Exploring the role of Profilin 1 in axon formation, growth and regeneration

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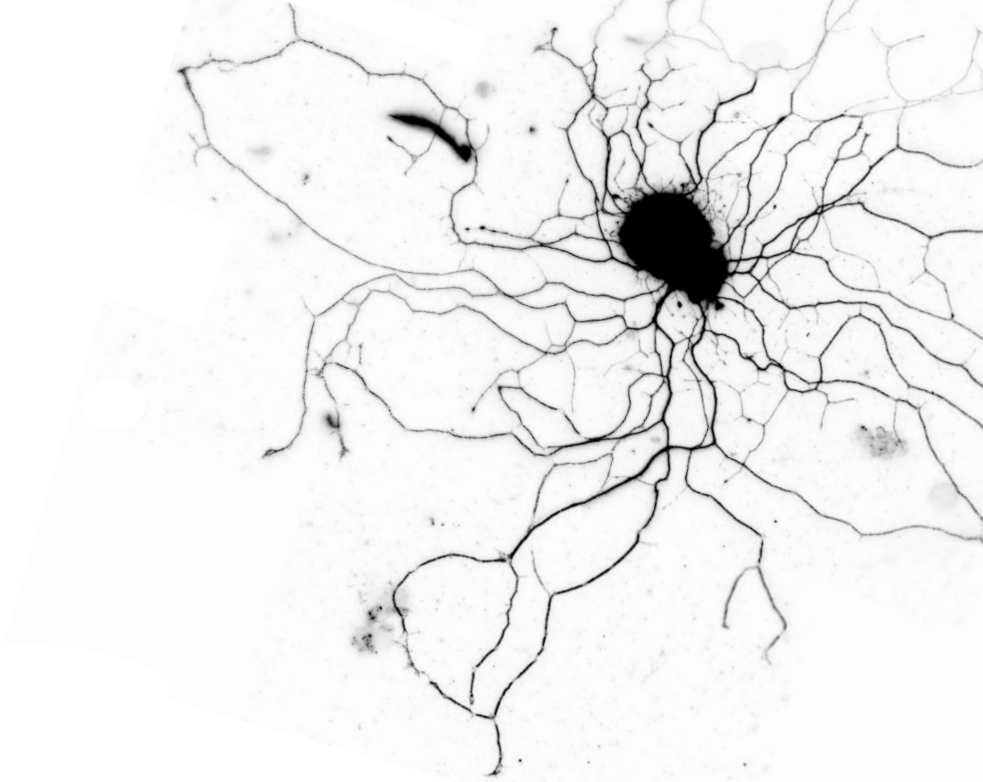

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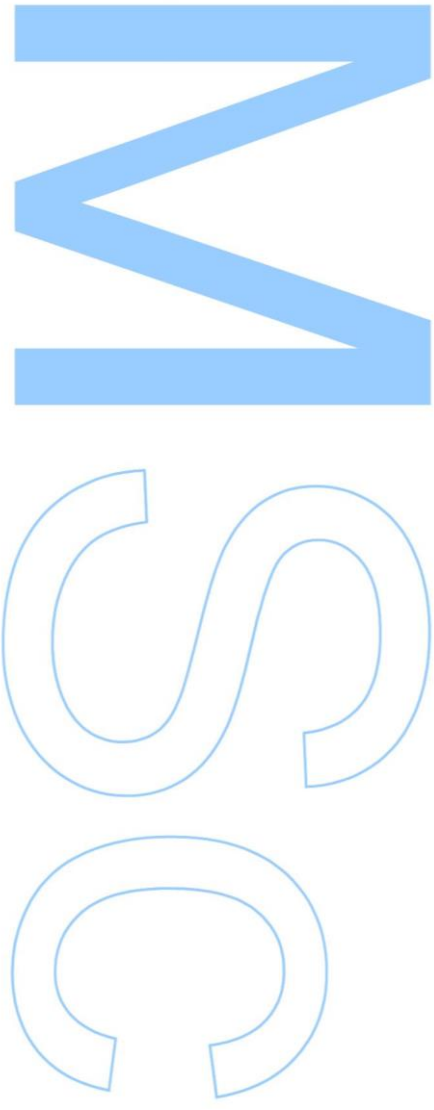

The greatest obstacle to discovery is not ignorance - it is the illusion of knowledge.

Daniel Joseph Boorstin

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Abstract

In the mammalian adult CNS, axons present a very low regenerative capacity after injury, in contrast to PNS neurons. Despite that the cytoskeleton is known to play a crucial role in axon growth and regeneration, the underlying mechanisms are still not fully understood. Actin is extremely important in these processes, as well as the various actin-binding proteins with which it interacts. One of these proteins is profilin 1 (Pfn1), which promotes the exchange of ADP for ATP in actin monomers, increasing the rate of polymerization of the actin filament. Previous results obtained in our group demonstrate that neurons with a high intrinsic regenerative capacity have increased activity of Pfn1, and that acute deletion of Pfn1 precluded axon formation in hippocampal neurons and impaired neurite outgrowth in DRG neurons. Collectively, these results point to an important role of this actin-binding protein in axon regeneration and neuritogenesis. Therefore, in this project we aimed to explore the role of Pfn1 in axon formation, growth and regeneration.

Knowing that axon growth is decreased when Pfn1 is absent, we tested whether Pfn1 overexpression would result in increased axon growth in both hippocampal and dorsal root ganglion (DRG) neurons. Two Pfn1 forms were overexpressed, wild-type (WT) Pfn1 and a phosphorylation-resistant mutated form, which is constitutively active (S137A), and subsequently we evaluated neurite outgrowth, actin and microtubule (MT) dynamics. Our results showed that, *in vitro*, the overexpression of either Pfn1 WT or Pfn1 S137A, which cannot be negatively regulated by phosphorylation at serine 137, consistently increased neurite outgrowth, actin dynamics and microtubule growth speed. Interestingly, the effect of Pfn1 S137A was generally stronger than that of Pfn1 WT. Taking into account the robust effect of Pfn1 S137A *in vitro*, we will test the effectiveness of this Pfn1 mutant in enhancing axon regeneration *in vivo*. This will be achieved by using a viral approach to deliver Pfn1 S137A to DRG neurons of rats with a sciatic nerve injury, followed by evaluation of axon regeneration in the sciatic nerve. If Pfn1 S137A increases axon regeneration *in vivo*, Pfn1 may become a good target for future therapies to improve axon regeneration following nerve injury.

Moreover, previous data from our group has shown that DRG neurons from mice lacking Pfn1 in neurons (*Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ - Pfn1 cKO) have impaired neurite outgrowth, decreased actin retrograde flow and impaired MT dynamics. To determine if there is a compensatory effect following Pfn1 deletion *in vivo*, the levels of Pfn2 in the brain of Pfn1 cKO mice were evaluated. In the brains of Pfn1 cKO mice, no statistical difference was observed in Pfn2 levels when comparing to WT mice. To further determine possible

compensatory mechanisms, we assessed neurite outgrowth after the acute deletion of Pfn2 (by shRNA) in Pfn1 cKO DRG neurons and found that the absence of Pfn2 had no effect on neurite growth, whether Pfn1 was absent or not.

Besides profilin role as an actin-binding protein, it may also interact with phosphatidylinositol lipids, in particular PIP_2, which is involved in the PI3K/AKT pathway. This fundamental pathway is involved in several cellular processes, such as MT dynamics. To further understand the molecular role of Pfn1 in this pathway and how it modulates MT dynamics, Pfn1 WT and Pfn1 S137A were overexpressed in a neuronal cell line and the expression levels of some players of the PI3K/AKT pathway were quantified. Pfn1 S137A overexpression led to increased levels of activated AKT and inactivated GSK3β, which suggests that Pfn1 not only affects the actin cytoskeleton, but may also regulate MT dynamics by interfering with an intracellular signalling pathway.

In summary, this work provides important evidences supporting the pivotal role of Pfn1 in axon formation, growth and regeneration.

Keywords: actin dynamics; profilin; axon formation; axon growth; axon regeneration; spinal cord injury; microtubule dynamics.

Resumo

No sistema nervoso central adulto dos mamíferos, os axónios apresentam uma capacidade regenerativa diminuída após uma lesão, contrariamente aos neurónios do sistema nervoso periférico. Apesar de o citoesqueleto ter um papel crucial no crescimento e regeneração axonal, os mecanismos subjacentes ainda não são totalmente compreendidos. A actina é extremamente importante nestes processos, bem como as diversas proteínas de ligação à actina com as quais esta interage. Uma destas proteínas de ligação à actina é a profilina 1 (Pfn1), que promove a permuta de ADP por ATP nos monómeros de actina, aumentando a taxa de polimerização dos filamentos de actina. Resultados preliminares provenientes do nosso grupo de investigação demonstraram que neurónios, cuja capacidade intrínseca de crescimento se encontra aumentada, exibem um aumento da atividade de Pfn1. Por outro lado, a deleção aguda desta proteína resultou numa diminuição do crescimento de neurites, indicando que a Pfn1 tem um papel importante no contexto de regeneração axonal. Assim, neste projeto, o principal objetivo consistiu em investigar o papel da Pfn1 na formação, crescimento e regeneração axonal.

Dado que o crescimento axonal diminui na ausência de Pfn1, foi testado se a sua sobreexpressão resultaria num aumento do crescimento de neurites, tanto em neurónios de hipocampo como em neurónios dos gânglios da raiz dorsal (GRD). Para tal, foi efetuada a sobreexpressão de duas formas de Pfn1, a forma selvagem (Pfn1 WT) e uma forma mutada fosforesistente, constitutivamente ativa (Pfn1 S137A), e, posteriormente, avaliou-se o crescimento de neurites, bem como a dinâmica do citoesqueleto de actina e microtúbulos. Os resultados obtidos demonstraram que, *in vitro*, a sobreexpressão de qualquer uma das duas formas de Pfn1 levou a um aumento consistente do crescimento de neurites, bem como da dinâmica de actina e dinâmica de microtúbulos. Curiosamente, o efeito da Pfn1 S137A foi, em geral, mais pronunciado do que o da Pfn1 WT. Tendo em conta o efeito robusto da Pfn1 S137A *in vitro*, iremos testar a eficiência deste mutante em promover a regeneração axonal *in vivo.* Para tal, será utilizada uma abordagem viral para sobreexpressar a Pfn1 S137A *in vivo,* em neurónios de GRD de ratazanas que sofreram uma lesão no nervo ciático, seguida de uma avaliação da regeneração axonal no nervo ciático. Se a Pfn1 S137A levar a um aumento da regeneração axonal *in vivo*, a Pfn1 poderá constituir um bom alvo em futuras terapias para promover a regeneração após uma lesão de um nervo.

Além disso, resultados preliminares obtidos no nosso grupo demonstraram que neurónios de GRD de murganhos que não possuem Pfn1 (*Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ - Pfn1 cKO)

apresentavam uma redução no crescimento neuronal, bem como diminuição da dinâmica do citoesqueleto de actina e microtúbulos. Por forma a determinar se existe um efeito compensatório da Pfn2 nos neurónios acima mencionados, os níveis de Pfn2 foram avaliados em amostras de cérebro de murganhos Pfn1 cKO. Verificou-se que, em murganhos Pfn1 cKO, não há diferença estatisticamente significativa nos níveis de Pfn2 quando comparado com murganhos WT. Adicionalmente, também se avaliou o crescimento de neurites após a deleção aguda de Pfn2 (através de shRNA) em neurónios de GRD de murganhos Pfn1 cKO e observou-se que a deleção de Pfn2 não tem qualquer efeito sobre o crescimento de neurites, tanto na presença como ausência de Pfn1.

Além do papel da profilina como proteína de ligação à actina, esta proteína também pode interagir com lípidos fosfatidilinositol, em particular com PIP_2, que está envolvido na via de sinalização PI3K/AKT. Esta via está envolvida em diversos eventos celulares, tal como na dinâmica de microtúbulos. Para se tentar perceber melhor o papel da Pfn1 nesta via e como poderá influenciar a dinâmica de microtúbulos, a Pfn1 WT e a Pfn1 S137A foram sobreexpressas numa linha celular neuronal e os níveis de expressão de algumas moléculas intervenientes na via PI3K/AKT foram avaliados. A sobreexpressão da Pfn1 S137A conduziu a um aumento dos níveis da forma ativada da proteína AKT e da forma inativada da proteína GSK3β, o que sugere que a Pfn1 não só afeta o citoesqueleto de actina, como também pode regular a dinâmica de microtúbulos através de uma via de sinalização intracelular.

Em suma, este trabalho fornece importantes evidências que suportam o papel essencial da Pfn1 na formação, crescimento e regeneração axonal.

Palavras-chave: dinâmica de actina; profilina; formação axonal; crescimento axonal; regeneração axonal; lesão da espinal medula; dinâmica de microtúbulos.

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List of Abbreviations

AAV	Adeno-Associated Virus
ABP	Actin-Binding Protein
ADF	Actin-Depolymerizing Factor
ADP	Adenosine Diphosphate
AKT	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
APC	Adenomatous Polyposis Coli protein
Arg1	Arginase 1
Arp2/3	Actin-Related Protein 2 and 3 complex
ATF3	Activating Transcription Factor 3
ATP	Adenosine Triphosphate
BBB	Blood–Brain Barrier
BSA	Bovine Serum Albumin
CAD	Cath.-a-differentiated
cAMP	cyclic Adenosine Monophosphate
CAP	Cyclase-Associated Protein
CGN	Cerebellar Granule Neurons
cKO	Conditional Knockout
CL	Conditioning Lesion
CMV	Cytomegalovirus
CNS	Central Nervous System
CRMP2	Collapsin Response Mediator Protein 2
CSPG	Chondroitin Sulphate Proteoglycan
DAPI	4',6-Diamidino-2-Phenylindole
DIV	Day(s) *in vitro*
DLK-1	Dual Leucine Kinase 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DRG	Dorsal Root Ganglia
DsRed	*Discosoma* sp. red fluorescent protein
E	Embryonic day(s)
EB3	End-Binding protein 3
ECM	Extracellular Matrix

EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced GFP
Elk-1	ETS domain-containing protein
ERK	Extracellular signal-Regulated Kinase
ERM	Ezrin–Radixin–Moesin
F-actin	Actin filament
FBS	Fetal Bovine Serum
G-actin	Globular actin
GAP-43	Growth-Associated Protein-43
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate
HBSS	Hanks' Balanced Salt Solution
HDAC5	Histone Deacetylase 5
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
HRP	Horseradish Peroxidase
IF	Intermediate Filaments
Ig	Immunoglobulin
IL-6	Interleukin-6
IRES	Internal Ribosome Entry Site
ITR	Inverted Terminal Repeat
JNK	c-Jun N-terminal Kinases
KD	Knockdown
LIMK	LIM domain Kinase
MAG	Myelin-Associated Glycoprotein
MAP	Microtubule-Associated Protein
MT	Microtubules
mTor	mammalian Target of rapamycin
N-cadherin	Neural cadherin
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
NMRI	Naval Medical Research Institute
NPY	Neuropeptide Y
OMgp	Oligodendrocyte Myelin glycoprotein
ori	origin of replication

PAR1	Protease-Activated Receptor 1
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDK1	3-Phosphoinositide Dependent protein Kinase-1
PenStrep	Penicillin-Streptomycin
PFA	Paraformaldehyde
Pfn	Profilin
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-Kinase
PIP_2	PI-(4,5)-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLL	Poly-L-Lysine
PLP	Poly-L-Proline
PMGS	Plasma Membrane Ganglioside Sialidase
PNS	Peripheral Nervous System
PTEN	Phosphatase and Tensin Homologue
Rac1	Ras-related C3 botulinum toxin substrate 1
RAG	Regeneration-Associated Gene
RhoA	Ras homolog family member A
RNA	Ribonucleic Acid
ROCK	Rho-associated protein kinase
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SCI	Spinal Cord Injury
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
shRNA	small hairpin RNA
SLICK	Single-neuron Labeling with Inducible Cre-mediated Knockout
SMA	Spinal Muscular Atrophy
SMN	Survival of Motor Neuron
snRNP	small nuclear Ribonucleoprotein
STAT3	Signal Transducer and Activator of Transcription 3
TBS	Tris Buffered Saline
VASP	Vasodilator-Stimulated Phosphoprotein

VIP	Vasointestinal Peptide
WASP	Wiskott–Aldrich Syndrome Protein
WAVE	WASP-family Verprolin-homologous protein
WT	Wild-Type
YFP	Yellow Fluorescent Protein

Introduction

Neurons are probably one of the most striking examples of polarization, having two distinct compartments: the somatodendritic compartment - comprising the cell body (soma) and the dendrites - and the axonal compartment. Neurons rely on this highly polarized shape to perform their functions that depend on dendrites to receive information that will be integrated in the cell body and then transmitted through the axon. Dysfunction in any of these mechanisms has been associated with neurodegenerative diseases that, nowadays, increasingly affect the human population given the increased life expectancy. Thus, having a deeper insight into the intracellular machinery and cellular events involved in the establishment and maintenance of neuronal polarity may be part of the key to thrive in discovering new therapeutics for neurodegenerative disorders.

