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Dissecting the role of profilin-1 in axon formation, growth and regeneration

Ana Rita Costa

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Ana Rita Costa

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto e ao
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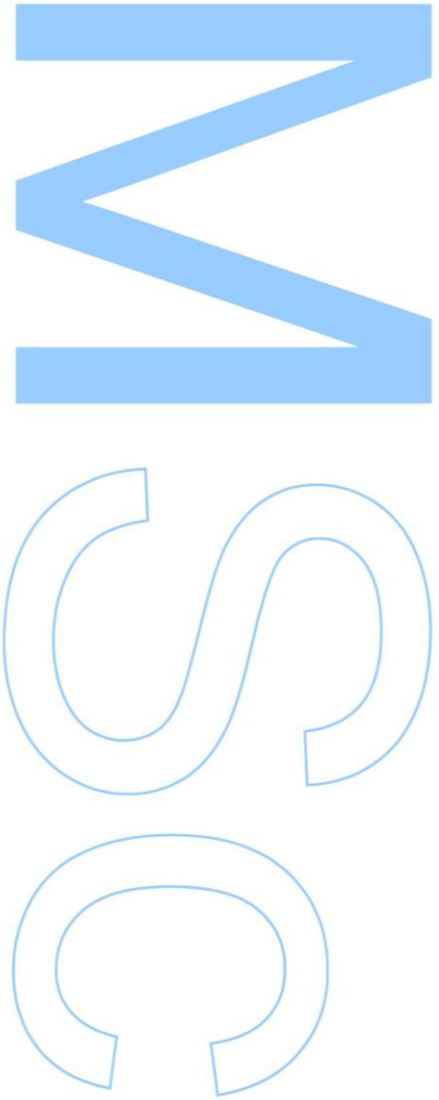
Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



Try not to become a man of success but rather try to become a man of value.

Albert Einstein

À doutora Mónica Sousa, minha orientadora, cujo profissionalismo, dedicação e ambição me inspira, todos os dias. Por ser um exemplo como investigadora, pela exigência em cada detalhe, pelo conhecimento partilhado. Sobretudo, por acreditar em mim.

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À minha família, o meu pilar omnipresente. A Vós dedico todos os meus frutos, pois sois a minha fonte do Ser e Estar. Vocês são o meu tesouro, a relíquia sagrada que sustenta a base da minha pirâmide. Mãe e Pai, a Flor que nasceu, hoje cresce, robusta, no Vosso jardim.

Abstract

Emerging evidence suggests that regulators of cytoskeleton dynamics might be attractive targets to control axon formation, growth and regeneration. Specifically, actin dynamics in the growth cone is thought to play a major role in generating the force required for axon extension. As such, interventions aimed at promoting axonal regeneration may converge on the manipulation of this mechanism. Although actin is well recognized as a key player in axonal growth, how different actin-binding proteins control its dynamics is still not fully understood. Using the conditioning lesion, a model in which the axonal regeneration capacity of spinal dorsal column axons is increased following a priming lesion to the sciatic nerve, we determined that the actin-binding protein profilin-1 (Pfn1) is increased in regenerating axons. Profilins provide the pool of competent ATP-actin monomers that can be added to free filamentous actin ends to support their polymerization and growth. Previous data from our lab further demonstrated that acute *in vitro* ablation of Pfn1 precluded axon formation in hippocampal neurons and significantly decreased neurite outgrowth in DRG neurons. Collectively, these evidences point to a critical role of Pfn1 during early neuritogenesis and axonal regrowth following injury. To unravel the importance of Pfn1 during axonal regeneration, we generated mice with an inducible neuronal deletion of Pfn1 using cre-lox technology. Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ neurons, lacking Pfn1, displayed impaired actin dynamics and defective neurite outgrowth upon a conditioning lesion. Besides, *in vivo* regeneration of both peripheral and central axons tended to be diminished. Thus, Pfn1 emerges as a potential determinant for axonal regeneration capacity. In the future, exploring the mechanisms by which Pfn1 acts in neurons would enable to set the foundations for drug screens unveiling modulators of Pfn1 activity with therapeutic potential for spinal cord injury treatment. Aiming at dissecting the molecular mechanisms that underlie Pfn function in neurons, Pfn1 mutants in key residues were used. A phospho-resistant mutant of Pfn1, the constitutively active form of the protein, strongly enhanced *in vitro* neurite outgrowth. Hence, specific modulation of regulatory regions of Pfn1 could represent a useful approach for future therapeutic applications. Moreover, this work also revealed that Pfn1 is capable of linking actin and the microtubule cytoskeleton by interfering with intracellular signaling namely with the PI3K/AKT/GSK pathway that is crucial for axon growth and survival. The relevance of Pfn1 in the biology of motor neurons was recently highlighted by the finding that missense mutations in this protein are linked to cases of familial amyotrophic lateral sclerosis, a late-onset neurodegenerative disorder resulting from destruction of neuromuscular junctions (NMJs) and consequently motor neuron death. By generating DCX-cre$^{+/-}$Pfn1$^{fl/fl}$, this work intended to characterize the consequences of

