helical mDia1 rotation along the long-pitch helix during the elongation of F-actin, which results in the twisting of F-actin in the opposite direction to that induced by cofilin. This twisting converts F-actin into a state of resistance to cofilin (Mizuno et al., 2018). In this study, we investigated whether the protein Fmn2 interacts with capping protein Eps8 to regulate the dynamic balance of actin patches. FH2 domains of formins have a short amphipathic helix attached to a hydrophobic pocket on actin's barbed face in the cleft of subdomains 1 and 3. The capping protein's β-tentacle competes with the FH2 domain's knob for binding to actin, while other regions of these proteins maintain their association with other actin sites. The partial overlap of binding sites on the barbed end of actin allows for the potential formation of a ternary complex when two distinct regulators, i.e., forming and capping proteins, are present.

 The dissociation of formin from barbed ends is facilitated by capping protein, which leads to the formation of a transient ternary complex consisting of the barbed end, formin, and capping protein. This complex reduces the affinity of both formin and capping protein for the barbed end promotes rapid displacement of each other. Thus, capping protein regulates formin-mediated actin filament elongation by restricting the duration of formin's association with the barbed end. Conversely, formin can bind a capped barbed end and reinstate its rapid growth by displacing capping protein (Shekhar et al., 2015). This supports our results that the co-expression of Eps8 and Fmn2 results in a significant increase in protrusion density and actin patch size when compared to the overexpression of Eps8 alone. Live imaging of cells expressing fluorescently tagged Eps8 and Fmn2 proteins has revealed that both proteins assemble at the same time prior to protrusion formation. This observation suggests that the coordinated expression of these two proteins may be critical for the

formation of collateral protrusions. In order to obtain conclusive evidence, further experimentation is required.

In summary, we demonstrate that the Eps8 capping activity regulates specific Actin patch architecture in the axon shaft that is crucial for the initiation of axon collateral branching. Moreover, Capping activity of Eps8 have a crucial role in regulating growth cone dynamics. We identify the interaction between Fmn2 and Eps8 in regulating the actin patch dynamics that are essential for axonal branching.

The forthcoming experiment will be focused on the function of endogenous Eps8 in the regulation of F-actin patch dynamics. To achieve this, knockdown experiments will be conducted to reduce the levels of Eps8 protein in spinal neurons. Moreover, to strengthen the evidence implicating the interaction of Fmn2 and Eps8 in the regulation of F-actin patch dynamics, knockdown studies of both Eps8 and Fmn2 will be performed.To study the spatiotemporal dynamics of Eps8 and Fmn2 during the formation of axonal filopodia, Opto-TrkA, an optogenetic construct of TrkA, will be used. This approach will allow for the localized activation of the receptor, resulting in branch formation at a predetermined location.
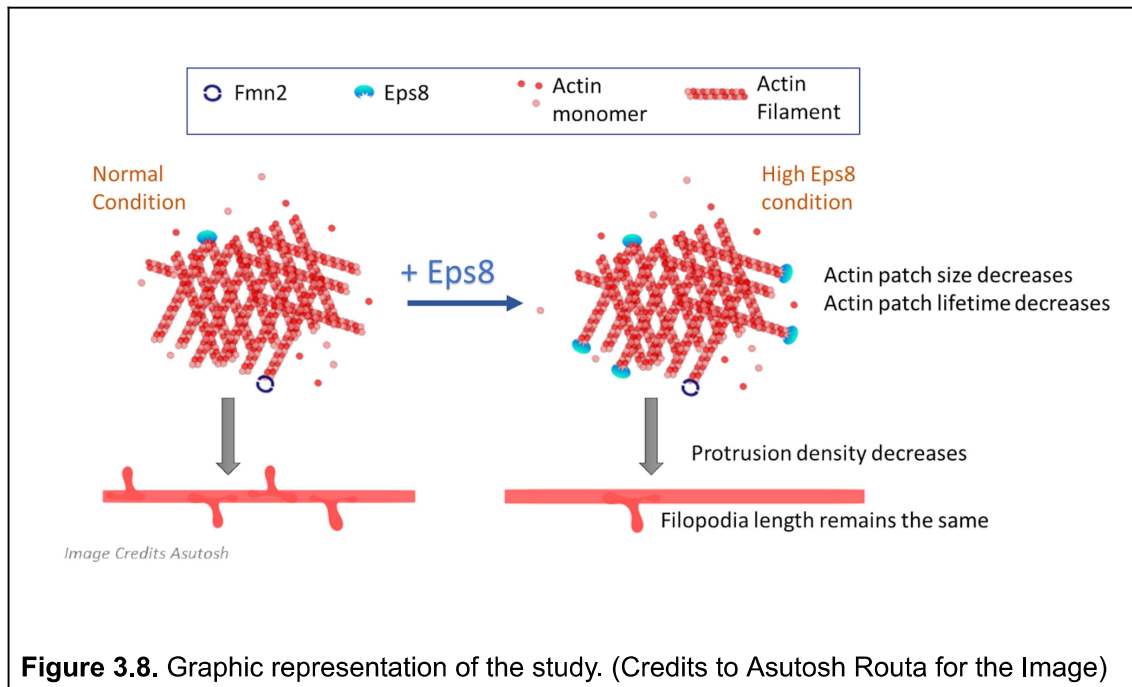
Figure 3.8. Graphic representation of the study. (Credits to Asutosh Routa for the Image)