1. Neuronal cytoskeleton and polarization

The cytoskeleton consists of a dense and highly dynamic network formed by three distinct classes of interacting structural complexes, with quite different properties, namely: microtubules (MTs), intermediate filaments (IFs) and microfilaments (or actin filaments) (Figure 1). Each of these elements exists concurrently and interdependently in overlapping cellular domains and each one presents a characteristic composition, structure and organization. [1] The cytoskeleton is involved in a plethora of critical functions across the cell, from determining its shape and architecture to being involved in intracellular transport (such as organelle trafficking), but also plays a role in neuronal growth and secretion, cell motility, interaction with the extracellular environment or in the formation of presynaptic and postsynaptic specializations, among other cellular events.[2] Despite the word ‘skeleton’, cytoskeleton is not a stiff or static structure, but rather a dynamic and adaptive one, which can be arranged into bundles or networks. Due to its adaptability and tight regulation, it allows cells to sense external mechanical and/or chemical signals and respond accordingly, functioning as a link between the intracellular and extracellular environments. [3]

As aforementioned, the cytoskeleton’s main components although structurally different interact with one another in a variety of ways. Briefly, microtubules, which are the stiffest of the three polymers, consist of hollow tube-like structures (24 nm in diameter), whose walls are typically formed by 13 protofilaments. [4] In turn, these

protofilaments are composed of α- and β-tubulin heterodimers that align in a head-to-tail fashion and possess GTPase activity. Tubulin dimers can bind two molecules of GTP, one of which can be hydrolysed to GDP subsequent to incorporation in the microtubule filament. This allows MTs to switch between growing and shrinking phases and this 'dynamic instability' enables both individual microtubules to search the cellular space quickly and the microtubule cytoskeleton to reorganize rapidly. [2, 5] Actin filaments (F-actin) are especially enriched in certain subcellular neuronal regions, such as the growth cone or dendritic spines and consist of two strands of polymerized globular actin monomers (G-actin) arranged in a helix. Actin polymerization is tightly regulated by actin-binding proteins (ABPs) and occurs preferentially at the fast growing end (or 'barbed end'), while depolymerization occurs in the opposite end of the filament (the 'pointed end'). [6] Unlike actin filaments and microtubules, which are assembled from one or two proteins, intermediate filaments (or neurofilaments, in neurons) may be assembled from a large diverse family of proteins. Despite this, intermediate filaments are structurally similar, regardless of the type, forming 8–10 nm rope-like filaments that may be several micrometres long. Furthermore, IFs are the less rigid of the three cytoskeleton components and, as they are not polarized structures (unlike actin and MTs), IFs cannot support directional movement of molecular motors. [6, 7]

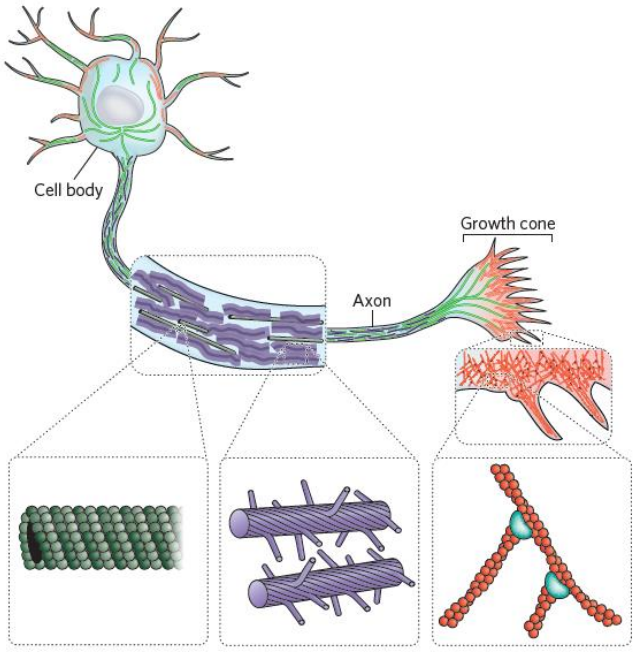


Figure 1 – Cytoskeleton components. Neurons have a cytoskeleton that consists of three main polymers: microtubules (green), neurofilaments (purple) and actin filaments (red). [3]

Because of the central role of cytoskeleton in cell structure and in many cellular events, as previously mentioned, even a slight alteration in a cytoskeleton component or

binding protein might have a profound impact in cells, which can eventually lead to marked pathological conditions. There are some reports in the literature of cytoskeleton alterations which can lead to a variety of phenotypes, such as: tumorigenic phenotypes, in which the regulation of the actin cytoskeleton is unbalanced [8, 9], myopathies, skin and epithelial diseases and also neurodegenerative disorders, which are typically characterized by abundant abnormal aggregates of cytoskeletal proteins. [10, 11]

Furthermore, the cytoskeleton plays a crucial role in the establishment and maintenance of cell polarity, which is essential in many cell types, but especially important in neurons, in order to allow its correct functioning. [12, 13] Interestingly, two of the three cytoskeleton components (namely actin and MTs) present an intrinsically polarized structure, as previously referred, which results in distinct polymerization and depolymerization rates in each end. This structural difference is crucial for the cytoskeleton to perform its role in neuronal polarization and therefore, the comprehension of how actin and MTs contribute to cell polarity and its regulation is of particular importance.

Of note, during neuronal polarity, neurons also rely on several signalling pathways and respective signal transduction molecules, which are activated during axon specification, formation and elongation and regulate cytoskeleton dynamics, such as phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue (PTEN) or glycogen synthase kinase 3 beta (GSK3β). [14] Several reports revealed that PI3K is implicated in axon specification, since its pharmacological inhibition prevented axon formation. [14, 15] Briefly, PI3K catalyzes PIP_2 phosphorylation, originating PIP_3, which was found to be selectively accumulated within a single neurite of hippocampal neurons at stage 2 of development. [15] On the other hand, PTEN catalyzes the opposite reaction, promoting PIP_3 dephosphorylation into PIP_2 and consequently limiting PIP_3 signalling. The formation of PIP_3 by PI3K recruits AKT to the membrane and promotes its activation, which once activated, may phosphorylate its downstream targets. [14] Curiously, the active form of AKT has been reported to be enriched in the growth cones of polarized neurons, [16] highlighting the importance of this molecule and the signalling pathways in which it is involved. A common AKT target is GSK3β, which becomes inactive when phosphorylated in serine 9 residue, therefore preventing phosphorylation of its targets, some of which are potential effectors of neuronal polarity and are involved in cytoskeleton regulation (such as adenomatous polyposis Coli protein (APC), collapsin response mediator protein 2 (CRMP2), tau and other microtubule-associated proteins (MAPs)). [17]

1.1. Stages of neuronal polarization

Polarity enables cells to carry out specialized functions and it is characterized by the asymmetric accumulation of mobile components, such as regulatory molecules, in opposite poles of the cell and by the oriented organization of the inherently polarized cytoskeletal filaments (actin and MTs) along the axis of polarity. [13] Neurons are among the most polarized cell types, which classically present a round cell body and two morphologically and functionally distinct types of processes - axon and dendrites. The axon is a long and thin structure, whereas dendrites are relatively short and tapered, becoming thinner with increased distance from the cell body. [6]

This polarized morphology is fundamental for the transmission of neuronal information, as the dendrites receive synaptic input from other neurons, which is integrated at the soma and propagated along the axon to the presynaptic terminal, where the signal is transmitted to target neurons through synapses. [18, 19] Therefore, polarization is an indispensable event during neuronal development and also for the integration and transmission of information in the entire nervous system.

During polarization, neurons experience different developmental stages, starting from a small, round and symmetric sphere, which will develop into a highly polarized mature neuron. However, the timing of each step may vary greatly among the different neuron types. [18] This process has been extensively studied, predominantly in rat embryonic hippocampal neurons [20, 21] and postnatal cerebellar granule neurons (CGN) [22], which allowed to establish a well-characterized model for the *in vitro* neuronal polarization process. The morphological events that occur during embryonic hippocampal neurons polarization can be divided in five robust stages (Figure 2).

Shortly after being plated, neurons attach to the substrate as round spheres surrounded by lamellipodia and filopodia, which are actin-rich structures (stage 1). A few hours later, they start forming various minor processes, which are termed immature neurites that are the precursors of the future axon and dendrites (stage 2). This process is called neuritogenesis and at this stage, the neuron is still similar to an unpolarized, symmetric sphere. These immature neurites are morphologically identical and display repeated, random cycles of extension and retraction. After neuritogenesis, one of the neurites, which will develop into the future axon, begins to extend rapidly, becoming much longer than the other neurites (stage 3). [20] Simultaneously, the growth of the remaining neurites is inhibited and they continue to undergo alternating spurts of growth and retraction, maintaining their length until they develop into dendrites, at later stages (stage 4). Finally, the maturation is achieved by the formation of dendritic spines and the

establishment of synapses, which will enable the transmission of signals between neurons (stage 5). [23, 24]

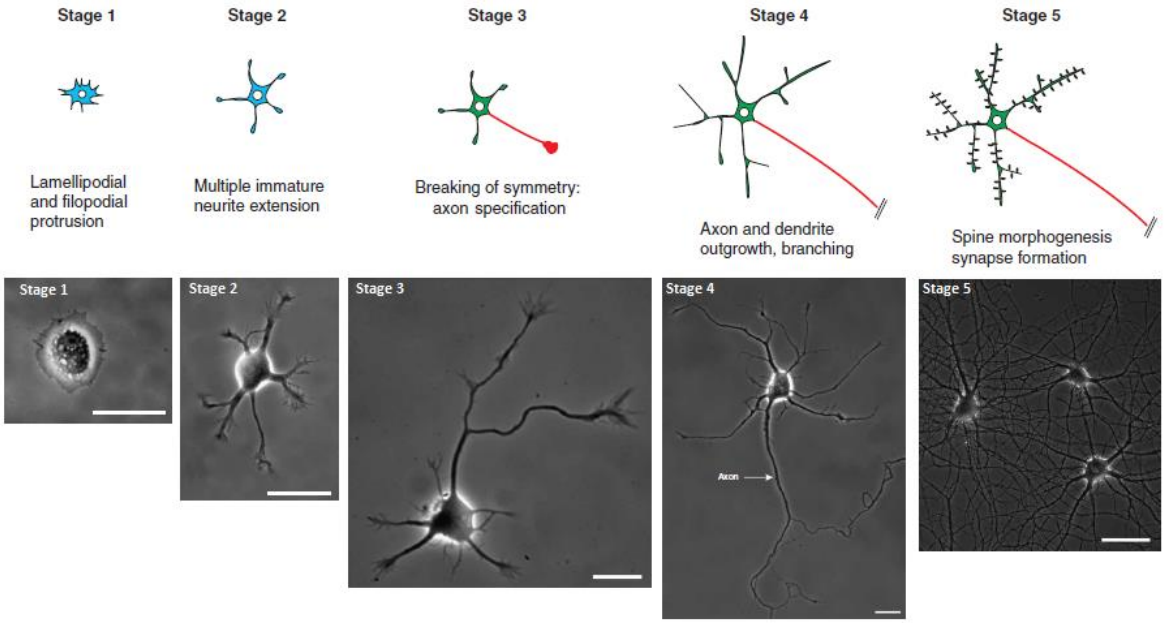


Figure 2 – Stages of neuronal polarization *in vitro*. Representation of morphological changes that occur during neuronal polarization. Right after plating, neurons adhere to the substrate and exhibit intense lamellipodial and filopodial protrusive activity (Stage 1), which eventually leads to the formation of multiple immature neurites (Stage 2). Then, there is a break of the neuronal symmetry when one of the neurites starts to grow more rapidly than the others, originating the axon (in red) (Stage 3), while the remaining neurites will become dendrites. At stage 4, axon and dendritic growth occur, as well as branching and subsequently, after several days in culture, neurons develop dendritic spines and synapses are established (stage 5). (Below) Phase-contrast images of hippocampal neurons during the first 13 d of culture. Scale bars: 25 µm. Adapted from [23] and [25].

It is important to mention that this *in vitro* model of polarization is based on cultures of embryonic rat hippocampal neurons, in which most plated cells are polarized postmitotic neurons prior to dissociation that may preserve some aspects of the original polarization. For this reason, neuronal polarization using this *in vitro* model likely corresponds to the repolarization of previously polarized neurons *in vivo* and does not reproduce exactly the conditions that neurons face during development. [23] Notwithstanding, hippocampal neuron cultures are still extremely useful to understand the cell biology events that lead to polarization and, undoubtedly, the formation and elongation of the axon is the crucial event in breaking cell symmetry and establishing neuronal polarity.

1.2. Cytoskeleton dynamics during axon growth and neuronal polarization

It is known that between stages 2 and 3 of polarization, symmetry is broken when one of the immature neurites starts to grow faster and becomes the axon, whereas the

growth of other processes is inhibited, as previously mentioned. Even though external stimuli play a role in the establishment of neuronal polarity, neurons definitely possess intrinsic mechanisms that govern symmetry breaking and allow the maintenance of that morphology over time. [18] Those intrinsic mechanisms greatly depend on actin and MTs dynamics. [26]

In fact, it was observed in cultured hippocampal neurons that the axon formation is preceded by an enlargement and increased dynamics of the growth cone in one of the neurites, showing an unstable and loose actin meshwork. [27] The increased actin turnover in one of the neurites is enough to confer axonal identity and as it is less dense, allows MTs to more easily and rapidly protrude into distal regions of the axonal growth cone, sustaining axonal extension. [28] Contrary to the actin cytoskeleton, MTs stabilize in that particular neurite before axon formation and become even more stable in the future axon shaft of the polarized neuron. [29] Consequently, that specific neurite will elongate rapidly, originating an axon that grows at a rate between five to ten times faster than the other neurites. [20]

On the other hand, the other neurites remain small and in a state of undynamic quiescence, presenting a denser actin meshwork. This creates a non-permissive environment that sterically hinders microtubule advance, characterized by stable and condensed actin filaments. Therefore, MTs are not able to protrude and stabilize in those neurites, which will eventually develop into dendrites at later stages. [28]

Even though the above reported evidences clearly demonstrate that actin and MTs work together in the establishment of polarity, the underlying mechanisms that trigger initial actin dynamics and MTs stabilization are still not fully understood. However, a variety of factors have already been implicated in MTs stabilization in axons, namely GSK3β, protease-activated receptor 1 (PAR1), CRMP2, tau and MAP1b. Nevertheless, it is still unclear which of these factors are involved in the initial stabilization of MTs in the axon and how they might be recruited to the presumptive axon. [13, 18] Interestingly, it was observed that when the actin depolymerizing reagents Cytochalasin D or Latrunculin B were added to neuronal cultures, neurons had multiple axon-like processes, emphasizing the importance of the destabilization of the actin cytoskeleton in axon formation and extension. [27]

Furthermore, it has also been reported that even after the axon is specified, other neurites have the potential to change their identity during development. It was observed that the transection of axons of hippocampal neurons early in development [21] or even of mature neurons [30], could cause the polarity to change and thus a minor neurite would become the axon, instead of the transected axon. In immature neurons, after

transection of the axon, if one of the neurites was at least 10 μm longer than the others, it would invariably begin to grow and become the axon. However, in mature neurons this change in polarity depended on the length of the cut axon. If the axon was cut at a distance inferior to 35 μm from the cell body, polarity would change and another dendrite would become the axon; if the cut was further than 35 μm, the axon would regenerate. Therefore, these observations demonstrate that the neuronal polarization is a plastic process, not only early in development, but also at later stages, when neurons are integrated in functional networks.

1.3. The Growth Cone

As previously stated, the cytoskeleton plays a crucial role in the establishment of neuronal polarity, in particular in the intense rearrangements it undergoes at the tip of the neurite, which is also denominated growth cone (Figure 3). The growth cone is a highly dynamic and specialized structure, which is responsible for the axon growth and allows neurons to sense and explore the extracellular environment, in response to multiple cues. Regarding axon growth in neurons, the growth cone plays an essential role in controlling both the rate and the direction of growth. [31]

Growth cones may assume different shapes and sizes and they constantly extend and retract membrane protrusions, in order to sense the extracellular environment. The structure of the growth cone is fundamental for it to perform its function and it is composed of three main domains that can be distinguished by their characteristic cytoskeletal organization, namely: the central (C) domain, the transition (T) zone and the peripheral (P) domain. [32]

The P domain consists of dynamic finger-like protrusions (termed filopodia) that explore the surrounding environment, separated by sheets of membrane between the filopodia, which are called lamellipodia. Filopodia are formed by long, bundled actin filaments (F-actin bundles), whereas flat mesh-like branched F-actin networks confer support and structure to lamellipodia. In addition, individual dynamic microtubules explore the P domain, usually along the F-actin bundles of filopodia, which may act as guidance sensors. In fact, filopodia may also have a major role in establishing growth cone–substrate adhesive contacts during environmental exploration, by functioning as points of attachment to the substrate and therefore producing tension that can be used for growth cone progression. [33] In turn, the C domain comprises stable, bundled MTs that enter the growth cone from the axon shaft, which support the constant shuttling of organelles and vesicles. Lastly, the T zone lies between the C and P domains. It is

enriched in actomyosin contractile structures (actin arcs), which are oriented perpendicular to the F-actin bundles, forming a hemicircumferential ring that in a certain extent hinders MTs protrusion to the P domain. [32, 34]

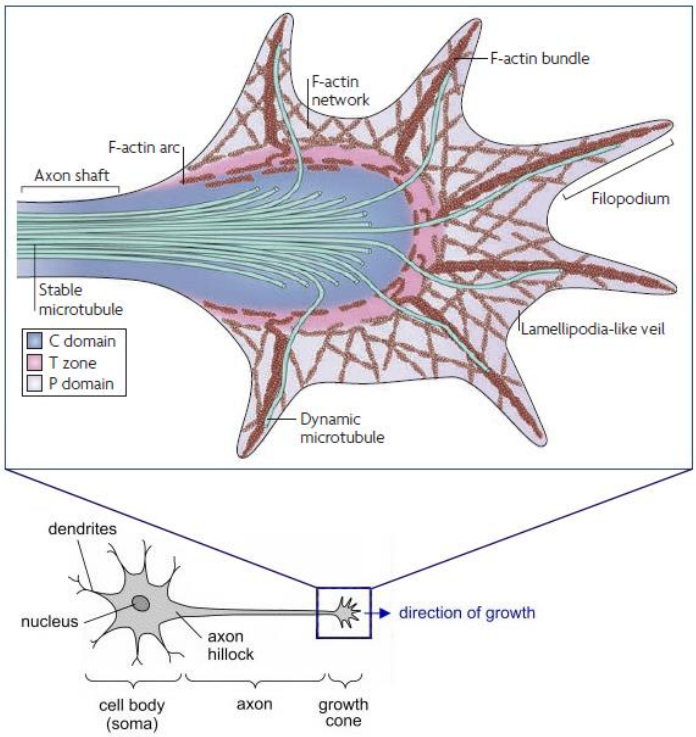


Figure 3 – **The growth cone structure.** The cytoskeletal elements in the growth cone underlie its shape, and the growth cone can be separated into three distinct domains based on cytoskeletal distribution: central (C) domain, transition (T) zone and peripheral (P) domain. The P domain consists of long F-actin bundles (filopodia) separated by flattened mesh-like branched F-actin networks (lamelipodia). Additionally, individual dynamic microtubules explore this region, along the F-actin bundles. The C domain comprises stable, bundled microtubules that enter the growth cone from the axon shaft, allowing the transport of numerous organelles and vesicles. Regarding the T zone, it lies in the interface between the C and P domains, with actomyosin contractile structures (actin arcs) perpendicular to F-actin bundles, forming a hemicircunferential ring. Adapted from [32]