early developmental lack of Pfn1 to axon formation and growth and to the formation of neuromuscular junctions. Our initial analysis of the innervated area in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice did not show significant differences in relation to controls but further detailed analysis of the structure of NMJs remains to be performed. In summary, our work uncovered Pfn1 has an important determinant in axon formation, growth and regeneration.

Keywords: axon formation, axon growth, axon regeneration, actin dynamics, spinal cord injury, amyotrophic lateral sclerosis.

Resumo

Evidências recentes sugerem que os reguladores da dinâmica do citoesqueleto poderão constituir alvos para o controlo da formação, crescimento e regeneração axonal. Especificamente, a dinâmica do citoesqueleto de actina, no cone de crescimento, assume um papel crucial na produção da força necessária à extensão de um axónio. Assim, estratégias que visam a promoção da regeneração axonal convergem na manipulação deste mecanismo. Embora o citoesqueleto de actina seja considerado um elemento crucial para o crescimento axonal, a influência das proteínas de ligação à actina na dinâmica do citoesqueleto ainda não se encontra descrita. Utilizando o modelo de lesão condicionada, no qual a capacidade regenerativa dos axónios da coluna dorsal da medula espinal se encontra aumentada após uma lesão prévia no nervo ciático, o nosso grupo reportou um aumento notável dos níveis totais da proteína de ligação à actina, profilina-1 (Pfn1), no local de lesão na medula espinal. A Pfn1 é uma proteína responsável pela polimerização e crescimento dos filamentos de actina uma vez que promove a adição de monómeros de actina às extremidades livres dos mesmos. Recentemente, o nosso grupo demonstrou que a depleção aguda de Pfn1 em neurónios de hipocampo impossibilitou a formação axonal. Observações similares foram constatadas em neurónios dos gânglios da raiz dorsal nos quais se verificou uma diminuição significativa do crescimento de neurites. Estas evidências apontam assim para um papel peculiar da Pfn1 durante os estadios iniciais da neuritogénese, bem como no processo de regeneração após lesão. Com o objetivo de desvendar o impacto da função da Pfn1 durante a regeneração axonal, o nosso grupo gerou modelos animais nos quais se procedeu à indução da deleção da Pfn1, em idade adulta, utilizando o sistema tecnológico cre-lox. Constatou-se que neurónios Thy1cre$^{+/-}$ Pfn1$^{fl/fl}$, não portadores de Pfn1, possuem anomalias na dinâmica da actina e defeitos na capacidade de extensão de neurites, após lesão condicionada. Além disso, a regeneração *in vivo* de axónios periféricos e centrais é tendencialmente menor no caso de murganhos Thy1cre$^{+/-}$Pfn1$^{fl/fl}$. Desta forma, a proteína Pfn1 emerge como um potencial determinante da capacidade regenerativa dos axónios. No futuro, a exploração dos mecanismos através dos quais a Pfn1 atua em neurónios irá permitir o estabelecimento dos fundamentos básicos necessários à pesquisa de fármacos moduladores da atividade da Pfn1 com potencial terapêutico, no caso de lesões medulares. Com o objetivo de dissecar os mecanismos moleculares que subjazem a ação da Pfn1, efetuaram-se mutações específicas em resíduos chave na sequência aminoacídica da proteína. Constatou-se que mutantes da Pfn1 fosforesistentes, ou seja, constitutivamente ativos, possuem a capacidade de aumentar, de forma robusta, o crescimento de neurites *in vitro*. Desta forma,