References

1. Ahuja, R., Pinyol, R., Reichenbach, N., Custer, L., Klingensmith, J., Kessels, M.M., and Qualmann, B. (2007). Cordon-bleu is an actin nucleation factor and controls neuronal morphology. Cell 131, 337–350.
2. Armijo-Weingart, L., and Gallo, G. (2017). It takes a village to raise a branch: Cellular mechanisms of the initiation of axon collateral branches. Mol. Cell. Neurosci.
3. Bhattacharya, N., Ghosh, S., Sept, D. and Cooper, J. A. (2006). Binding of myotrophin/V-1 to actin-capping protein: implications for how capping protein binds to the filament barbed end. J. Biol. Chem.
4. Billault-Chaumartin, I., & Martin, S. G. (2019). Capping Protein Insulates Arp2/3-Assembled Actin Patches from Formins. *Current biology.*
5. Brandt, Dominique T., Sabrina Marion, Gareth Griffiths, Takashi Watanabe, Kozo Kaibuchi, and Robert Grosse. 2007. "Dia1 and IQGAP1 Interact in Cell Migration and Phagocytic Cup Formation." Journal of Cell Biology .
6. Cao, L. G., Babcock, G. G., Rubenstein, P. A., & Wang, Y. L. (1992). Effects of profilin and profilactin on actin structure and function in living cells. *The Journal of cell biology, 117*(5), 1023–1029.
7. Carlier M-F, Pantaloni D (1997). Control of actin dynamics in cell motility. J Mol Biol 269, 459–467.
8. Campellone, Kenneth G, and Matthew D Welch. 2010. "A Nucleator Arms Race: Cellular Control of Actin Assembly," no. March.
9. Castrillon, D H, and S A Wasserman. 1994. "Diaphanous Is Required for Cytokinesis in Drosophila and Shares Domains of Similarity with the Products of the Limb Deformity Gene." Development 120 (12): 3367–77
10. Chen, B., Chou, H.-T., Brautigam, C.A., Xing, W., Yang, S., Henry, L., Doolittle, L.K., Walz, T., and Rosen, M.K. (2017). Rac1 GTPase activates the WAVE regulatory complex through two distinct binding sites. Elife 6.
11. Chesarone, M., Gould, C. J., Moseley, J. B., & Goode, B. L. (2009). Displacement of formins from growing barbed ends by bud14 is critical for actin cable architecture and function. *Developmental cell, 16*(2), 292–302.
12. Croce A, Cassata G, Disanza A, Gagliani MC, Tacchetti C, et al. (2004) A novel actin barbed-end-capping activity in EPS-8 regulates apical morphogenesis in intestinal cells of Caenorhabditis elegans. Nat Cell Biol 6: 1173–1179. 17.
13. Disanza A, Mantoani S, Hertzog M, Gerboth S, Frittoli E, et al. (2006) Regulation of cell shape by Cdc42 is mediated by the synergic actin-bundling activity of the Eps8-IRSp53 complex. Nat Cell Biol 8: 1337–1347.

14. Disanza A, Carlier MF, Stradal TE, Didry D, Frittoli E, et al. (2004) Eps8 controls actin-based motility by capping the barbed ends of actin filaments. Nat Cell Biol 6: 1180–1188.

15. Di Fiore, P. P. & Scita, G.(2002). Eps8 in the midst of GTPases. Int. J. Biochem. Cell Biol. 34, 1178–1183.

16. Dong, Yuqing, David Pruyne, and Anthony Bretscher. 2003. "Formin-Dependent Actin Assembly Is Regulated by Distinct Modes of Rho Signaling in Yeast." Journal of Cell Biology 161 (6): 1081–92.

17. Drees F, Gertler F. 2008. Ena/VASP: proteins at the tip of the nervous system. Curr Opin Neurobiol 18: 53–59.

18. Flynn KC, Bradke F (2020) Role of the cytoskeleton and membrane trafficking in axon–dendrite morphogenesis. In: Cellular Migration and Formation of Axons and Dendrites, pp 21–56. Elsevier.