Regarding cytoskeleton's orientation in the growth cone, in particular actin cytoskeleton, it is known that the barbed ends of actin filaments of both filopodia and lamellipodia are oriented towards the leading edge of the growth cone, where incorporation of G-actin monomers in F-actin occurs, whilst the pointed ends face the T zone, where depolymerization takes place. The continuous addition and disassembly of actin monomers (a process called actin treadmilling) ensures that the polymer maintains a constant length and at the same time promotes protrusion of the leading edge membrane and growth cone motility. These dynamic properties of actin, namely F-actin treadmilling combined with F-actin retrograde flow (the continuous movement of F-actin from the leading edge towards the centre of the growth cone) lie in the basis of the growth cone movement in response to directional cues. [31] It has been demonstrated that F-

actin retrograde flow is driven both by the contractility of myosin II and the push force exerted by actin polymerization in the leading membrane of the P domain. [35] Briefly, myosin II compression in the T zone combined with the pushing force from leading edge actin polymerization may cause buckling and severing of F-actin bundles in the proximal ends, which might also involve the actin-depolymerization factor (ADF)/cofilin. Consequently, the actin fragments can be recycled into actin monomers, which become available for the continuous actin polymerization at the leading edge. However, the difference between the rates of actin polymerization/depolymerization and retrograde flow is not enough to determine if the growth cone extends or retracts. In fact, a hypothesis, called the 'clutch' hypothesis has been proposed. Authors suggested that when a growth cone receptor binds to an adhesive substrate this leads to the formation of a complex that acts like an 'anchor' or molecular clutch, mechanically coupling the receptors and F-actin flow. Therefore, F-actin becomes anchored, preventing retrograde flow and promoting actin-based forward progression of the growth cone. [32]

The growth cone progression process upon the encountering of attractive adhesive substrates can be divided into three main phases: protrusion, engorgement and consolidation. [36] Briefly, as the receptors at the distal end of the growth cone bind to an adhesive substrate, intracellular signalling cascades are activated and so a molecular 'clutch' that links the substrate to the actin cytoskeleton is formed. This will counterbalance the actin retrograde flow and as F-actin polymerization continues in front of the clutch site, filopodia and lamellipodia will move forward and extend. Subsequently, engorgement occurs after F-actin bundles between the adhesion site and the C domain disaggregate and the F-actin arcs reorganize from the C domain towards the site of new growth. [37] This will allow the invasion of C domain MTs into the protruding area, also supporting the transport of organelles and vesicles into this region. Finally, consolidation occurs when the proximal part of the growth cone compacts to form a new segment of the axon shaft. This is promoted by the compression exerted in MTs by myosin II-containing actin arcs, followed by MT-associated protein stabilization. These three stages take place both during the formation of nascent axons or during axon branching.[38]

1.4. Actin-Binding Proteins (ABPs)

In cells, the assembly and disassembly of actin filaments, as well as their organisation into functional networks, is regulated by a plethora of actin-binding proteins. Therefore, the relative abundance of the different classes of ABPs will dictate the actin architecture at a given place. ABPs participate in a wide range of events involving actin, such as actin polymerization and depolymerization, capping, severing, cross-linking or nucleation of actin filaments (Figure 4). [39]

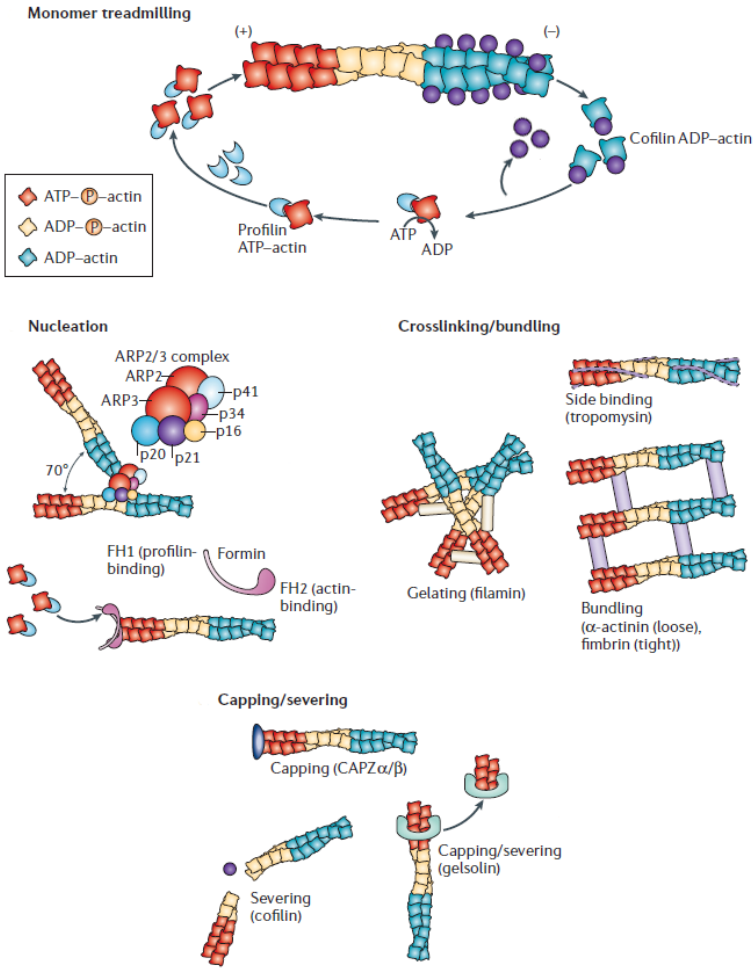


Figure 4 – An overview of some actin-binding proteins and their functions. Actin-binding proteins (ABPs) interact with actin in different ways. In the actin treadmilling, cofilin promotes F-actin severing while profilin supports ADP to ATP exchange in G-actin monomers, so that they can be incorporated in free barbed ends. On the other hand, there are other ABPs that promote nucleation, such as Arp2/3 complex (made up of two Arp proteins and five associated subunits) that interacts with the sides of existing actin filaments, forming branched F-actin arrays and formins that capture actin monomers to nucleate a new filament. Additionally, some proteins facilitate the formation of F-actin structures, by promoting cross-linking or bundling, such as filamin, fimbrin or α-actinin. Actin filaments can also become capped by some ABPs, such as CapZ or gelsolin, therefore blocking incorporation of new actin monomers. Adapted from [40]

Actin is an extremely abundant protein in the growth cone, as it can reach a cytoplasmic concentration of up to 100 µM, a value much higher than the 0.1 µM critical

concentration needed for actin spontaneous polymerization. Interestingly, about half of the actin in the growth cone is in the monomeric form (i.e. unpolymerized), due to the abundance of ABPs that regulate several facets of actin dynamics. [41]

As mentioned before, during axon growth, actin polymerization takes place at the growth cone, pushing the plasma membrane forward. This actin polymerization requires availability of G-actin monomers, which is essentially regulated by two proteins, namely profilin and thymosin β4. Profilin's main function is to accelerate the ADP to ATP exchange in G-actin monomers released from F-actin pointed ends, thus providing a pool of ATP-actin monomers that can be readily available for polymerization. [42] In turn, thymosin β4 binds and sequesters ATP-G-actin monomers, preventing their incorporation in actin filaments. However, when the free concentration of ATP-G-actin monomers drops, thymosin β4 readily releases them, thus functioning as a 'buffering system'. As profilin shows a higher affinity for actin than thymosin β4, this allows profilin to maintain a pool of actin monomers available for polymerization, while thymosin β4 holds the rest of the monomers in reserve. [43]

Besides availability of ATP-G-actin monomers, accessible free F-actin barbed ends are also required for actin polymerization. In order to produce new barbed ends, there are three possible mechanisms regulated by different ABPs, namely: nucleation, severing or uncapping of existing filaments. [44] Specifically, actin depolymerizing factor (ADF) and cofilin, which are abundant at the growth cone leading edge, promote actin severing and depolymerization by binding to ADP-F-Actin. Therefore, in combination with profilin, they promote actin treadmilling, by increasing both the number of available free barbed ends and G-actin monomers. [41, 45] There is also another ABP that promotes F-actin severing, named gelsolin, but its role in growth cone dynamics is minor when compared with ADF/cofilin. This protein is able to promote severing of actin filaments in the presence of micromolar calcium and caps the resulting free barbed ends, preventing monomer addition. [46]

There are other ABPs that also regulate the availability of free F-actin barbed ends by capping them, such as CapZ that binds barbed ends and blocks monomer addition, preventing polymerization. However, this capping activity may be inhibited by Ena/VASP (vasodilator stimulated phosphoprotein) proteins. The process of capping may seem counterproductive, since it inhibits F-actin elongation and consequently cell motility or axon growth. However, capping serves as a mechanism to control the length of actin filaments and also avoids non-productive consumption of actin monomers, thus channelling efforts to a limited number of barbed ends. [43]

Moreover, there are also ABPs that nucleate G-actin to create new barbed ends for polymerization, for example the actin-related protein (Arp) 2/3 complex and formins. The Arp2/3 complex, made up of two Arp proteins and five associated subunits, binds at the side of an existing actin filament and nucleates a new filament that branches from a pre-existing one, supporting the formation of branched actin networks. [47] Formins capture several actin monomers to nucleate a new filament and remain bound to the barbed end to stabilize the nascent filament and facilitate polymerization of long filaments. Both Arp2/3 complex and formins individually bind profilin-ATP-G-actin, enhancing G-actin incorporation. [48]

Additionally, the effective transformation of actin polymerization into protrusion requires that F-actin interacts with membrane components. These interactions can be mediated by ABPs, such as the ezrin–radixin–moesin (ERM) proteins that can bind F-actin to several membrane proteins (for example, L1 - a neuronal adhesion molecule). F-actin can also interact with N-cadherin through α- and ß-catenin [49] or with integrins (which mediate growth cone adhesion to extracellular matrix) via talin, vinculin and α-actinin. These connections between F-actin and adhesive molecules are crucial in order to allow growth cone migration and axon elongation. [43]

2. Regeneration in the Central and Peripheral Nervous System

It is widely known that adult mammalian central nervous system (CNS) neurons present a very restricted regenerative capacity and any attempts to regenerate upon an injury are usually abortive. The regenerative process may be regarded as a recapitulation of neuronal development, since neurons with severed axons must form a new growth cone in order to allow axonal extension and re-establish neuronal connections with their targets. In fact, during development neurons execute a transcription-dependent program of axonal growth that relies in signalling pathways, which allows neurons to successfully perform axon elongation until reaching their post-synaptic targets. However, following this initial phase, the developmental axon growth capacity drops. [50, 51] In contrast to the CNS, after an injury, adult peripheral nervous system (PNS) neurons can reactivate this cell intrinsic program and spontaneously regrow to a significant extent. For that reason, PNS neurons are frequently used as a model to study the process of regeneration and to dissect the underlying mechanisms and players. Interestingly, some studies demonstrated that the removal or neutralization of extracellular inhibitory molecules is not enough to allow successful regeneration of CNS neurons, since they display an incomplete axon regeneration *in vivo*. [52] Therefore, this highlights the

importance of both extrinsic factors and cell intrinsic mechanisms in regeneration after injury and the need to better understand them. It is also important to mention that, although PNS regeneration may seem quite remarkable, frequently regenerating axons are misdirected and reinnervate inappropriate targets, leading to incomplete functional recovery. [53]

After an axotomy, neurons present typical morphological changes, referred to as chromatolysis. The elicited acute reactions are analogous in both PNS and CNS neurons, namely: dispersal of the Nissl substance, displacement of the nucleus to the cell's periphery, swelling of the cell body and loss or retraction of synaptic terminals. Regarding long-term responses, these vary significantly between regeneration-competent (PNS) and incompetent (CNS) neurons. In the first ones, the cell bodies remain hypertrophic and exhibit signs of protein synthesis and increased metabolism, whereas in CNS neurons cell bodies appear atrophic, with reduced cell volume and dendritic arborization. [52, 54] On the other hand, there are also alterations in the axonal compartment, specifically the distal part of the axon endures Wallerian degeneration and the proximal lesion site reseals the damaged axonal membrane. [55] This is the critical step for regeneration, since the transformation of the severed axonal stump into a competent growth cone that can integrate intra- and extracellular signals is of the most importance, in order to allow growth and extension. [56] Remarkably, whereas peripheral axons can restore a growth cone and start to regrow in the first 24 hours post-injury, CNS axons form a retraction bulb (also called frustrated growth cone). [57] This structure is a hallmark of degenerating axons and typically displays an accumulation of anterogradely transported vesicles and mitochondria and a disorganized network of MTs. [52]

2.1. Extrinsic factors

As previously mentioned, there are substantial differences between the conditions that PNS and CNS neurons encounter upon axonal injury. One such difference is the highly inhibitory glial scar that is formed after CNS injury and which creates a mechanical and chemical barrier to axonal regeneration. The glial scar is particularly rich in reactive astrocytes and proteoglycans, but microglia and oligodendrocytes can also be recruited. [58] Astrocytes can become hypertrophic and produce higher quantities of intermediate filaments and ECM proteins, such as chondroitin sulphate proteoglycans (CSPGs), which are extremely inhibitory to axon outgrowth. Despite the strong detrimental effect of the glial scar, it may also provide important beneficial functions for stabilizing fragile

CNS tissue after injury, such as promoting repair of the blood–brain barrier (BBB) to prevent an overwhelming inflammatory response and limit cellular degeneration. [59]

In addition, if the myelin structure is damaged during CNS injury, axons also become exposed to myelin-associated inhibitors of regeneration. Several classes of these proteins have already been identified, such as Nogo [60], oligodendrocyte myelin glycoprotein (OMgp) [61] and myelin-associated glycoprotein (MAG) [62], which are all expressed in CNS oligodendrocytes, except MAG that is expressed in PNS Schwann cells as well. Furthermore, other molecules that are upregulated in the core of the lesion and also contribute to the growth retarding effects of the glial scar have been described. For example, semaphorin 3, whose expression increases in fibroblasts that penetrate deeply in the lesion, and ephrin E3, produced by oligodendrocytes.

2.2. Intrinsic Factors

After axonal injury, neurons trigger a complex injury signalling cascade in order to reprogram the neuronal cell body to enter a pro-regenerative program. When the axon is severed, the axon's interior is exposed to the extracellular ionic concentrations, which leads to calcium entrance and consequently the intracellular free calcium concentration rises. This increase in calcium at this point is extremely important for the formation of a new growth cone, as it triggers several cellular mechanisms fundamental for successful axon growth, namely: membrane sealing; local cytoskeleton transformation (typically actin and MT depolymerization) in order to form a growth cone; activation of local protein synthesis; activation of calpains (which proteolytically cleave submembrane spectrin) to allow vesicles fusion with plasma membrane; and activation of long-range retrograde molecular signalling. [56] This cytoskeleton remodelling is crucial to allow the transformation of a severed axon into a functional growth cone. Briefly, after calcium influx, MTs and actin depolymerize and calpain proteolitically digests submembraneous spectrin, allowing the plasma membrane to collapse. [63] Thereafter, there is an accumulation of vesicles in the ruptured site that will eventually fuse with the membrane and form a sealing patch. [64] After sealing, the excess intracellular calcium is removed, allowing for actin and MTs polymerization and therefore formation of a new growth cone. MTs repolymerize in random directions and anterogradely transported vesicles accumulate at their tips, whereas actin filaments assemble to generate the mechanical force at the leading edge of the lamellipodium. Of note, in the immediate moments after injury, the proteins needed come from recycling of the pre-existent material, while in later stages, the proteins needed in the reconstruction of the growth cone can either be

synthesized in the cell body and then anterogradely transported or locally synthesized. [56, 65]

In addition to calcium influx through the cut axonal end, axotomy also leads to calcium release from internal storages and to membrane depolarization, which activates voltage-gated calcium channels and further promotes calcium inflow. This calcium influx elicits a back-propagating calcium wave that reaches the soma and triggers epigenetic changes that will induce the expression of several genes, such as regeneration-associated genes (RAGs). [66] Interestingly, while PNS neurons can support long periods of high calcium concentrations and these are important for axonal regeneration, these are damaging for CNS neurons and therefore fail to upregulate RAGs, which may, in part, explain their lack of regeneration capacity. [51]

Furthermore, the early calcium influx also activates calcium dependent enzymes, such as adenylate cyclase, which leads to an increase in cAMP levels, which potentiate axonal regrowth. The increased levels of cAMP induce downstream dual leucine zipper kinase (DLK-1) expression, promoting local remodeling of the cytoskeleton needed for growth cone assembly. [67] Besides, when the calcium wave propagates into the cell body, protein kinase Cμ (PKCμ) is elicited and activates nuclear export of histone deacetylase 5 (HDAC5), which will increase histone acetylation and activate the pro-regenerative transcription program. Interestingly, in rodent sensory neurons, HDAC5 accumulates at the end of injured axons, where it promotes local tubulin deacetylation, inducing growth cone MT dynamics and therefore axon regeneration. [66] Although the calcium influx is critical for the establishment of the regeneration machinery, its duration and intensity depends on the neuron type and species. [56]

After the calcium dependent early phase, several injury signals, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and signal transducer and activator of transcription 3 (STAT3), are locally activated and retrogradely transported into the soma, more specifically to the nucleus. Some of these injury signals can activate a variety of transcription factors, for example: ERK activates ETS domain-containing protein (Elk-1), while JNK activates c-Jun and activating transcription factor 3 (ATF3). [68] Whereas JNK signalling has been implicated in the reorganization of the axonal cytoskeleton, STAT3 activation is important for neuronal survival after injury and both contribute to neuronal regeneration. Besides, the activated transcription factors can also induce the expression of several RAGs, like arginase 1, neuropeptide Y (NPY), vasointestinal peptide (VIP), interleukin-6 (IL-6), growth-associated protein-43 (GAP-43), among others. [51, 52] Some of the intracellular mediators involved in the post-injury response in PNS neurons are summarized in Figure 5. In summary, these proteins have

the capacity to alter the transcriptional profile of injured neurons in order to promote their survival and regeneration.