a modulação específica de regiões reguladoras da Pfn1 poderá representar uma abordagem inteligente e útil para a delineação de tratamentos de lesões medulares. Além disso, este trabalho revela também que a proteína Pfn1 é capaz mediar a comunicação entre o citoesqueleto de actina e o de microtúbulos uma vez que participa em cascatas de sinalização intracelulares, nomeadamente na via PI3K/AKT/GSK, já descrita como essencial à sobrevivência e crescimento axonal. Recentemente, foi estabelecida uma conexão sólida entre mutações da Pfn1 e casos hereditários de esclerose lateral amiotrófica, o que enfatiza a relevância desta proteína na biologia dos neurónios motores. A esclerose lateral amiotrófica é um distúrbio neurodegenerativo de início tardio que culmina na destruição das junções neuromusculares e, consequentemente, na morte dos neurónios motores. Deste modo, e através da criação murganho modelo DCX-cre$^{+/-}$Pfn1$^{fl/fl}$, este projeto visou caracterizar as consequências da deleção da Pfn1, em estadios precoces do desenvolvimento, na formação e crescimento axonal bem como na formação das junções neuromusculares. Análises preliminares da área de inervação no músculo diafragma em murganhos DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ não revelaram diferenças significativas relativamente aos controlos, embora futuras análises no que respeita à estrutura das junções neuromusculares devam ser realizadas de forma detalhada. Concluindo, este trabalho enfatizou a importância da Pfn1 na formação, crescimento e regeneração axonal.

Palavras-chave: formação axonal, crescimento axonal, regeneração axonal, dinâmica da actina, lesão medular, esclerose lateral amiotrófica.

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Abbreviations list

ADP	Adenosine diphosphate
AKT/PKB	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAD	Cath.a-differentiated
CGN	Cerebellar granule neurons
CL	Conditioning lesion
CNS	Central nervous system
CT-B	Cholera toxin subunit B
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DCX	Doublecortin
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal root ganglia
F-actin	Actin filaments
G-actin	Globular-actin
GSK-3	Glycogen synthase kinase 3
HBSS	Hanks’ balanced salt solution
KO	Knock-out
NGF	Nerve growth factor
NMJ	Neuromuscular junction
PB	Phosphate buffer
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
Pfn	Profilin
PI3K	Phosphoinositide 3-kinase
PtdIns(3, 4, 5)P_3	Phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(4, 5)P_2	Phosphatidylinositol (4,5)-bisphosphate
PLP	Poly-L-proline
PNS	Peripheral nervous system
RNA	Ribonucleic acid
ROCK	Rho-Kinase
SCI	Spinal cord injury

SDS	Sodium dodecyl sulphate
Sh	Small hairpin
SNI	Sciatic nerve injury
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline – 1%Tween 20
WT	Wild-type
YFP	Yellow fluorescent protein

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Introduction

1. Cytoskeleton

The cytoskeleton is a highly dynamic network of filamentous proteins that shapes the morphology of cells. This network is multifunctional, providing structural support and intracellular organization by keeping together many cellular components. As it mediates communication across the entire cell, the cytoskeleton has a profound impact on functional specialization. Combining mechanical and dynamic features, the architecture of the cytoskeleton exists as a bridge linking the intracellular space and the extracellular environment. Due to the fact that cytoskeleton components are not rigid or static scaffolds, but rather they are continuously dynamic and tightly regulated, this close connection enables the cell to sense and respond quickly and effectively to external mechanical or chemical signals transducing and integrating information from the environment.