19. Fujiwara, I., Remmert, K., & Hammer, J. A., 3rd (2010). Direct observation of the uncapping of capping protein-capped actin filaments by CARMIL homology domain 3. *The Journal of biological chemistry, 285*(4), 2707–2720.

20. Gallo, G. (2011). The cytoskeletal and signaling mechanisms of axon collateral branching. Dev. Neurobiol. 71, 201–220.

21. Gallo, G. (2015). Localized regulation of the axon shaft during the emergence of collateral branches. Neural Regen. Res. 10, 1206–1208.

22. Higgs, Henry N. 2005. "Formin Proteins: A Domain-Based Approach." Trends in Biochemical Sciences 30 (6): 342–53.

23. Hu, J., Bai, X., Bowen, J.R., Dolat, L., Korobova, F., Yu, W., Baas, P.W., Svitkina, T., Gallo, G., and Spiliotis, E.T. (2012). Septin-driven coordination of actin and microtubule remodeling regulates the collateral branching of axons. Curr. Biol. 22, 1109–1115.

24. Joy, M. E., Vollmer, L. L., Hulkower, K., Stern, A. M., Peterson, C. K., Boltz, R. C., Roy, P., & Vogt, A. (2014). A high-content, multiplexed screen in human breast cancer cells identifies profilin-1 inducers with anti-migratory activities. *PloS one, 9*(2), e88350.

25. Kerstein PC, Nichol RH, Gomez TM (2015) Mechanochemical regulation of growth cone motility. Front Cell Neurosci 9:244.

26. Ketschek, A., and Gallo, G. (2010). Nerve growth factor induces axonal filopodia through localized microdomains of phosphoinositide 3-kinase activity that drive the formation of cytoskeletal precursors to filopodia. J. Neurosci. Off. J. Soc. Neurosci. 30, 12185–12197.

27. Ketschek, A., Spillane, M., and Gallo, G. (2011). Mechanism of NGF-induced formation of axonal filopodia: NGF turns up the volume, but the song remains the same. Commun. Integr. Biol. 4, 55–58.

28. Ketschek, A., Jones, S., Spillane, M., Korobova, F., Svitkina, T., and Gallo, G. (2015). Loudon Robert P. , Silver Lee D., Yee Jr. Hal F., Gallo Gianluca. 2006. "RhoA-Kinase and Myosin II Are Required for the Maintenance of Growth Cone Polarity and Guidance by Nerve Growth Factor" 66 (8): 847–67.

29. Ketschek, A., Spillane, M., Dun, X.P., Hardy, H., Chilton, J., and Gallo, G. (2016). Drebrin coordinates the actin and microtubule cytoskeleton during the initiation of axon collateral branches. Dev. Neurobiol. 76, 1092–1110.

30. Kovar, D. R., Wu, J. Q., & Pollard, T. D. (2005). Profilin-mediated competition between capping protein and formin Cdc12p during cytokinesis in fission yeast. *Molecular biology of the cell*, *16*(5), 2313–2324.

31. Littlefield, R. & Fowler, V. M. (1998)Defining actin filament length in striated muscle: rulers and caps or dynamic stability? Annu. Rev. Cell Dev. Biol. 14, 487–525.

32. Loisel TP, Boujemaa R, Pantaloni D, Carlier M-F (1999). Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401, 613–616.

33. Lowery LA, Van Vactor D (2009) The trip of the tip: understanding the growth cone machinery. Nat Rev Mol Cell Biol 10:332–343

34. Martelli, A.M., Evangelisti, C., Chappell, W., Abrams, S.L., Bäsecke, J., Stivala, F., Donia, M., Fagone, P., Nicoletti, F., Libra, M., et al. (2011). Targeting the translational apparatus to improve leukemia therapy: roles of the {PI3K/PTEN/Akt/mTOR} pathway. Leukemia 25, 1064–1079

35. Mizuno H, Tanaka K, Yamashiro S, Narita A, Watanabe N (2018) Helical rotation of the diaphanous-related formin mDia1 generates actin filaments resistant to cofilin. Proc Natl Acad Sci U S 115:E5000–E5007.

36. McGough, A. M., Staiger, C. J., Min, J. K. & Simonetti, K. D. (2003)The gelsolin family of actin regulatory proteins: modular structures, versatile functions. FEBS Lett. 552, 75–81. 21.