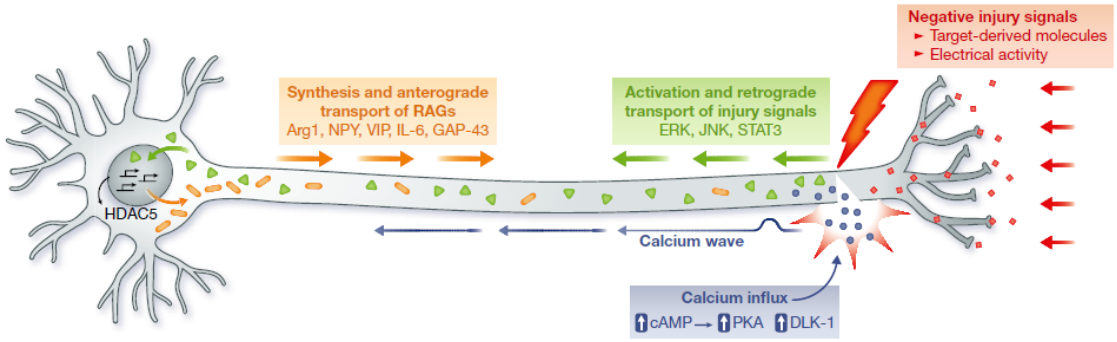


Figure 5 – Intrinsic mechanisms of axonal regeneration in PNS neurons after injury. After axonal severing, target-derived negative signals are interrupted and repression of axonal elongation is relieved. Therefore, after calcium influx into the axoplasm, cAMP and PKA become activated, signalling to DLK-1, promoting membrane sealing, local protein synthesis and growth cone formation. When the calcium wave propagates to the soma, this leads to nuclear export of HDAC5 that together with retrogradely transported injury signals (such as ERK, JNK and STAT3) will activate the proregenerative transcription program and promote expression of RAGs. RAGs (Arg1, NPY, VIP, IL-6, GAP-43, among others) are important to establish an effective regenerative response and after synthesis are then anterogradely transported. [51]

2.3. The Conditioning Lesion Model

Adult mammalian CNS neurons usually are not capable of regenerating. However, under certain conditions regeneration can occur, specifically by conditioning dorsal root ganglion (DRG) neurons, through an initial lesion of their peripheral axons that will allow regeneration of their central axons within the spinal cord. This is called the conditioning lesion effect. [69]

DRG neurons are a class of sensory neurons and present a pseudo-unipolar morphology with a peripheral axon branch, that is able to regenerate when injured, and a central axon branch that enters the spinal cord and does not regenerate upon injury. [52] As DRG neurons have a peripheral and a central branch, they have been extensively explored to study the regeneration process, given the possibility of evaluating differences in neuronal behaviour depending on the CNS and PNS environment. Therefore, in DRG neurons, if injury to the central branch is preceded by a lesion in the peripheral one, the central axon gains regenerative capacity and grows beyond the hostile lesion site. [70] This conditioning effect may be due to the previous activation of the regenerative machinery in that neuron, allowing to an effective and faster response after the second injury. In fact, it has been observed that there is an upregulation of RAGs and some proteins synthesized in the cell body, such as cytoskeletal proteins, are transported along

the regenerating axon where they may contribute to a reservoir of pre-made material that is at the disposal of the growth cone. Additionally, some proteins can also be synthesized near the tip of the axon, since specific mRNAs may be transported into the regenerating axon. [69, 71]

3. Profilin

Preliminary data from our group has demonstrated that the active form of profilin is increased in regenerating axons *in vivo*, which supports its relevance in axon formation and growth. Therefore, in this section, this actin-binding protein and its characteristics will be further described.

Profilin (Pfn) comprises a 14-17 kDa family of actin-binding proteins, expressed in all eukaryotes investigated [72] and in some viruses [73]. It was first described forty years ago, by Carlsson and colleagues [74] and so far four distinct profilin genes have been identified in mammals. [75] Profilin 1, whose gene is located in chromosome 17, is ubiquitously expressed, whereas the other forms have tissue-specific expression patterns. Profilin 2 (located in chromosome 3) has been reported to be alternatively spliced (in human, bovine, mouse and rat) into two isoforms, namely a major Pfn2A isoform, which is primarily expressed in neuronal cells and a minor Pfn2B isoform, which is mainly found in kidneys [76]. Pfn3 (chromosome 5) is kidney and testis specific, while Pfn4 (chromosome 2) is only testis specific, both playing a major role in acrosome formation and sperm morphogenesis. [77]

Curiously, despite having moderate amino acid sequence homology between the different profilin isoforms and also between profilins from distinct species, they arrange in quite similar three-dimensional structures. The ubiquitous distribution of profilin through eukaryotes, as well as the high level of conservation of the structure indicates an important role of profilin in cells [72, 78]. Typically, profilins display a compact centre of seven β-strands, surrounded by four α-helixes. Despite being a small protein, they have three main domains that allow interaction with other molecules, as depicted in Figure 6, namely: the actin-binding domain, responsible for its interaction with actin [79]; the phosphatidylinositol (PI) lipid-binding site, which allows interaction with PI-(4,5)-bisphosphate (PIP_2) and other PI lipids [80]; and the poly-L-proline (PLP) binding domain, which allows to interact with a plethora of proteins that contain PLP sequences [81]. Profilin contains two PI lipid-binding sites of which one overlaps the actin-binding domain and the other overlaps the PLP-binding site, meaning that interaction with PI lipids precludes interaction with either actin or PLP sequences. [82] These binding

domains are crucial for profilin functions and given the overlapping disposal of some of these domains, as mentioned above, this creates some regulatory complexity. Importantly, different profilin forms display distinct affinities to their ligands, for example, in humans, Pfn1 affinity for actin is higher than that for Pfn2, due to differential exposure of hydrophobic residues. [83]

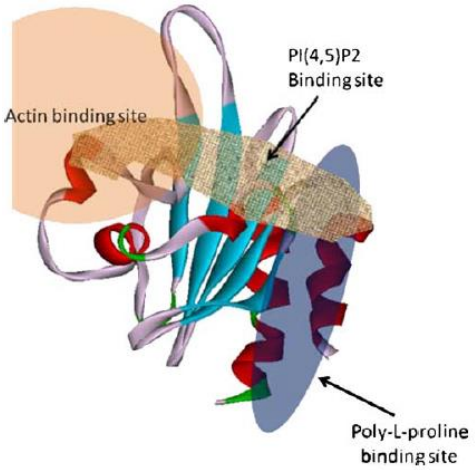


Figure 6 – Profilin structure and its binding domains. Representation of human profilin 1 structure, showing actin (orange), PIP_2 (light brown) and PLP (blue) binding domains. [72]

3.1. The role of profilin in actin dynamics

As previously mentioned, profilin can interact with actin and therefore participate in the actin treadmilling. Profilin's main function is to promote the transition of ADP-G-actin to ATP-G-actin, so that it can be incorporated in actin filaments and also mediates the release of actin monomers in F-actin barbed ends. Consequently, profilin is able to promote actin polymerization and increase actin dynamics. [42] Interestingly, profilin increases the nucleotide exchange rate in G-actin by 1000-fold, when compared with the rate based on simple diffusion, [84] which stresses its important role in actin dynamics and the associated cellular events. Importantly, it has been reported that the open nucleotide-site in G-actin is unstable in the absence of profilin, but is stabilized in its presence, promoting nucleotide exchange. [85] After the incorporation of G-actin in actin filaments, ATP is progressively hydrolysed by the intrinsic actin ATPase activity, generating ADP-actin in the older part of the filament, where other ABPs, such as cofilin, can operate, thus restarting a new cycle of actin polymerization. [42]

Given the above mentioned role of profilin in actin dynamics and the importance of actin dynamics in a plethora of cellular events, such as growth cone protrusion and axonal growth, it is expectable that its loss might have a pronounced impact in neurons.

Recent reports have demonstrated that Pfn1 depletion may cause significant defects in membrane extension and slower velocity of protrusion in both neuronal and non-neuronal migrating cells. [86, 87] This striking phenotypes can be explained by the fact that membrane protrusion depends on *de novo* actin nucleation or elongation of pre-existing filaments, which require the intervention of certain ABPs (Ena/VASP, WASP/WAVE, Arp2/3 complex and formins) that possess PLP regions. Besides profilin alone, some ABPs may also bind to profilin-G-actin-complex, enhancing F-actin elongation. [88] Therefore, this feature allows the interaction between these specific ABPs and profilin, further regulating actin dynamics. Specifically, Ena/VASP proteins promote elongation of actin filaments and tend to create unbranched and longer bundles of F-actin and by recruiting profilin-G-actin complexes, actin elongation is promoted. [89] Furthermore, the Arp2/3 complex, which can be recruited by WASP/WAVE proteins, promotes actin nucleation and may interact with profilin-G-actin complex to perform its function. [43] The same happens in formin-mediated actin nucleation, where profilin-G-complexes are also required. [90] Taken together, these reports support the view that profilin can interact concomitantly with actin and PLP regions, which further enriches the range of functions and resultant effects that Pfn may exert.

Besides the ability to interact with actin and PLP sequences, profilin can bind to PI lipids as well, being PIP_2 one of best characterized PI lipids in terms of profilin interactions and also the one with which it interacts with greater affinity. When profilin binds to PIP_2, its hydrolysis by phospholipase C-γ (PLCγ) is inhibited. [91] However, when PLCγ is phosphorylated in certain tyrosine residues, PIP_2 is hydrolysed into second messengers [92] and Pfn is released from the membrane to the cytosol, where it can interact with actin and other ligands. Therefore, given the PIP_2 involvement in signalling events, this suggests that Pfn may function as bridge between intracellular signalling and cytoskeleton remodelling.

It is also important to mention that profilin can be regulated by phosphorylation. Specifically, in the presence of PIP_2, protein kinase C zeta (PKCζ) phosphorylates Pfn at serine 137. [93] In addition, profilin may also be phosphorylated by Rho-associated protein kinase 1 (ROCK1). [94] This serine phosphorylation was reported to increase the affinity for G-actin and PLP stretches, while no effect was observed regarding its affinity to PIP_2. [95] This increased actin affinity of phosphorylated profilin may result in increased actin sequestering time and consequently lower actin incorporation in filaments.

3.2. Profilin in neuritogenesis

As aforementioned, during neuronal development, the symmetry of the initial cellular round spheres is broken, ensuing establishment of polarity and neuritogenesis, which greatly depend on actin dynamics. It has been previously proposed that both Pfn1 and Pfn2 participate in neuritogenesis. Specifically, it was observed that Pfn2a is a negative regulator of neuritogenesis, via ROCK, which is a downstream effector of RhoA. [96] When activated, ROCK may phosphorylate either Pfn1 or Pfn2 [94, 96], thus inactivating them and it may activate LIMK-1 which, in turn, inhibits cofilin [97], preventing actin severing. In fact, it was observed that an excess of intracellular Pfn2a blocks neurite formation and extension, whereas Pfn2a suppression does the opposite. [96] These effects seem to be due to increased and decreased actin-filament stability, respectively. Also, this implies that breaking the neuronal sphere requires a mechanism of inactivation of RhoA/ROCK/Pfn2 pathway, in order to reduce intracellular Pfn2 levels. A mechanism was proposed stating that, at early stages, there is a growth-positive stimuli capable of inactivating RhoA, which in turn dissociates from the membrane, reducing the interaction between ROCK and Pfn2a, avoiding Pfn2a phosphorylation. This translates into a localized shift in the local F/G-actin ratio, favouring the monomeric form and leading to actin instability and sprout formation. [96] It has also been reported that plasma membrane ganglioside sialidase (PMGS), which asymmetrically accumulates at the tip of the future axon in stage 2 neurons, can trigger PI3K- and Rac1-dependent inhibition of RhoA signalling. [98] Therefore, this would result in local actin instability and consequently axon specification.

Regarding Pfn1, it is known that it localizes at the leading edges of growth cones and it was proposed that this isoform also has a role in neuritogenesis. High-level overexpression of Pfn1 has been reported to inhibit neurite outgrowth, while low-level expression resulted in increased filopodia formation. [99] Interestingly, in a Pfn1 knockdown (KD) situation, studies revealed that there is a marked reduction of lamellipodia size, which were not able to efficiently extend. Therefore, Pfn1 KD leads to decreased actin dynamics and growth cone extension, [87] suggesting its importance in neuritogenesis. These differences between the role of Pfn1 and Pfn2 in neurite outgrowth could be explained, at least in part, by their different affinities for ligands.

Besides profilin function in the growth cone, it also has a significant role in regulating actin dynamics in dendritic spines. Dendritic spines are small actin-rich protrusions from neuronal dendrites that form the postsynaptic part of most excitatory synapses. Remarkably, modulation of actin dynamics drives morphological changes in

dendritic spines that are associated with alterations in synaptic strength. [100] Studies revealed that Pfn, through its PLP-binding site, is targeted to spine heads once postsynaptic NMDA (N-methyl-D-aspartate) receptors are activated, thus inducing suppression of actin dynamics and long-term stabilization of spine morphology, which is related to memory consolidation. [101] Fascinatingly, it was observed that fear conditioning in rats drives Pfn into dendritic spines and these spines undergo enlargements in their postsynaptic densities, which is important in synaptic plasticity. [102] Besides accumulation in dendritic spines, Pfn is also targeted to the nucleus following NMDA receptors activation, via a process involving actin rearrangements. [103] Additionally, there is a specific exporting mechanism for Pfn-actin complexes from the nucleus, involving exportin 6, which allows to regulate actin and/or Pfn nuclear and cytoplasmic concentrations. [104] In the nucleus, Pfn1 has also been identified in small nuclear ribonucleoprotein (snRNP)-core proteins and Cajal bodies, suggesting a role of Pfn in mRNA processing. [105]

3.3. Implications of Profilin *in vivo*

Profilin has already been described and implicated in a variety of diseases, ranging from diabetes [106] or cardiovascular disorders [107] to cancer [108] or neurodegenerative diseases [109]. Given the plethora of molecules and signalling pathways with which profilin may interact and consequently the wide range of effects it can have, its biological role is still not fully understood.

It has been reported that, in homozygous Pfn1 knockout mice, embryos fail to develop and this results in a pre-implantation embryonic lethal phenotype, whereas heterozygous embryos showed reduced survival during embryogenesis [110], thus pointing to an essential role of this protein in the early stages of mouse development. In fact, Pfn1 is highly expressed during all stages of embryonic development. Despite Pfn2 being weakly expressed at early stages, it was not sufficient to rescue the absence of Pfn1. [110] In addition, Pfn1 deletion in the brain, during brain development, led to cerebellar hypoplasia, abnormal organization of cerebellar cortex layers and ectopic cerebellar granule neurons (CGN), [111] which further demonstrates that Pfn1 is indispensable for normal mouse brain development. It is also documented that Pfn1 is required for lamellipodia formation of Schwann cells and myelination during PNS development. [112] On the other hand, in mice that lack Pfn2, the embryos are viable and the development of the nervous system proceeds normally, with some alterations in neurotransmitter release (and consequent behavioural alterations), [113] indicating that

Pfn2, contrary to Pfn1, is not essential for general embryonic development. Regarding profilin role in other systems than the nervous system, studies revealed that mice with homozygous Pfn1 deletion in cartilage developed progressive chondrodysplasia and defective chondrocyte cytokinesis (as complete abscission normally fails). [114]

Given its vital function, it is not hard to understand that profilins have been implicated in several pathological conditions. In cancer, it has been observed that depletion of Pfn1 results in slower but more stable lamellipodial protrusions, enhancing overall cellular motility, which is crucial in cancer metastasis. [108] Also, it was observed that, when compared to normal epithelial breast cells, a human breast cell line expressed lower levels of Pfn1. However, when Pfn1 was transfected in these cells, tumor growth was suppressed. [115] Therefore, these observations suggest that Pfn1 might act as tumorigenic suppressor in breast cancer cells, contrasting the conventional pro-migratory functions of Pfn1 in other physiological contexts.

In the context of cardiovascular diseases, it was reported that Pfn1 expression was significantly increased in human atherosclerotic plaques, when compared to the normal vessel wall, and also its serum levels were correlated with the degree of atherosclerosis in human patients, suggesting that Pfn1 might contribute to atherogenesis. [107]

Concerning neurodegenerative disorders, some Pfn1 mutants have been shown to have a role in the development of familial amyotrophic lateral sclerosis (ALS). [109] Briefly, ALS is a late-onset fatal disorder, in which a progressive degeneration of upper and lower motor neurons occurs. This results in a gradual weakening of muscles, eventually leading to overall paralysis, breathing difficulties and subsequent death, within 3-5 years since the diagnosis. [116] Specifically, the Pfn1 mutations reported to be linked to familial ALS lie within the actin-binding domain of Pfn1 and neurons carrying these mutations show decreased affinity for actin, resulting in reduced neurite outgrowth capacity and small sized growth cones. [109] These observations clearly demonstrate there might be a link between ALS pathogenesis and cytoskeleton dynamics. Besides, these neurons also display insoluble, ubiquitinated aggregates, which is a common hallmark in other neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. [109]

Besides profilin involvement in ALS, it has also been implicated in other neurodegenerative disorders, such as spinal muscular atrophy (SMA). [117] SMA is an autosomal recessive disorder characterized by the progressive degeneration and loss of motor neurons in the spinal cord, caused by reduced levels of functional survival of motor neuron (SMN) protein. This protein can interact with profilin through PLP stretches [118] and it has been reported that when SMN levels are decreased, Pfn2 becomes hyper

phosphorylated by ROCK, leading to inhibition of neurite outgrowth. This supports the idea of competition between SMN and ROCK for binding to Pfn2 and also establishes a bridge between ROCK pathway and SMA. [117]

In summary, these few examples of profilin role and impact in different physiological contexts clearly demonstrate that this small actin-binding protein has numerous ligands and intervenes in a wide variety of mechanisms, which suggests that more profilin functions will probably emerge in the future.