Given the widespread role of cytoskeleton in cellular functions, any sort of subtle alteration on its basic components or binding partners leads to pathological abnormalities, including tumorigenic transformation. To date, it has been reported that differential remodelling of actin cytoskeleton architecture leads to malignant phenotypes [1] [2]. A dysfunctional actin cytoskeleton, on the other hand, is often linked to muscular defects. Moreover, abundant abnormal aggregates of cytoskeletal proteins are neuropathological signatures of many neurodegenerative diseases [3]. Additionally, cytoskeleton is crucial to ensure pathogen' survival. For example, pathogens such as *Listeria monocytogenese* exploit the host machinery that controls actin cytoskeleton and generates actin tails to improve the pathogen invasion [4].

The cytoskeleton is composed by three main components which exhibit their intrinsic characteristics but interdependent functions: intermediate filaments, microtubules and actin polymers. Several biological processes rely on those components. As mentioned above, cytoskeleton executes and regulates differentiation into specialized compartments inside the cell leading to cellular asymmetry and consequently cell polarity.

Generally, cell polarity is defined by the asymmetric accumulation of mobile components between opposite poles of a cell and by the orientated organization of polar cytoskeletal filaments [5]. Actin filaments and microtubules, unlike intermediate filaments, are inherently polarized polymers since their globular actin (G-actin) subunits and α- and β-tubulin heterodimeric subunits, respectively, align in the same direction creating distinct ends that structurally differ from each other. Regarding the structural differences, each end exhibits different polymerization and depolymerization rates which are also dependent on

free subunits concentration. The dynamics is also enhanced through nucleotide hydrolysis by actin (ATP hydrolysis) and tubulin (GTP hydrolysis) which is translated into an asymmetry in the state of nucleotide that is bound along the filament. Concerning this asymmetry and structural differences, distinct binding partners might interact with each end. Additionally, a class of cytoskeleton-associated proteins that are particularly important for cell polarity are the motor proteins. For example, the motor protein myosin moves exclusively towards actin barded ends.

Therefore, it is essential the comprehension of how actin and microtubules contribute to the different phases of cell polarity through their dynamic assembly and transport functions.

2. Axonal Elongation during development

Among the better characterized polarized cells are neurons. Neurons are compartmentalized into two functional and molecular domains: the axon and the dendrites. This highly polarized arrangement underlies the basis of unidirectional signal propagation. Dendrites have the ability to receive synaptic inputs that are processed and integrated at the level of the soma. Thereafter, the electrical signal is propagated along the axon until it reaches presynaptic terminals which contact onto target cells. At terminals, the electrical signal is converted into a chemical signal by the release of synaptic vesicles containing neurotransmitters. Despite the sophisticated morphology of mature polarized neurons, developing neurons exhibit a quite distinct structure emerging as round neuronal spheres. Several studies have been providing new insights on the mechanisms that govern symmetry breaking.

Current knowledge indicates that, throughout development, neural progenitor cells divide asymmetrically [6], giving rise to a new progenitor daughter cell and a neuron, a process that is regulated by the cytoskeleton. Moreover upon cell cycle exit, the newborn unpolarized neuron must migrate over long distances before reaching its functional location. Appropriate migration is essential for construction of functional synaptic circuitry in the brain. During this process, neurons become polarized forming an axon and dendrites [7]. Interestingly, neurons possess a high intrinsic ability to elongate which is transcription dependent and relies on the activity of several signalling pathways. Specific signalling pathways are known to be activated during axonal elongation process such as phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue (PTEN) and glycogen synthase kinase 3 beta (GSK3β) [7]. Besides intracellular signalling, other events

contribute to the correct axonal elongation like local protein synthesis and degradation [7, 8].

2.1 Stages of neuronal polarization

Establishment of neuronal polarization has been subject of intense scrutiny for many years. *In vitro* studies led to the notion that this polarization was mainly due to intrinsic neuronal factors. Two major well-described *in vitro* systems are commonly used to study neuronal polarity and its inherent molecular mechanisms, namely primary cultures of dissociated rodent embryonic hippocampal neurons (embryonic day 18) [9] and postnatal cerebellar granule neurons (CGN, postnatal day 4-10) [7, 10]. Pioneering work established that those neurons, once dissociated and separated from the complexity of the CNS environment, have the ability to recapitulate and develop their functional polarity generating the axon-dendrite morphology [11, 12]. The sequence of polarity events follows well characterized morphological changes [10, 13].