37. Menna E, Disanza A, Cagnoli C, Schenk U, Gelsomino G, et al. (2009) Eps8 Regulates Axonal Filopodia in Hippocampal Neurons in Response to BrainDerived Neurotrophic Factor (BDNF). PLoS Biol 7(6): e1000138. doi:10.1371/journal.pbi

38. Menna E, Disanza A, Cagnoli C, Schenk U, Gelsomino G, et al. (2009) Eps8 Regulates Axonal Filopodia in Hippocampal Neurons in Response to BrainDerived Neurotrophic Factor (BDNF). PLoS Biol 7(6): e1000138. doi:10.1371/journal.pbi

39. Menna, E., Zambetti, S., Morini, R., Donzelli, A., Disanza, A., Calvigioni, D., Braida, D., Nicolini, C., Orlando, M., Fossati, G., Cristina Regondi, M., Pattini, L., Frassoni, C., Francolini, M., Scita, G., Sala, M., Fahnestock, M., & Matteoli, M. (2013). Eps8 controls dendritic spine density and synaptic plasticity through its actin-capping activity. *The EMBO journal*, *32*(12), 1730–1744.

40. Menon S, Gupton S (2018) Recent advances in branching mechanisms underlying neuronal morphogenesis.

41. Narita, A., Takeda, S., Yamashita, A., & Maéda, Y. (2006). Structural basis of actin filament capping at the barbed-end: a cryo-electron microscopy study. *The EMBO journal*, *25*(23), 5626–5633.

42. Patel, R.K., and Hardy, R.W. (2012). The role for the Phosphatidylinositol {3-KinaseAkt-TOR} Pathway during Sindbis Virus Replication in Arthropods. J. Virol. 86, 3595–3604

43. Paul, Aditya S., and Thomas D. Pollard. 2009. "Energetic Requirements for Processive Elongation of Actin Filaments by FH1FH2-Formins." Journal of Biological Chemistry 284 (18): 12533–40.

44. Pollard TD, Borisy GG (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell 1

45. Qiang L, Yu W, Andreadis A, Luo M, Baas PW. Tau protects microtubules in the axon from severing by katanin. J Neurosci. 2006; 26:3120–3129. [PubMed: 16554463]

46. Sanes, D H, T A Reh, and W A Harris. 2005. Development of the Nervous System. Elsevier Science

47. Shekhar, S., Kerleau, M., Kühn, S. *et al.* (2015) Formin and capping protein together embrace the actin filament in a ménage à trois. *Nat Commun* 6, 8730 .

48. Sofroniew, M.V. (2018). Dissecting spinal cord regeneration. Nature 557, 343–350

49. Spillane, M., Ketschek, A., Donnelly, C.J., Pacheco, A., Twiss, J.L., and Gallo, G. (2012). Nerve Growth Factor-Induced Formation of Axonal Filopodia and Collateral Branches Involves the Intra-Axonal Synthesis of Regulators of the Actin-Nucleating Arp2/3 Complex. J. Neurosci. 32, 17671–17689.

50. Spillane, M., Ketschek, A., Merianda, T.T., Twiss, J.L., and Gallo, G. (2013). Mitochondria Coordinate Sites of Axon Branching through Localized Intra-Axonal Protein Synthesis. Cell Rep. 5, 1564–1575.

51. Stamatakou E, Hoyos-Flight M, Salinas PC (2015) Wnt Signalling Promotes Actin Dynamics during Axon Remodelling through the Actin-Binding Protein Eps8. PLoS ONE

52. Sweeney, M.O., Collins, A., Padrick, S.B., and Goode, B.L. (2015). A novel role for {WAVE1} in controlling actin network growth rate and architecture. Mol. Biol. Cell 26, 495–505.

53. Wang, C.L., L. Zhang, Y. Zhou, J. Zhou, X.J. Yang, S.M. Duan, Z.Q. Xiong, and Y.Q. Ding. 2007. Activity-dependent development of callosal projections in the somatosensory cortex. J. Neurosci. 27:11334–11342.

54. Welch, H.C.E., Coadwell, W.J., Stephens, L.R., and Hawkins, P.T. (2003). Phosphoinositide 3-kinase-dependent activation of Rac. FEBS Lett. 546, 93–97.

55. Yang, C., and Svitkina, T. (2011). Filopodia initiation: focus on the Arp2/3 complex and formins. Cell Adh. Migr. 5, 402–408.

56. Yu, W., Ahmad, F.J., and Baas, P.W. (1994). Microtubule fragmentation and partitioning in the axon during collateral branch formation. J. Neurosci. 14, 5872– 5884.

57. Zhuravlev, P. I., & Papoian, G. A. (2009). Molecular noise of capping protein binding induces macroscopic instability in filopodial dynamics. *Proceedings of*

the National Academy of Sciences of the United States of America, 106(28), 11570–11575.

58. Zwolak, A., Fujiwara, I., Hammer, J. A., 3rd, & Tjandra, N. (2010). Structural basis for capping protein sequestration by myotrophin (V-1). The Journal of biological chemistry.