4. Preliminary Data

It is well established that while adult mammalian PNS neurons can regenerate after injury, CNS neurons do not. As previously mentioned, some players in regeneration have already been identified, for example, the increase in cAMP levels after injury has been shown to potentiate axonal regrowth. [119] However, none of the players identified so far in this process has demonstrated an effect strong enough to mimic the high intrinsic regenerative capacity of neurons in a conditioning lesion. Therefore, the conditioning lesion model was used in our group to further study and identify the underlying mechanisms of the enhanced regenerative capacity of DRG neurons. For that, a proteomic analysis was performed and the protein expression levels in the spinal cord injury site of rats after a spinal cord injury (SCI) or a conditioning lesion (CL) were assessed. Fascinatingly, the SCI site of conditioned animals had increased levels of some actin-binding proteins, namely cofillin-1 and Pfn1, emphasizing the importance of regulating actin dynamics for optimal axon growth and regeneration. Specifically, the levels of Pfn1 were 4.5-fold increased in the SCI site after a CL when compared to animals with only SCI. This result was later validated by western blot, as represented in Figure 7.

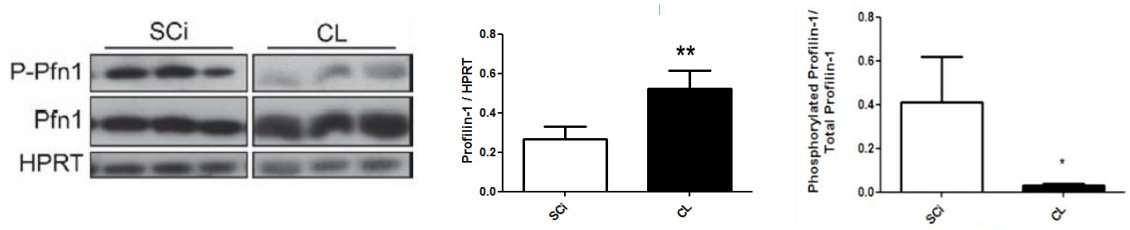


Figure 7 – Pfn1 activity increases after a conditioning lesion. Western blot analysis (left) and quantification (right) of P-Pfn1 and Pfn1 levels in spinal cord injury samples of rats with either spinal cord injury (SCI) or conditioning lesion (CL). *p*-value: * <0.05; ** <0.01.

The increase of Pfn1 levels in the SCI site is consistent with its function in axon regeneration, as this ABP promotes actin polymerization which may provide the required force for growth cone motility and ultimately axonal extension. Additionally, the levels of the inactive form of Pfn1 (P-Pfn1), i.e. phosphorylated at serine 137, were 10-fold decreased in spinal cord samples from conditioned rats, further demonstrating the importance of the active form of Pfn1 for axon regeneration.

Furthermore, following Pfn1 acute deletion, more than 90% of axon formation and growth was inhibited in hippocampal neurons (Figure 8A) and in DRG neurons the neurite outgrowth was decreased (Figure 8B). To evaluate whether a similar effect would be observed *in vivo*, a mouse strain lacking Pfn1 specifically in neurons (*Thy1cre*$^{+/-}$ *Pfn1*$^{fl/fl}$) was generated by crossing *Pfn1* floxed mice [114] with SLICK-H mice, a strain that co-expresses an inducible form of Cre recombinase and YFP, under the control of the neuronal promoter *Thy1* [120]. As expected, Pfn1 depleted DRG neurons (from *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ mice) showed decreased neurite outgrowth and branching *in vitro* when compared to *Thy1cre*$^{+/-}$*Pfn1*$^{wt/wt}$ mice (data not shown).

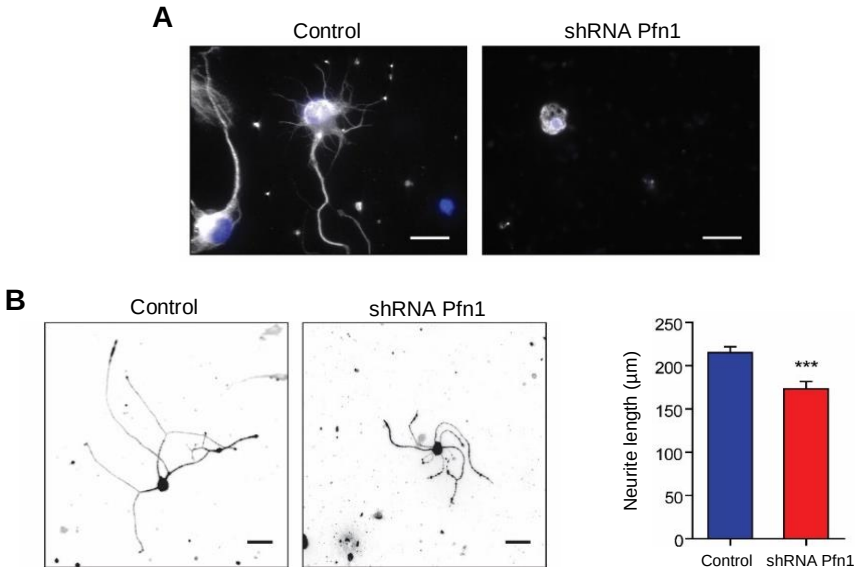


Figure 8 – Pfn1 is a regulator of neuritogenesis and axon growth. (A) Representative images of βIII-tubulin immunofluorescence (in grey) in DIV2 (post-replating) hippocampal neurons transduced with either an empty lentivirus (control) or a lentivirus targeting Pfn1 (shRNA Pfn1). DAPI - blue. Scale bars: 200µm. (B) Representative images of βIII-tubulin immunofluorescence in DRG neurons grown for 12-hour post-replating treated with either an empty lentivirus (control) or a lentivirus targeting Pfn1 (shRNA Pfn1) (left) and neurite outgrowth quantification (right). Scale bars: 100µm. *p*-value: *** <0.001.

To evaluate if the loss of Pfn1 also had an effect on actin and MT dynamics, actin retrograde flow and MT growth speed were evaluated in the growth cones of DRGs isolated from *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ and *Thy1cre*$^{+/-}$*Pfn1*$^{wt/wt}$ mice. In the absence of Pfn1 both

actin and MT dynamics were significantly decreased (Figure 9), further supporting the crucial role of this actin-binding protein in growth cone protrusion and extension. Besides, in order to assess how the absence of Pfn1 would impact the regenerative capacity of axons *in vivo* after a lesion, *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ and *Thy1cre*$^{+/-}$*Pfn1*$^{wt/wt}$ mice were subjected to CL and the axons that were capable of entering in the glial scar were quantified. The results obtained showed that, in the absence of Pfn1, the distance that axons can travel within the glial scar is statistically diminished (data not shown), suggesting an important role of Pfn1 in axon regeneration.

Taken together, these evidences further support the concept that the regulation of cytoskeleton dynamics, namely through Pfn1, is a pivotal requirement for effective axon growth and regeneration.

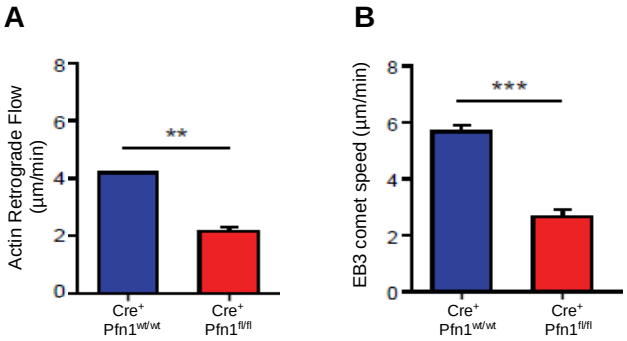


Figure 9 – Depletion of Pfn1 impairs actin and microtubule dynamics in the growth cones of DRG neurons. Quantification of (A) actin retrograde flow and (B) microtubule growth speed in DRG neurons from *Thy1cre*$^{+/-}$*Pfn1*$^{wt/wt}$ and *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ transfected with lifeAct-GFP and EB3-GFP, respectively. *p*-value: ** <0.01; *** <0.001.

Objectives

The main goal of this project was to delve and dissect the role of the actin-binding protein Profilin 1 in neurons, namely:

I) Evaluate the role of Pfn1 in axon growth and regeneration;

II) Assess the role of Pfn2 in axon growth, using the *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ mouse model;

III) Study the molecular and signalling mechanisms behind Pfn1 function in neurons, using a neuronal cell line.

Materials and Methods

Animals

Homozygous mice for the *Pfn1* floxed allele (*Pfn1*$^{fl/fl}$; a kind gift from Prof. Dr Reinhard Fässler, Max Planck Institute of Biochemistry) and SLICK-H mice (kindly provided by Dr Guoping Feng, Duke University) were used to breed neuron-specific *Pfn1* knockout mice (*Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$). SLICK-H mice co-express tamoxifen-inducible CreER^{T2} [1] and YFP, under the control of the neuron-specific *Thy1* promoter [120]. After tamoxifen administration, the CreER^{T2} migrates to the nucleus, where it excises *Pfn1*, leading to the depletion of this protein only in neurons. To achieve this, *Pfn1*$^{fl/fl}$ and SLICK-H mice were crossed. Then, *Thy1cre*$^{+/-}$*Pfn1*$^{fl/wt}$ mice were crossed with *Pfn1*$^{fl/fl}$ or *Pfn1*$^{wt/wt}$ mice to generate *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ (hereafter called Pfn1 cKO mice) and *Thy1cre*$^{+/-}$*Pfn1*$^{wt/wt}$ mice (used as control and hereafter termed as WT mice), respectively. Tamoxifen administration to induce Cre expression was performed by intraperitoneal injection (75 mg/Kg; Sigma-Aldrich) at weaning (3-4 weeks of age).

Mice and rats were bred and housed at the i3S animal facility with *ad libitum* access to water and standard rodent food. The animals were kept under a 12-hour light/dark cycle, with 45-65% humidity and temperatures between 20 and 24 ºC. All genotypes were determined by PCR on genomic DNA extracted from tail, by the i3S CCGen facility. In all experiments animals of either sex were used arbitrarily and they were euthanized by carbon dioxide inhalation.

All animals involved in the experiments were handled in strict accordance with good animal practice, as defined by the European Union Directive 2010/63/EU and the national Decreto-Lei nº 113/2013. The animal experimental procedures described in this work were approved by the i3S Animal Ethics Committee and by the Portuguese National Authority for Animal Health.

[1] CreER^{T2} consists of Cre recombinase fused to a mutated hormone-binding domain of the estrogen receptor, in order to be activated by tamoxifen and not by endogenous estrogens. [121]

Primary Neuron Cultures

Dorsal Root Ganglia Neuron Culture

DRG were dissected aseptically from WT and Pfn1 cKO mice or Wistar rats (at 6-8 weeks of age), freed of roots and collected in DMEM:F12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (PenStrep; Invitrogen). Ganglia were enzymatically treated with 0.125% collagenase-IV-S (Sigma-Aldrich) for 90 minutes (mice) or 2 h (rats), at 37 ºC in a 5% CO_2 atmosphere. After the treatment, a single-cell suspension was readily obtained by dissociation of the ganglia with a flame-polished Pasteur pipette. Then, the single-cell suspension was centrifuged in a 15% bovine serum albumin (BSA; Sigma-Aldrich) gradient for 10 minutes at 1000 rpm and the pellet obtained was resuspended in complete medium: DMEM:F12 medium (Sigma-Aldrich) supplemented with 1% PenStrep (Invitrogen), 1× B-27 (Invitrogen), 5.6 mM L-Glutamine (Invitrogen) and 50 ng/mL NGF (Millipore). Both rat and mouse DRG neurons were subjected to transfection using the 4D-Nucleofector™ System, which will be described below.

Hippocampal Neuron Culture

Pregnant rats or NMRI mice at embryonic day (E) 18 were euthanized and the embryos were removed. Then, the embryonic hippocampi were dissected aseptically and enzymatically treated with 0.06% porcine trypsin (Sigma-Aldrich) in HBSS (Sigma-Aldrich) for 15 minutes at 37 °C in a 5% CO_2 atmosphere. After the treatment, hippocampi were washed, resuspended in Neurobasal medium (Invitrogen) and dissociated by passing the solution up and down in a micropipette and through a 70 µm cell strainer, to remove any undissociated tissue. As for the DRG neurons, hippocampal neurons also underwent transfection using the 4D-Nucleofector™ System, which will be explained below.

Restriction analysis of pAAV-hPfn1S137A-IRES-EGFP

An AAV-based vector for the *in vivo* expression of Pfn1 S137A in DRG neurons was designed and custom-made by Vector Builder (www.vectorbuilder.com). In order to ensure the intactness of ITRs and expression cassette, the pAAV-hPfn1S137A-IRES-EGFP plasmid DNA was digested with either SmaI (Roche) or HindII (Fermentas) for

5 h (at 25 ºC and 37 ºC, respectively). Subsequently, the digestion products were loaded (1.5 µg of DNA per lane) into a 1% agarose gel containing GreenSafe Premium (NZYTech) and the gel was visualized under UV light.

Overexpression of Pfn1 in DRG and hippocampal neurons

After obtaining the single-cell solution of rat DRG or hippocampal neurons, as aforementioned, cells were counted and divided (at least 200,000 cells per condition for DRG and 750,000 cells per condition for hippocampal neurons) and were then centrifuged for 5 minutes at 800 rpm (for DRG neurons) or 10 minutes at 1000 rpm (for hippocampal neurons). The pellets obtained were resuspended in Opti-MEM medium (Invitrogen) and neurons were nucleofected using the 4D-Nucleofector™ System (Lonza; CM-138 program for DRG and CU-110 program for hippocampal neurons). To test the AAV-based vector designed for Pfn1 *in vivo* delivery, DRG neurons were nucleofected, using the 4D-Nucleofector™ System, with 900 ng of empty pAAV-IRES-EGFP (a kind gift from Dr Antonio Velayos-Baeza, University of Oxford) or pAAV-hPfn1S137A-IRES-EGFP (Vector Builder). For Pfn1 overexpression, 600 ng of the pCMV-SPORT6 vector, coding the full length Pfn1 open reading frame (Addgene, clone IRATp970C034D) or the mutant plasmid of phosphorylation-resistant Pfn1 (Pfn1 S137A), were co-transfected with either 400 ng of pmaxGFP™ (Lonza), 750 ng of LifeAct-GFP [122] or 500 ng of EB3-GFP [123] per condition, according to the parameter to be analysed (neurite outgrowth, actin or microtubule dynamics, respectively). The Pfn1 mutation was generated by PCR-based site-directed mutagenesis using QuickChange II XL (Agilent Technologies). After nucleofection, cells underwent a recovery step: rested for 10 minutes, RPMI medium (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) was added and cells rested for another 10 minutes. Then, DRG neurons were transferred to complete medium, left in suspension for 24 h (at 37 °C in a 5% CO_2 atmosphere) and after that, cells were counted and plated. Concerning hippocampal neurons, after the recovery step, they were transferred to Neurobasal medium (Invitrogen) supplemented with 1x B-27 (Invitrogen), 1% PenStrep (Invitrogen) and 2 mM L-Glutamine (Invitrogen) and plated. Afterwards, neurite outgrowth, actin and microtubule dynamics were assessed, as will be described below.

Acute deletion of Pfn2 in DRG neurons

After obtaining the single-cell suspension of DRG neurons (from WT and Pfn1 cKO mice), as previously described, cells were counted and divided (200,000 cells per condition) and were then centrifuged for 5 minutes at 800 rpm. The pellets obtained were resuspended in Opti-MEM medium (Invitrogen) and neurons were nucleofected using the 4D-Nucleofector™ System (Lonza; CM-137 program). To perform the acute shRNA-mediated depletion of Pfn2, 1500 ng of the pLKO.1 vector coding the shRNA for Pfn2 (TRCN0000071642, Sigma-Aldrich) or the empty pLKO.1 vector (Sigma-Aldrich), as control, and 500 ng of DsRed were used. After nucleofection, cells underwent a recovery step, as described above, and were then transferred to complete medium. After 24 h in suspension at 37 °C in a 5% CO_2 atmosphere, cells were counted and plated, and neurite outgrowth was assessed, as will be described below.

Neurite outgrowth evaluation

For neurite outgrowth analysis, an immunocytochemistry against ßIII-tubulin was performed. After being left in suspension, DRG neurons were plated in poly-L-lysine (PLL, 20 µg/mL, Sigma-Aldrich) and laminin (5 µg/mL, Sigma-Aldrich) coated 13 mm coverslips and maintained for 12 h at 37 °C in a 5% CO_2 atmosphere. Regarding hippocampal neurons, they were plated in PLL (20 µg/mL, Sigma-Aldrich) coated 13 mm coverslips and maintained for 96 h at 37°C in a 5% CO_2 atmosphere.

Neurons were fixed 12 h or 96 h post-plating (for DRG and hippocampal neurons, respectively) with 2% paraformaldehyde (PFA) for 10 minutes, permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich) for 5 minutes at room temperature (RT), blocked with 5 % FBS (in phosphate-buffered saline - PBS) for 1 h at RT and incubated with the primary antibody, mouse anti-ßIII tubulin (1:2,000 in blocking buffer; Promega), overnight at 4 °C. After washing with PBS, the secondary antibody, donkey anti-mouse IgG Alexa Fluor® 568 or Alexa Fluor® 647 (1:1,000; Invitrogen), was diluted in blocking buffer and incubated for 1 h at RT. The coverslips were then washed and mounted on microscope slides with Fluoroshield™ with DAPI (Sigma-Aldrich). The slides were analysed and images acquired with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR 3.0 camera and Axiovision software (4.7 version) (all from Carl Zeiss, Germany). Only GFP^+/βIII-$tubulin^+$ neurons (from rat) or YFP^+/βIII-$tubulin^+$ neurons (from WT and Pfn1 cKO mice) were considered and the respective neurites were traced, using the

SynD software [124]. Concerning hippocampal neurons, the length of the longest neurite was determined using NeuronJ plug-in for ImageJ.