Shortly after plating, hippocampal neurons attach to the substrate as round uniform spheres (stage 1) and transform into multipolar cells (12-24 hours; stage 2) containing several neurites decorated with dynamic growth cones at their tips. The critical polarity event is initiated when one of the minor neurites enlarges its growth cone and elongates rapidly to become an axon (24-48 hours, stage 3) whereas the concomitant growth of the future dendrites is inhibited. The other minor neurites remain short and only develop into dendrites at later stages (> 3-4 days, stage 4). Functional maturation is achieved later when the formation of synapses and spines is observed (> 2 weeks, stage 6). Undoubtedly, axonal specification lays the foundation for the process of polarity.

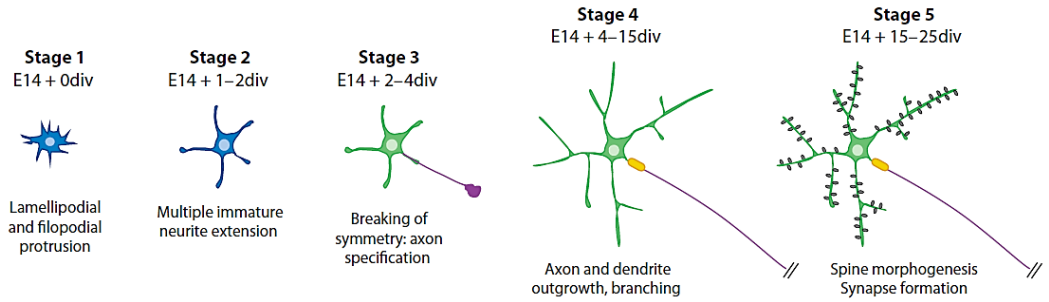


Fig. 1 – **Neuronal polarization of cortical neurons *in vitro*.** In dissociated cultures of postmitotic cortical neurons, stage 1 neurons present an intense lamellipodia and filopodia, which leads to the emergence of multiple immature neurites, stage 2. Then a critical step occurs, neurons become assymetric and a single neurite grows rapidly originating the axon (purple), stage 3. Subsequently, stage 4 is characterized by a rapid elongation of both axon and dendrites, and finally neurons present dendritic spines and the axonal initial segment (stage 5). Taken from [7].

2.2 The growth cone

During development, each neuron must extend a polarized axon through a changing and challenging environment in order to reach its final destination. The axon growth takes place at the tip of growing axons where a specialized highly motile structure exists, termed growth cone (fig. 2). The dynamic behaviour of the growth cone is responsible for the extension capacity by sensing and exploring the extracellular environment. Growth cones are accurate sensors that interpret multiple extracellular cues transducing signals to the cytoskeleton. It is currently understood that cytoskeleton dynamics underlies axonal formation and the establishment and maintenance of neuronal polarity [14] since it generates the force that drives the direction of elongation process.

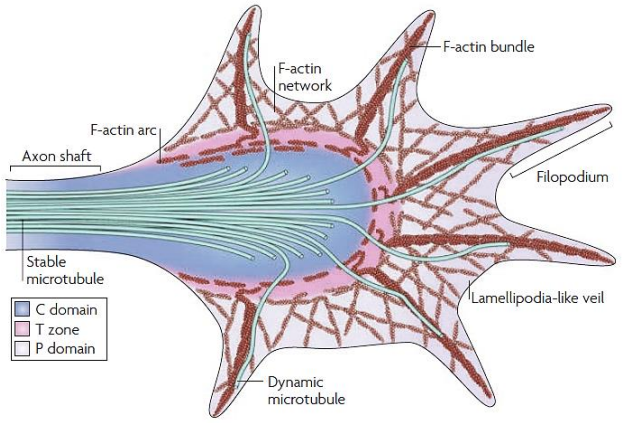


Fig. 2 – **The structure of the growth cone.** The peripheral domain contains long F-actin bundles forming filapodia along which dynamic microtubules explore the extracellular environment. In addition, a mesh-like branched F-actin network gives structure to lamellipodia-like veils. The central domain encloses stable, bundled microtubules that enter the growth cone from the axon shaft. Finally, the transition domain is decorated by actomyosin contractile structures termed actin arcs. Taken from [15].