Analysis of actin retrograde flow and microtubule dynamics

In order to evaluate the effect of Pfn1 overexpression on actin and microtubule dynamics at the growth cone, rat DRG and hippocampal neurons were cultured and nucleofected, as previously described. After being left in suspension for 24 h, DRG neurons were counted and plated for 12 h at 37 ºC in a PLL (20 µg/mL, Sigma-Aldrich) and laminin (5 µg/mL, Sigma-Aldrich) coated 8 well µ-Slide (Ibidi), previously treated with 2 M HCl (Sigma-Aldrich). One hour before time-lapse recording, the medium was changed to Neurobasal medium without phenol red (Invitrogen). Concerning hippocampal neurons, after nucleofection they were plated in Neurobasal medium without phenol red (Invitrogen), supplemented as mentioned above, in a PLL (20 µg/mL, Sigma-Aldrich) coated 8 well µ-Slide (Ibidi), previously treated with 2 M HCl (Sigma-Aldrich).

Time-lapse recordings were performed at 12 h post-plating and at DIV4 (for DRG and hippocampal neurons, respectively) at 37 ºC in a Spinning Disk Confocal System Andor Revolution XD equipped with an iXon^{EM}+ DU-897 camera and IQ 1.10.1 software (all from ANDOR Technology, UK). For actin retrograde flow analysis, neurons were imaged for 40 frames, captured each 5 seconds (in a total of 200 seconds) and for the analysis of microtubule dynamics, neurons were imaged for 100 frames, captured each 2 seconds (in a total of 200 seconds). Only GFP^{+} neurons were considered and kymographs were generated using the Kymograph plug-in for ImageJ software.

Overexpression of Pfn1 in CAD cells

CAD cells were maintained in DMEM medium (Sigma-Aldrich) supplemented with 8% FBS (Sigma-Aldrich) and 1% PenStrep (Invitrogen). For Pfn1 overexpression, when CAD cells reached 70% confluence, they were plated in a 24-well plate (with 190,000 cells per well) for 24 h and were then transfected, using Lipofectamine® 2000 (Invitrogen), with 500 ng of pmaxGFP™ (Lonza) and 500 ng of the pCMV-SPORT6 vector coding the full length Pfn1 open reading frame (Addgene, clone IRATp970C034D) or the mutant plasmid of phosphorylation-resistant Pfn1 (Pfn1 S137A). After 48 h, the wells were washed with PBS and the cells were lysed with lysis buffer (PBS containing

0.3% Triton X-100 (Sigma-Aldrich), protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche) and 1 mM sodium orthovanadate (Sigma-Aldrich)).

Western Blotting

Protein lysates of brain (from WT and Pfn1 cKO mice) were prepared by homogenisation of the tissue followed by sonication, using the same lysis buffer as described before. CAD cell protein lysates were prepared by sonication only, also in lysis buffer. The protein lysates from brain samples were loaded (15 µg of protein per lane) into a 4–20% Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad), while CAD cell lysates were loaded (25 µg of protein per lane) into a 15% SDS-PAGE gel. After running the samples, gels were transferred for 2 h to Amersham™ Protran® Premium 0.45 µm nitrocellulose membranes (GE Healthcare Life Sciences), using a semi-dry system.

Membranes were washed in Tris buffered saline (TBS) with 0.1% Tween-20 (Sigma-Aldrich), blocked with 5% skim milk (Sigma-Aldrich) in TBS containing 0.1% Tween-20 for 1 h at RT, and incubated with primary antibodies in 5% BSA (Sigma-Aldrich) in TBS with 0.1% Tween-20, unless stated otherwise. Membranes containing brain lysates were probed with the following primary antibodies: rabbit anti-Pfn2 (1:2,000, diluted in 5% skim milk in TBS with 0.1% Tween-20; kindly provided by Dr Martin Rothkegel), mouse anti-ß-actin (1:5,000; Sigma-Aldrich) and mouse anti-α-tubulin (1:10,000; Sigma-Aldrich). Membranes containing CAD cell protein lysates were probed with the following primary antibodies: rabbit anti-Pfn1 (1:1,000; Abcam), rabbit anti-phospho-AKT (Thr308) (1:1,000; Cell Signalling), rabbit anti-phospho-AKT (Ser473) (1:1,000, Cell Signalling), rabbit anti-total-AKT (1:1,000; Cell Signalling), rabbit anti-phospho-GSK3α/β (Tyr216) (1:1,000; Santa Cruz Biotechnology), rabbit anti-phospho-GSK3ß (Ser9) (1:1,000; Cell Signalling) and mouse anti-total-GSK3α/ß (1:1,000; Santa Cruz Biotechnology). Incubations were done overnight at 4 ºC, except for anti-ß-actin and anti-α-tubulin which were incubated 1h at RT. Afterwards, the membranes were washed and incubated with the respective secondary antibody in 5% skim milk in TBS with 0.1% Tween-20 for 1h at RT. The secondary antibodies used were: donkey anti-mouse or donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:10,000 or 1:5,000, respectively) (both from Jackson ImmunoResearch Laboratories). Membranes were then incubated with Luminata Forte Western HRP (Millipore) for 5 min at RT and exposed to Fuji Medical X-Ray Film (Fujifilm). Films were scanned using a Molecular Imager GS800 calibrated densitometer (Bio-Rad) and quantified with Quantity One 1-D Analysis Software, version 4.6 (Bio-Rad).

Statistical Analysis

The results obtained are expressed as mean ± SEM (standard error of the mean) and the statistical analysis was performed with GraphPad Prism, version 6.01 for Windows (GraphPad Software, California, USA). In order to determine statistical significance, one-way ANOVA followed by Tukey's multiple comparisons test was performed. When comparison was only between two experimental groups, two-tailed Student's t-test was applied. A *p*-value of less than 0.05 was considered statistically significant.

Results

I) Overexpression of Pfn1 enhances axon growth, actin and microtubule dynamics

As previously mentioned, preliminary data showed that Pfn1 plays an important role in axon formation and growth, as in its absence, hippocampal neurons presented impaired neuritogenesis and DRG neurons displayed decreased neurite outgrowth (Figure 8). Taking this into account and given the crucial role of Pfn1 in actin treadmilling in the growth cone, we wanted to assess whether Pfn1 overexpression would result in increased axon growth, in both hippocampal and DRG neurons. For that, two Pfn1 forms were overexpressed, namely wild-type (WT) Pfn1 and a phosphorylation-resistant mutated form (Pfn1 S137A), which is constitutively active, and subsequently we evaluated neurite outgrowth.

As depicted in Figure 10, when Pfn1 S137A was overexpressed in hippocampal neurons, there was a significant increase of the axon length. Similarly, DRG neurons overexpressing Pfn1 WT showed increased neurite outgrowth and those overexpressing Pfn1 S137A demonstrated an even more pronounced effect, with a 2.5-fold increase when compared to the control group (Figure 11). This significant difference between the effect of the overexpression of the WT and mutated Pfn1 forms may be due to intracellular regulatory mechanisms that possibly try to counterbalance the excess of Pfn1 WT by phosphorylating it. In contrast, as the mutated form cannot be phosphorylated it can exert its full effect.

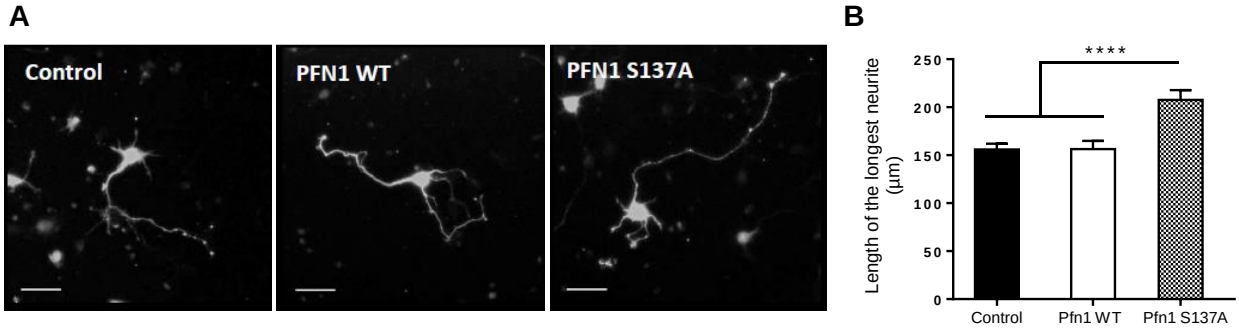


Figure 10 – Overexpression of Pfn1 S137A leads to increased neurite outgrowth of hippocampal neurons. (A) Representative images of βIII-tubulin immunofluorescence (in grey) in hippocampal neurons co-transfected with GFP and either an empty plasmid as control (left), a plasmid coding for Pfn1 WT (centre) or Pfn1 S137A (right). Scale bars: 50 µm. (B) Quantification of neurite outgrowth of $GFP^+/βIII\text{-}tubulin^+$ hippocampal neurons co-transfected with GFP and either and empty plasmid as control (n=126), a plasmid coding for Pfn1 WT (n=117) or Pfn1 S137A (n=118). All error bars are SEM. *p*-value: **** < 0.0001.

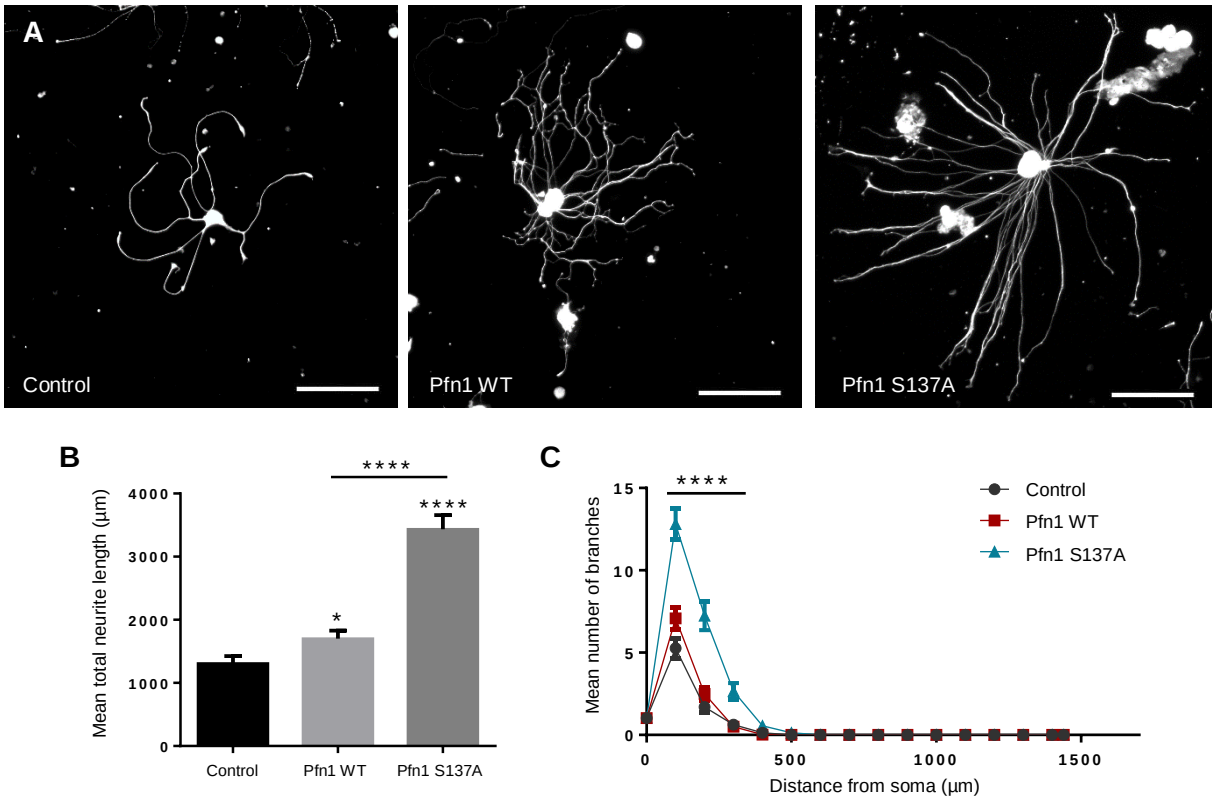


Figure 11 – Overexpression of Pfn1 WT and Pfn1 S137A leads to increased neurite outgrowth of DRG neurons. (A) Representative images of βIII-tubulin immunofluorescence (in grey) in rat DRG neurons co-transfected with GFP and either an empty plasmid as control (left), a plasmid coding for Pfn1 WT (centre) or Pfn1 S137A (right). Scale bars: 150 µm. Quantification (B) and Sholl analysis (C) of neurite outgrowth of GFP^+/$βIII\text{-tubulin}^+$ DRG neurons co-transfected with GFP and either an empty plasmid as control (n=91), a plasmid coding for Pfn1 WT (n=106) or Pfn1 S137A (n=95). All error bars are SEM. *p*-value: * <0.05; ****<0.0001.

Since cytoskeleton dynamics is crucial to provide the protrusion force and support for extension in axonal growth and given the observed striking effect of the constitutively active form of Pfn1 in neurite outgrowth (in both hippocampal and DRG neurons), we wanted to further dissect the impact of Pfn1 overexpression in this process. Therefore, actin and MT dynamics were assessed in both hippocampal (Figure 12) and DRG (Figure 13) neurons. In both hippocampal and DRG neurons, the overexpression of either Pfn1 WT or S137A significantly upregulated actin retrograde flow and MT growth speed, when compared to neurons from the control group, which were co-transfected with an empty vector. These results further highlight and support the pivotal role of Pfn1 activity in mediating the transition of ADP to ATP bound actin, required for the dynamism that growth cones present during axon extension. Regarding MT dynamics, the strong effect observed could be due to the interplay between the actin and MT cytoskeletons that have the ability to influence each other, or due to the participation of Pfn1 in intracellular

signalling cascades that regulate microtubule dynamics (as will be further explored), or to the combination of these two independent components.

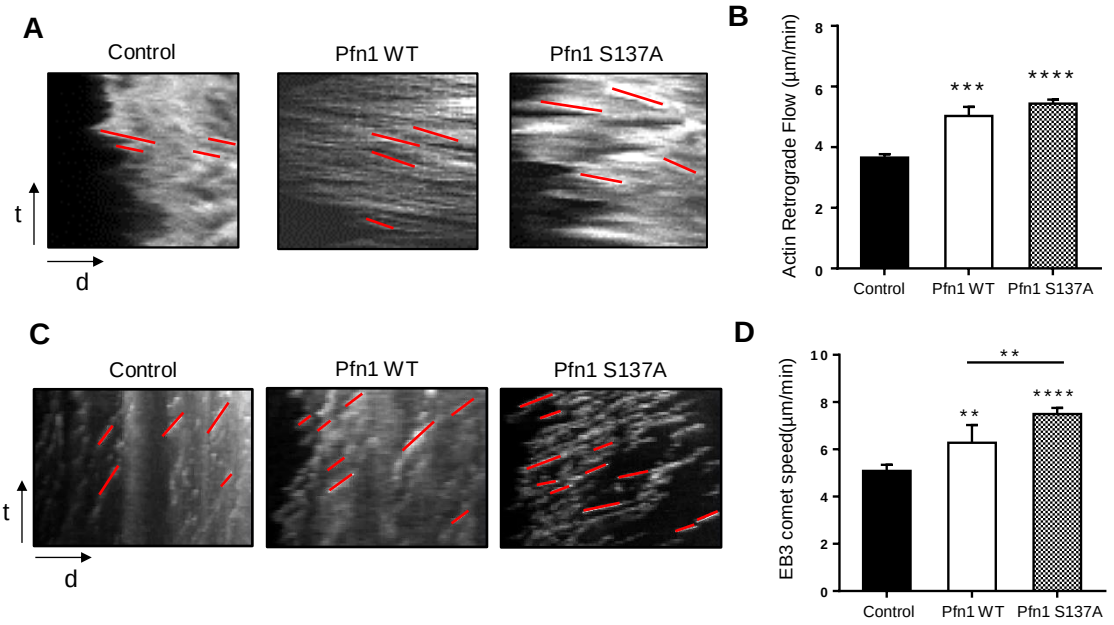


Figure 12 – Overexpression of Pfn1 WT and Pfn1 S137A increases actin and MT dynamics in hippocampal neurons. (A; C) Representative kymographs from growth cones of rat hippocampal neurons co-transfected with either an empty plasmid as control (left), a plasmid coding for Pfn1 WT (centre) or Pfn1 S137A (right) and with lifeAct-GFP to assess actin dynamics (A) or EB3-GFP to assess MT dynamics (C). Time (t) in minutes and distance (d) in µm. (B) Quantification of (A). (D) Quantification of (C). All error bars are SEM. *p*-value: ** <0.01; *** <0.001; ****<0.0001.