The axonal growth cone is composed by three domains concerning their cytoskeletal distribution. The central (C) domain encloses stable, bundled microtubules coming from the axon shaft which assist polarized transport of organelles and vesicles towards the growth cone. In addition, there is a peripheral (P) domain which is an actin-rich region containing filapodia – a thin transient actin protrusion formed by long bundled actin filaments (F-actin bundles) that extends from the cell surface – as well as mesh-like branched and flattened F-actin networks, termed lamellipodia [15]. Additionally, individual dynamic microtubules explore the P-domain, usually along F-actin bundles. In particular, filapodia emerge as guidance mechanosensors, stabilizing and turning towards an attractive guidance molecule or retracting and turning away when contacting with a repellent guidance molecule. When the P-domain connects with a substrate it creates points of attachment that produce tension used for growth cone progression. The barbed ends of actin filaments of both filapodia and

lamellipodia are oriented towards the leading edge of the growth cone whilst the pointed ends are located at the transition (T) domain – a region that covers the interface between the C- and P- domains decorated by actin arcs (actomyosin contractile structures). Continuous incorporation of G-actin takes places characteristically at the barbed ends whereas subunit disassembly occurs at the pointed ends. Both activities ensure the constant length of the actin polymer promoting at the same time its advance, a process termed actin treadmilling. F-actin treadmilling combined with F-actin retrograde flow– a process by which F-actin is constantly being transported away from the leading edge towards the centre of the growth cone – provides prompted availability to drive movement in response to directional cues [15]. It has been reported that F-actin retrograde flow is driven by contractility of the motor protein myosin II [16]. Specifically, myosin II contraction across the T-domain associated with the push of actin network assembly at the leading edge apply a mechanical strain to actin filaments which might potentiate bundle severing. Moreover, bundle severing involves the actin-depolymerization factor (ADF)/cofilin which ensures actin turnover maintaining a pool of actin monomers that allows the continual restructuring of the actin cytoskeleton.

Briefly, the elongation process is separated into three overlapping stages of advance that are influenced by environmental factors: protrusion, engorgement and consolidation. Following adhesive contacts establishment at the distal end of the growth cone, dramatic local actin remodelling occurs since it has been reported a local increase of actin assembly and a slowing of F-actin retrograde flow. Consequently, in the subsequent engorgement phase, F-actin bundles disagregate and F-actin arcs located at the transition domain reorientate from central domain towards the adhesion site creating a corridor which is rapidly invaded by central domain microtubules carrying vesicles and organelles and fixing the new axonal growth direction [17]. Finally, consolidation occurs as the proximal part of the growth cone assumes a cylindrical shape and the transport of organelles becomes bidirectional, thus forming a new segment of axon shaft [18]. These stages of advance occur either during the formation of nascent axons or during axonal branching [18].

2.3 Cytoskeleton dynamics in neuron polarization and axonal growth

As neurite outgrowth intrinsically depends on actin remodelling and on microtubules, distinct cytoskeleton dynamics could explain the different growth rate of the developing neurites and ultimately the axon specification. In fact, one of the minor neurites enlarges its growth cone which becomes highly dynamic. Such dynamics is characterized by unstable actin that allows microtubules to protrude distal regions of the growth cone sustaining the outgrowth. Accordingly, such neurite will elongate rapidly forming a specified axon that

grows at a rate approximately ten times faster than the other neurites. In contrast, a stable and rigid actin network in the remaining growth cones may delay microtubule and organelle protrusion. Consequently, microtubules do not become stabilized in order to form the axon shaft [19]. Therefore those neurites will develop into dendrites at the later stages of the polarization process. Notably, it is documented that the local stability of actin is regulated by Pfn and cofilin that promote actin polymerization and depolymerization, respectively [14].