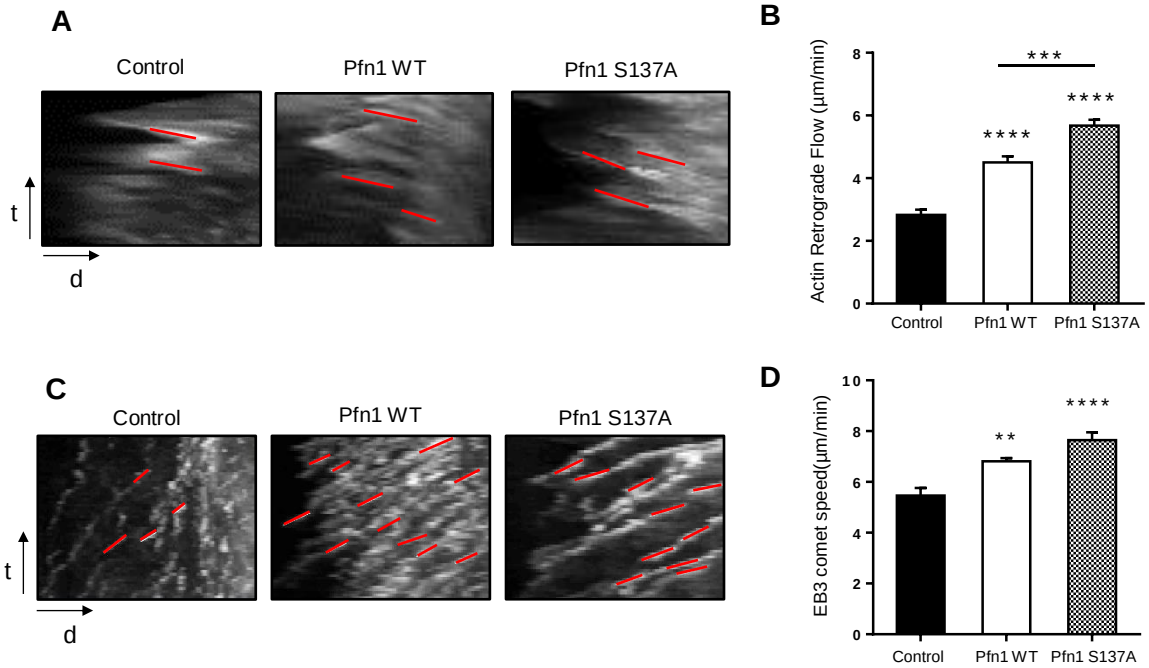


Figure 13 – Overexpression of Pfn1 WT and Pfn1 S137A increases actin and MT dynamics in DRG neurons. (A; C) Representative kymographs from growth cones of rat DRG neurons co-transfected with either an empty plasmid as control (left), a plasmid coding for Pfn1 WT (centre) or Pfn1 S137A (right) and with lifeAct-GFP to assess actin dynamics (A) or EB3-GFP to assess MT dynamics (C). Time (t) in minutes and distance (d) in µm. (B) Quantification of (A). (D) Quantification of (C). All error bars are SEM. *p*-value: ** <0.01; *** <0.001; ****<0.0001.

The above results support the possible positive effect of the *in vivo* delivery of Pfn1 S137A in the settings of axonal injury. In that respect, we have designed an AAV-based strategy to deliver Pfn1 S137A to the rat DRG after sciatic nerve injury. For that, the AAV-based vector displayed below - pAAV-hPfn1S137A-IRES-EGFP - was designed (Figure 14) and the construct was made as a service by Vector Builder (www.vectorbuilder.com). Given the low molecular weight of Pfn1 and the fact that tagging it with large molecules such as EGFP may affect its function, we opted by an IRES-based strategy with the independent translation of Pfn1 and EGFP.

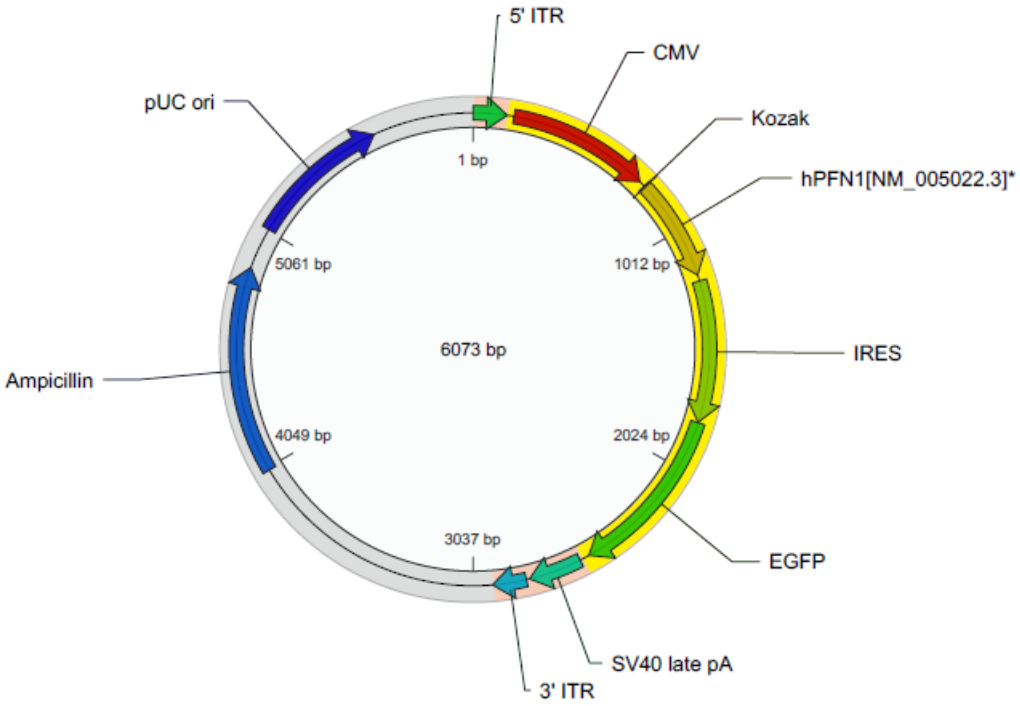


Figure 14 – Vector map designed for the *in vivo* delivery of Pfn1 S137A. ITR, Inverted Terminal Repeat; CMV, cytomegalovirus promoter; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; SV40 late pA, SV40 late polyadenation signal; pUC ori, pUC origin of replication.

A restriction analysis of the construct pAAV-hPfn1S137A-IRES-EGFP was performed to ensure correctness of expression cassette. As expected following analysis using the SnapGene software (www.snapgene.com), restriction of our construct with SmaI yielded visible fragments of 3201 bp, 2008 bp and 842 bp and HindII restriction yielded fragments of 4271 bp and 1802 bp (Figure 15). As a subsequent step, we analysed if transfection of the pAAV-hPfn1S137A-IRES-EGFP construct in DRG neurons elicited the observation of GFP-positive cells (as the length of GFP-positive axons will be used as the readout of the *in vivo* experiments). As depicted in Figure 16, neurons with high expression levels of GFP were clearly observed. Currently, evaluation of neurite outgrowth is being conducted to ensure that this AAV-based Pfn1 S137A

overexpressing construct has similar enhancing growth capacity as constructs that we have previously used.

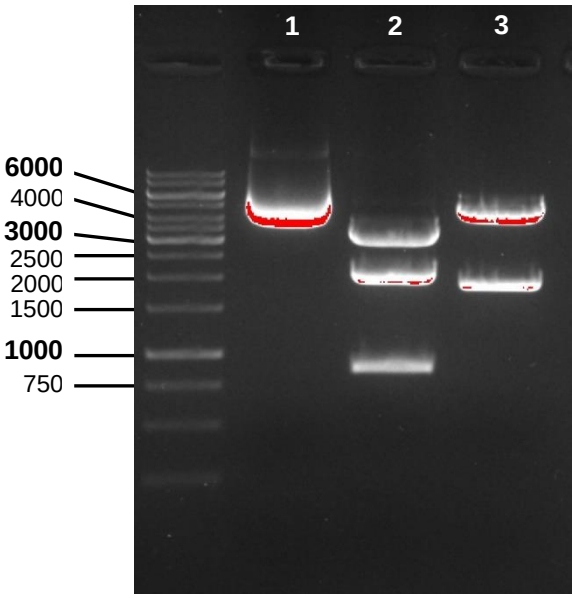


Figure 15 – Restriction analysis of pAAV-hPfn1S137A-IRES-GFP with either SmaI or HindII. 1 - undigested pAAV-hPfn1S137A-IRES-GFP; 2 - pAAV-hPfn1S137A-IRES-GFP digested with SmaI (fragments of 3201 bp, 2008 bp and 842 bp); 3 - pAAV-hPfn1S137A-IRES-GFP digested with HindII (fragments of 4271 bp and 1802 bp).

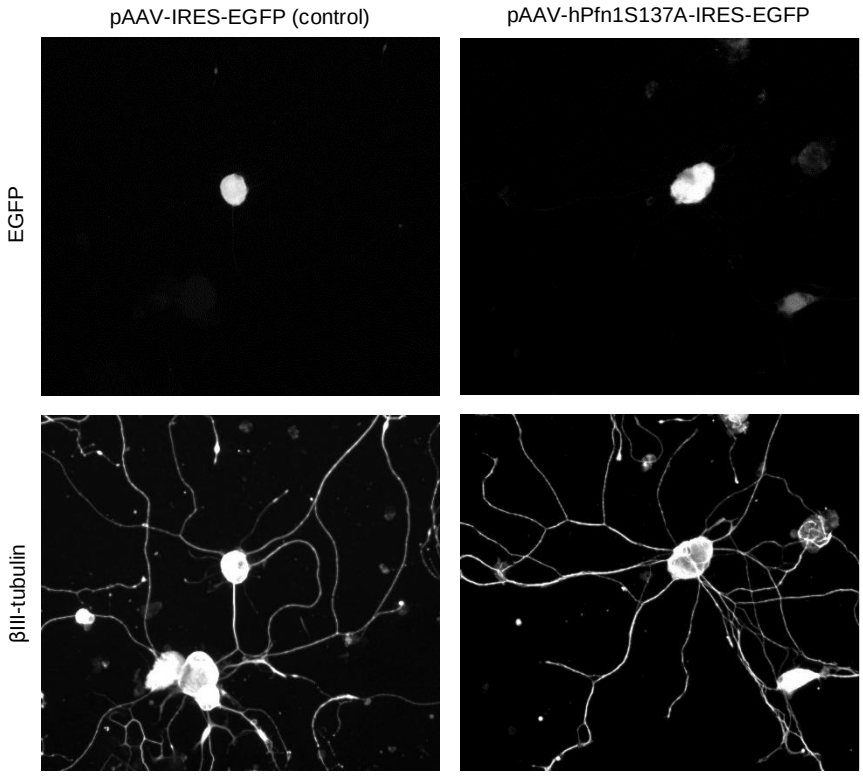


Figure 16 – DRG neurons show high expression levels of GFP after transfection with the AAV-based construct. Representative images of βIII-tubulin immunofluorescence and GFP in rat DRG neurons transfected with either an empty vector pAAV-IRES-EGFP as control (left) or pAAV-hPfn1S137A-IRES-EGFP (right).

II) Assessing the role of Pfn2 in axon growth

Given the redundancy of profilins, we next investigated the role of the brain-specific form of profilin (Pfn2) in axon growth. For that we used *Thy1cre*$^{+/-}$*Pfn1*$^{fl/fl}$ mice, which lack Pfn1 specifically in neurons. In these animals, a possible compensatory effect of Pfn2 may arise in the absence of Pfn1.

Firstly, the levels of Pfn2 were assessed by western blot in samples of brain tissue from WT and Pfn1 cKO mice. As can be seen in Figure 17, there is no statistical difference in the levels of Pfn2 in the brain tissue of these animals, suggesting that, at least at the level of expression, no compensation exists.

As this model represents a chronic scenario, some adaptations might occur and mask a possible role of Pfn2 in the absence of Pfn1. Therefore, to further assess if Pfn2 had an effect on axonal growth, the acute deletion of Pfn2 (by shRNA) was performed in DRG neurons from WT and Pfn1 cKO mice and neurite outgrowth was assessed (Figure 18). The results obtained showed that the absence of Pfn2 seems to have no effect on neurite length, whether Pfn1 is absent or not. Of note, as expected, there is a significant decrease in the neurite length and branching of Pfn1 cKO neurons when compared to WT, both in the presence or absence of Pfn2.

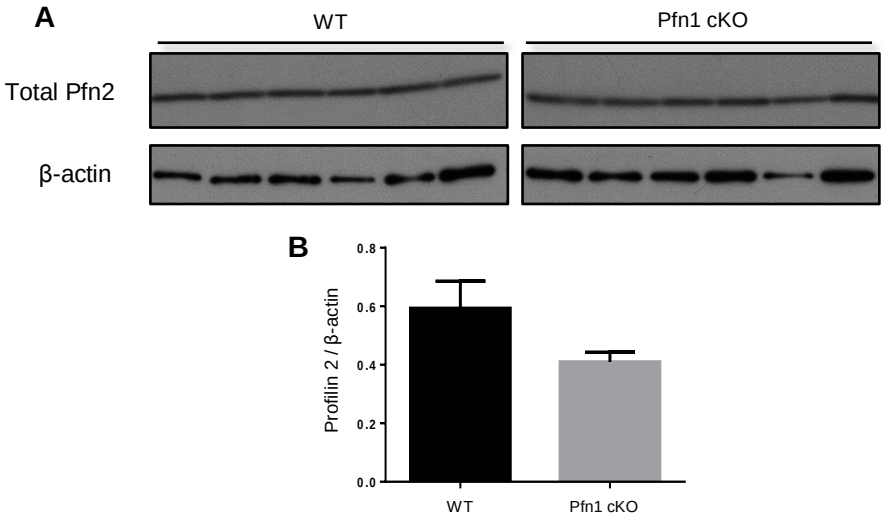


Figure 17 – Profilin 2 expression in brain from WT and Pfn1 cKO mice. (A) Western blot analysis of the expression levels of total Pfn2 and β-actin in brain tissue from WT and Pfn1 cKO mice. (B) Quantification of (A). All error bars are SEM.

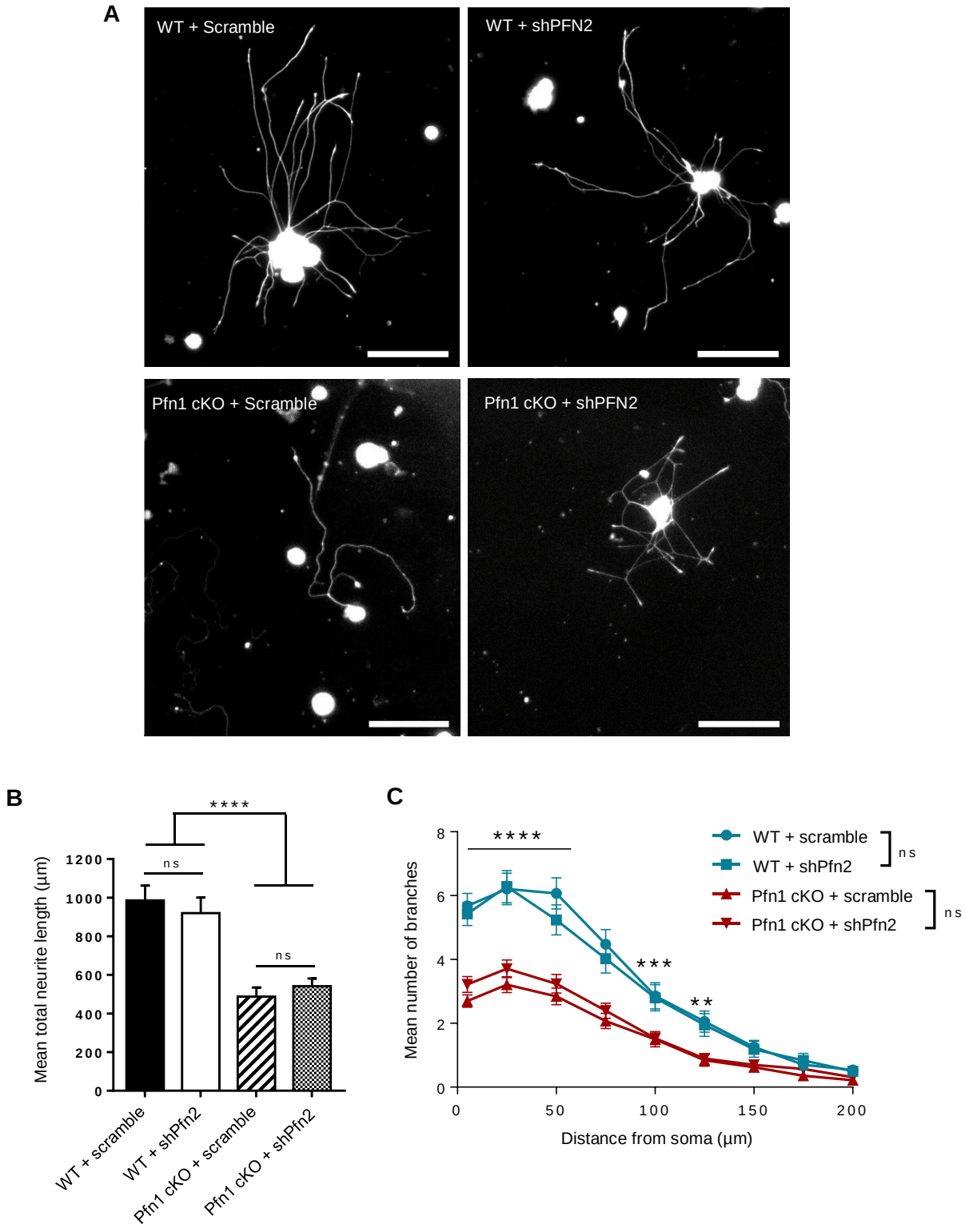


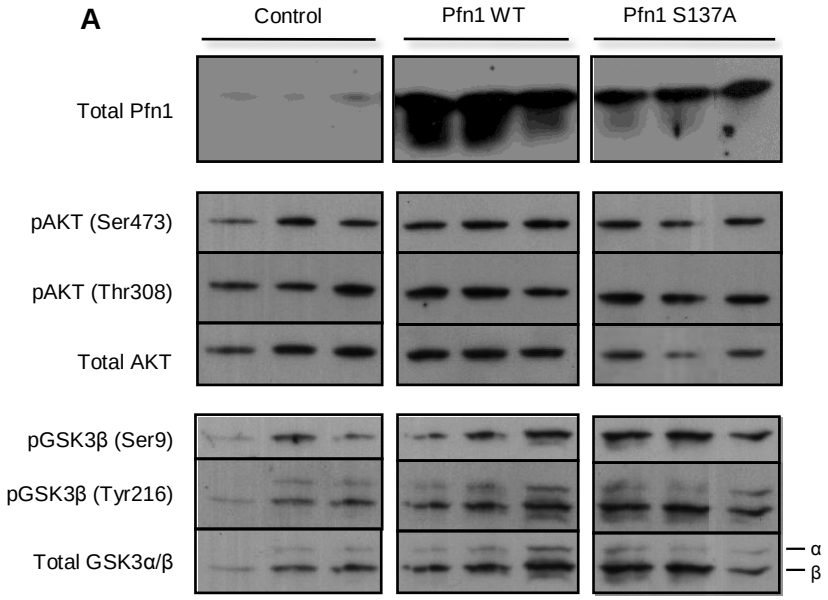
Figure 18 – Pfn2 acute deletion in DRG neurons from WT and Pfn1 cKO mice does not influence neurite outgrowth. (A) Representative images of βIII-tubulin immunofluorescence (in grey) in DRG neurons from WT and Pfn1 cKO mice co-transfected with DsRed and either an empty plasmid (scramble) or a plasmid coding the shRNA for Pfn2 (shPfn2). Scale bars: 200 µm. Quantification (B) and Sholl analysis (C) of neurite outgrowth of DsRed^{+}/ βIII-tubulin^{+} DRG neurons from WT and Pfn1 cKO mice, co-transfected with DsRed and either an empty plasmid (Scramble; WT: n = 71, Pfn1 cKO: n = 75) or a plasmid coding the shRNA for Pfn2 (shPfn2; WT: n= 82, Pfn1 cKO: n = 96). All error bars are SEM. ns, not statistically significant. *p*-value: ** <0.01; *** <0.001; ****<0.0001.