Taking into consideration all the reported evidences, it has been convincingly demonstrated that growth cone motility, which underlies the symmetry breakage of the neuronal spheres, depends on the dynamic properties of the actin cytoskeleton. Although actin dynamics and microtubule might jointly orchestrate neuronal polarization [17], microtubules mainly follow the trajectories of the actin cytoskeleton. Therefore, actin is considered to be a pivotal requirement for microtubules localization in the growth cone.

Interestingly, pioneering experiments revealed that even after the axon is specified, other neurites have the potential to acquire axonal identity meaning that polarity is a flexible and plastic process [11]. It is already demonstrated that, after transecting an axon from hippocampal neurons, if one process was at least 10 µm longer than the others, it invariably acquires the axon features regardless of its identity before transection. In addition, even mature neurons undergo such change when the original axon is cut closer to the cell body than a crucial length of approximately 35 µm [20] meaning that plasticity of polarity does not end during early neuronal development.

2.4 Orchestrating neuronal polarization and intracellular signalling

Although it is argued that the initial signal for breaking the symmetry is an intrinsic property of neurons regardless of the presence of extracellular cues [12], studies have shown that extracellular factors markedly guide growing axons to reach their targets. Extracellular matrix (ECM) is enriched by proteins that are potential cues for specifying axon formation. For example, laminin or neuron–glia cell adhesion molecule (NgCAM) are known to enhance neurite outgrowth and axon elongation. Additionally, although the earliest phases of axon growth may be neurotrophic-independent, several neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) are required for polarity regulation at later stages of axonal growth [21]. In summary, extracellular signals might therefore accelerate and modulate neuronal growth.

Significant progress has been made in the identification of intracellular mechanisms and signalling events that underlie axonal elongation relying on environmental factors. Importantly, multiple molecular counterparts extensively exhibit a tremendous impact in

actin remodelling during neuronal polarization namely several actin-binding proteins as well as Rho-GTPases and their downstream targets including Rho kinase (ROCK) and Pfn.

2.4.1 Actin-binding proteins and their relevance for neuronal polarity

Actin dynamics is regulated by actin nucleating, branching and bundling proteins that localize to the leading edge of growth cone through either lipid- or protein-dependent interactions. Among those proteins actin nucleators play a substantial role such as Arp2/3 complex and formins [22]. Specifically, Arp2/3 complex is a macromolecular complex that assists in the formation of branched actin superstructures either in lamellipodia or filapodia whereas formins enhance the rate of actin polymerization. Beside actin nucleators, other regulators of growth cone dynamics include proteins that directly modulate actin dynamics including WAVE (Wiskott-Aldrich syndrome protein [WASP]-family verprolin-homologous protein) [23], Ena (enabled)/VASP (vasodilator stimulated phosphoprotein) [24], Pfn [25], and severing proteins ADF/cofilin. Together, actin regulating proteins modulate the actin cytoskeleton to implement neuronal symmetry breakage.

2.4.2 Rho GTPases and their relevance for neuronal polarity

Rho GTPases regulate many cellular events, including cytoskeletal organization, proliferation and gene expression through specific effectors. They function as molecular switches in response to the stimulation of receptors such as adhesion receptors. Among the different types of Rho GTPase, RhoA, Cdc42 and Rac1 have been the most extensively characterized in neuronal development context providing insights relative to their *in vivo* functions [26, 27].

A tight regulation of the Rho GTPase Cdc42 activity is particularly important for axon specification and filapodia formation [27]. In particular, the major Cdc42 effectors in regulating actin dynamics are WASP, insulin-receptor substrate p53 (IRSp53) Tyr kinase, formins and p21-activated kinase [26] (PAK, which signals to actin cytoskeleton remodelling via cofilin [28]). The Rho GTPase Rac1 modulates actin cytoskeleton dynamics by controlling the formation of lamellipodia [27] via WAVE and also via PAK pathway. Together, Cdc42 and Rac1 seem to be implicated in promoting neurite formation and extension. By contrast, RhoA appears to act antagonistically to Cdc42 or Rac1 negatively regulating neurite extension through activation of Rho-associated protein kinase (ROCK) [29].