Therefore, our results suggest that Pfn2 does not have an essential role in neurite outgrowth, contrary to Pfn1.

III) Studying the molecular and signalling mechanisms behind Pfn1 function

The third goal of this project was to dissect the molecular and signalling mechanisms that underlie Pfn1 function during axon formation and growth. As mentioned before, Pfn1 has three distinct binding domains and consequently, besides its actin-binding activity, Pfn1 can also interact with PLP-rich regions and phosphatidylinositol lipids, with a preference for PIP_2 at the cellular membrane. Therefore, this interaction suggests a potential role of Pfn1 in modulating signalling cascades, such as the PI3K/AKT pathway, which is important in regulating MT dynamics [17]. In order to address this goal and assess whether Pfn1 interferes with this signalling pathway, Pfn1 WT and Pfn1 S137A were overexpressed in CAD cells and the expression levels of some proteins involved in this pathway were evaluated 48h post transfection (Figure 19).

As can be seen in Figure 19A, the expression levels of total Pfn1 significantly increased after transfection of both the WT and mutated forms. After overexpression of the constitutively active form of Pfn1, the levels of phospho-AKT S473 were increased and phospho-AKT T308 showed a similar trend, although not statistically significant. The downstream effector of AKT, GSK3β, showed increased phosphorylation at serine 9 residue when Pfn1 S137A was overexpressed. Notably, this phosphorylation is mediated by AKT and negatively modulates GSK3β activity. [125] On the other hand, phosphorylation at tyrosine 216 of GSK3β, which positively regulates its activity [126], was not altered with Pfn1 overexpression.



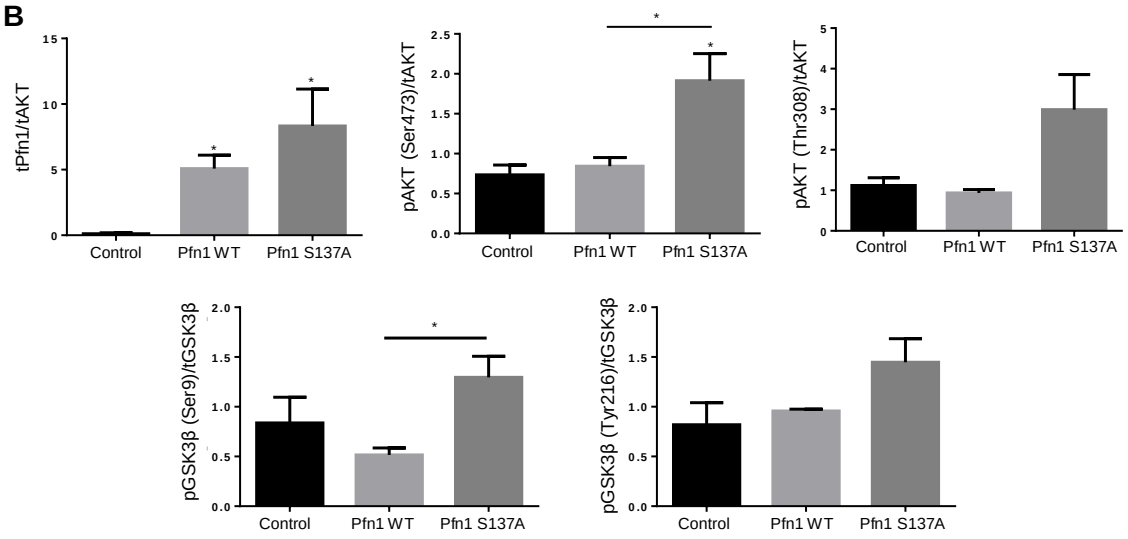


Figure 19 – Pfn1 may interfere with PI3K/AKT signalling pathway. (A) Western blot analysis (48h post transfection) of the expression levels of total Pfn1, pSer473-AKT, pThr308-AKT, total AKT, pSer9-GSK3β, pTyr216-GSK3β and total GSK3α/β in CAD cells (control) and CAD cells overexpressing either Pfn1 WT or Pfn1 S137A. (B) Quantification of (A). All error bars are SEM. *p*-value: * <0.05.

The levels of some downstream targets of GSK3β, namely CRMP2 and MAP1b, were also investigated but given technical limitations of the antibodies used, consistent results have not been obtained.

In summary, overexpression of the constitutively active form of Pfn1 may lead to AKT activation, followed by inactivation of GSK3β, through phosphorylation at serine 9 mediated by AKT. Therefore, these results support the view that Pfn1 has the capacity to modulate not only the actin cytoskeleton, but may also interfere with microtubule regulation, through the PI3K/AKT signalling pathway [127] (Figure 20).

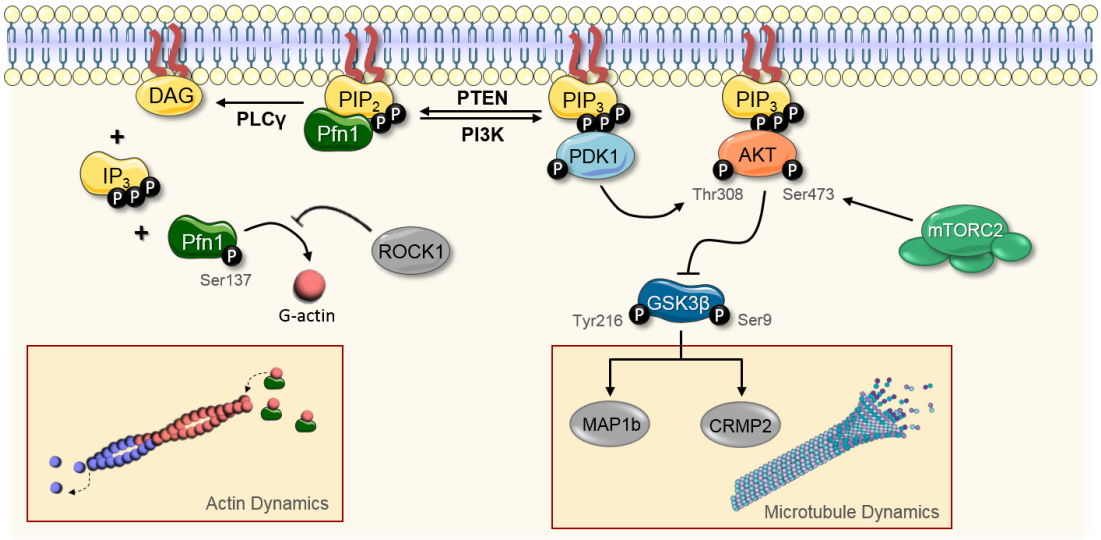


Figure 20 – Pfn1 may interfere with signalling cascades and function as bridge between actin and microtubules.

Discussion

Although actin and MTs are crucial and well recognized as key players for the generation of protrusion force and maintenance of axon growth [32], how the different ABPs and MT regulating proteins control these processes is still not fully understood. Our group has gathered evidence supporting that the ABP Pfn1 is an important player in mediating actin dynamics during axon growth and regeneration. Therefore, understanding how profilins (both Pfn1 and Pfn2) are involved in axon formation, growth and regeneration was the main focus of this project.

Our results showed that the overexpression of Pfn1 WT and of a constitutively active form of the protein, Pfn1 S137A, which cannot be negatively regulated by phosphorylation at serine 137 by ROCK [94] or PKCζ [93], consistently increased neurite outgrowth, actin dynamics and microtubule growth speed. Of note the effect of Pfn1 S137A was generally more robust than that of Pfn1 WT, emphasizing the importance of active Pfn1 for optimal axon growth to occur. This difference between the two Pfn1 forms is probably related to the fact that Pfn1 WT might be inactivated by phosphorylation after overexpression, inhibiting its interaction with actin. This hypothesis could be addressed in the future, by assessing through western blot the levels of Pfn1 phosphorylated at the serine 137 residue after Pfn1 WT overexpression.

The effect of Pfn1 overexpression in actin dynamics is consistent with the Pfn1 role in renewing the pool of ATP-actin monomers that can be added to actin filaments, promoting actin polymerization and overall dynamics at the leading edges of growth cones [99]. In addition, it has also been previously reported that Pfn1 depletion caused substantial defects in membrane extension and a slower velocity of protrusion in neuronal cells [87]. Thus, it was expected that Pfn1 overexpression would lead to opposite effects, as is here documented. These results clearly emphasize that Pfn1 plays an essential role in actin turnover at the leading edge of growth cones, a process that underlies not only axon growth and extension, but also axon regeneration following injury. The strong effect of Pfn1 overexpression in increasing MT growth speed was rather unexpected and should be the subject of further investigation. This effect could be caused either by the participation of Pfn1 in intracellular pathways that regulate MT dynamics (as is further discussed below) or be an indirect effect of the increase in actin dynamics, or a combination of both. It has been reported that interactions between MTs and actin filaments are required for axon branching and directed axon growth. [128] In fact, augmented actin retrograde flow was shown to organize the space for the protrusion and bundling of MTs, the backbone of neurites, thereby enabling increased neurite

outgrowth. [129] Additionally, Pfn1 was recently shown to connect actin assembly with MT dynamics [130]. In this report, Pfn1 was shown to be functionally linked to MTs through formins that act as major mediators of this association. However, the effect of Pfn1 in MT dynamics described in that report was the opposite from the one that we here show, thereby supporting that further studies are needed to clarify this subject.

Taking into account the strong effect of the constitutively active form of Pfn1 overexpression *in vitro*, we will test the effect of the *in vivo* delivery of this mutated form of Pfn1 in the settings of axonal injury. This will be achieved by using a viral approach to deliver Pfn1 S137A to DRG neurons of rats with a sciatic nerve injury, followed by evaluation of axon regeneration in the sciatic nerve. Given the previously mentioned results it is reasonable to expect that Pfn1 S137A might have a positive effect in regeneration of axons after injury. However, it is possible that this effect might not be so striking as the effect observed *in vitro*, as in an *in vivo* environment there are other factors, which are absent *in vitro*, that come into play. If Pfn1 S137A overexpression leads to increased axonal regeneration after injury, Pfn1 may become a good target for future therapies to improve axon regeneration following nerve injury.

Pfn1 is not the only profilin expressed in neuronal cells. Pfn2 is a neuronal-specific profilin form that has been implicated as a negative regulator in neuritogenesis, [96] in contrast to what we here show for Pfn1. In that report, Pfn2 KO hippocampal neurons exhibit multiple sprouting neurites with increased lengths. In our system, deletion of Pfn1 results in decreased neurite growth capacity. We challenged the previous results obtained for Pfn2 and tested whether this Pfn form could play a compensatory effect in the absence of Pfn1. Pfn2 knockout mice have a normal development of the nervous system with some alterations in neurotransmitter release [113], demonstrating that in early phases this profilin form is not crucial to neuronal polarization and axon extension, which does not mean that at later stages it could not assume a more important role in these processes. Curiously, while in early mouse embryos this isoform is only weakly expressed, in adult mouse brain Pfn2 accounts for two-thirds of the total profilin content [75], which could suggest that at later phases Pfn2 could have a more relevant role. In the brains of Pfn1 cKO mice, no statistical difference was observed in Pfn2 levels when compared to WT mice. Additionally, our data also show that the absence of Pfn2 has no effect on neurite growth, whether Pfn1 is absent or not. Taken together, these results suggest that Pfn2 does not have an important role in axon growth and regeneration. This remarkable differences in axon formation and growth between these two profilin forms, which fascinatingly present similar three dimensional structures, might be due to their different binding affinities to their plethora of Pfn ligands. [72] Of note, Pfn1 has a higher

affinity for actin and PIP_2 than Pfn2 [75], which might be the main reason for the observations reported above. To further explore the different contributions of Pfn1 and Pfn2 to axon growth and regeneration, it could be interesting to assess Pfn2 expression levels in the injury site after conditioning lesion, to check whether, similarly to Pfn1, its activity is altered in conditions of optimal axon regeneration. Furthermore, although neurite outgrowth is not impaired in the absence of Pfn2, actin and/or MT dynamics might be and thus it could be interesting to investigate the effect of the absence of Pfn2 in cytoskeleton dynamics.

Another goal of this project was to unravel the molecular and signalling mechanisms behind Pfn1 function in axon formation and extension. Interestingly, actin is not the only protein with which profilins can interact with. As aforementioned, profilins have three distinct binding domains, namely an actin-binding domain [79], PI lipid-binding site [80] and PLP-binding domain. Therefore, profilins are able to interact with a wide variety of ligands and possibly regulate several signalling pathways. Specifically, through its PI lipid-binding domain, Pfn1 can interact with PIP_2, which prevents Pfn1 interaction with either actin or PLP-containing proteins, due to overlapping of the binding domains. [82] Besides, when interacting with PIP_2, Pfn1 is interfering with its membrane availability and also hinders its hydrolysis by PLC, suggesting a possible role for Pfn1 in regulating the PI3K/AKT pathway, which has been previously reported to be involved in neurite outgrowth. [131] To study this hypothesis, Pfn1 WT and Pfn1 S137A were overexpressed in a neuronal cell line and the expression levels of some key molecules involved in this pathway were assessed. Our results show that after overexpression of the constitutively active Pfn1, there was a significant increase in the phosphorylation of AKT only at serine 473. This phosphorylation may represent a partial activation of AKT, as it is reported that maximal activity requires phosphorylation of both threonine 308, by PDK1, and serine 473, by mTor. [131] Interestingly, this partial AKT activation seems to be enough to phosphorylate GSK3β (one of its downstream targets), as a significant increase in phosphorylation of GSK3β at Ser9 was also observed. This phosphorylation strongly inhibits GSK3β activity, impeding phosphorylation of its downstream targets, such as APC, CRMP2 or MAPs. Consequently, these remain active and able to promote MT polymerization and stability in growing axons. [17, 132] In fact, it has been reported that phospho-AKT is localised at the tips of growth cones, which emphasizes a role for AKT in the regulation of neurite elongation. [131] Hence, these results suggest that the actin-binding protein Pfn1 not only affects actin cytoskeleton, but may also modulate MT dynamics, through GSK3β activity, thus creating a new indirect bridge between actin and microtubules. Besides, our results showing increased MT dynamics after overexpression

of Pfn1 WT and Pfn1 S137A in hippocampal and DRG neurons reinforce this idea of Pfn1 interfering with the microtubule cytoskeleton. To further dissect this topic, Pfn1 mutants with reduced binding affinity to PIP_2, but with a functional actin-binding site (such as R136D mutant) could be overexpressed in neuronal cells to assess the expression levels of the molecules previously evaluated and also to check MT dynamics. Thus, if the PIP_2 binding ability of Pfn1 was indeed in the basis of the link between actin and MTs, the expression levels of phosphorylated AKT and GSK3β would be decreased in the presence of this Pfn1 mutant, and MT dynamics would probably also be impaired.

In summary, this work strongly supports the important role of Pfn1 in axon formation and growth, and may be very relevant for future strategies aiming to promote axon regeneration. However, there are still some unanswered questions that should be further explored in the future.

Conclusion

Given the importance of actin and microtubules dynamics at the growth cone, numerous reports suggest that regulators of cytoskeleton dynamics are attractive targets to control axon formation, growth and regeneration. Specifically, actin dynamics is thought to be the major player in generating the force required for axon growth and extension. Nevertheless, how each ABP interacts and regulates this process is still not fully understood. Therefore, this work aimed at exploring and further understanding the role of Pfn1 in axon biology.

The present study clearly demonstrated a strong effect of Pfn1 in enhancing axon growth. This was evidenced by the robust increase in neurite outgrowth and both actin and microtubule dynamics, when the constitutively active form of Pfn1 (which is phosphorylation-resistant) was overexpressed. These observations also suggest a key role of the modulation of Pfn1 activity, by phosphorylation, in regulating actin dynamics at the growth cone. Given the solid effect of Pfn1 S137A overexpression *in vitro*, the next step will be to assess if this increased growth capacity is also observed *in vivo*, where a much more complex environment is present. Furthermore, it was also interesting to find that Pfn1 modulates not only the actin cytoskeleton, but might also regulate microtubules, further promoting axon growth and acting as a possible link between the actin and microtubule cytoskeletons. However, further studies are needed to clarify and corroborate this hypothesis. Additionally, a possible contribution of Pfn2 to axon growth was also evaluated. Although it needs to be further substantiated by additional experiments, our results suggest that this profilin form does not have a relevant role in this process.

Collectively, this work revealed a potential impact of Pfn1 in axon growth and regeneration and, consequently, a possible therapeutic application in circumstances of axonal injury, such as in spinal cord injury. Besides, a deeper understanding of Pfn1 biology in the nervous system could also have impact in other illnesses, as Pfn1 mutations and dysregulations are implicated in a plethora of pathological conditions.

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