3. CNS versus PNS regeneration

During development, neurons express a transcription-dependent program relying on multiple signalling pathways that allows a vigorous axonal elongation until reaching their post-synaptic targets. In a variety of forms, regeneration can be seen, at least in part, as a recapitulation of the developmental process, since axons need to regrow in order to reinnervate their targets. However following the establishment of connections, the intrinsic developmental growth capacity of CNS neurons declines [30]. In this sense, several studies support the notion that such switch, apart from the poor environment for axon extension, largely accounts for the failure of adult CNS axon regeneration. In fact, in CNS lesions as is the case of spinal cord injury (SCI), the disruption of either ascending sensory or descending motor tracts could lead to persistent sensorimotor dysfunctions below the lesion sites of varying degrees of severity [31]. Unravelling the reasons underlying such limited and abortive regenerative growth has been a major challenge. Although adult CNS neurons do not regenerate, those of peripheral nerves can reactivate the cell intrinsic program upon injury and thus spontaneously and successfully regrow to a significant extent. Curiously, eliminating extracellular inhibitory influences is insufficient to allow the majority of injured axons to regenerate emphasizing the importance of cell intrinsic mechanisms after injury [32, 33]. Hence, the regenerative ability of PNS neurons is supported by the combination of intrinsic and extrinsic factors. For these reasons, injured PNS neurons are often used as a model to study how axonal regeneration is achieved. However, whether intracellular signalling mechanisms in regenerating PNS axons resemble those in developing nervous system remains unknown. Of note, although PNS regeneration is described as a powerful process, usually such regrowth does not lead to a complete functional recovery since regenerating axons are misdirected [34].

3.1 Extrinsic Factors

Several differences have been suggested to explain the lack of regenerative capacity of CNS neurons, namely the inefficient Wallerian degeneration followed by the formation of the glial scar which creates a mechanical and chemical barrier to regenerating axons. Wallerian degeneration comprises non-neuronal cellular responses that occur in the distal part of the cut nerve allowing the clearance of the debris and the formation of a favourable environment for axonal regeneration to take place.

Following injury, damaged neurons are exposed to myelin which contains numerous proteins that are inhibitory to axonal growth. Among those myelin inhibitors, Nogo [35] and oligodendrocyte myelin glycoprotein (OMgp) [36] expressed in CNS and myelin-associated

glycoprotein (MAG) [37] expressed both in CNS oligodendrocytes and PNS Schwann cells are better characterized. Besides myelin components, repulsive guidance cues semaphorin 4D (Sema4D) and ephrin B3 may restrict axonal regeneration in the injured CNS. Myelin and its axonal growth inhibitors accumulates at the site of injury and, if not removed, impairs axonal regeneration. Importantly, soon after peripheral nerve injury, both the injured axon and its cell body display distinct responses. The cell body response is characterized by chromatolysis, displacement of the nucleus to a peripheral position and swelling of the cell body [32]. The proximal axon terminal reseals, assembles a growth cone and initiates a regenerative growth. On the other hand, the distal axonal segment undergoes Wallerian degeneration. Specifically, before axonal degeneration, Schwann cells start to dedifferentiate, proliferate, produce cytokines and chemokines (that recruit immune cells), and align in order to provide a supportive substrate and growth factors for regenerating axons [38]. Furthermore, Schwann cells are the major phagocytic cells responsible for myelin debris clearance. Such fast and efficient Wallerian degeneration in PNS injuries culminates in successful regeneration.

In contrast, CNS lesions induce oligodendrocyte apoptosis leading to an accumulation of myelin debris and an incomplete Wallerian degeneration, which together with astrocyte activation promotes the formation of the glial scar. The dense scar tissue that surrounds the injured area consists predominately of reactive astrocytes that release gradients of inhibitory extracellular matrix molecules such as chondroitin sulphate proteoglycans (CSPGs) [39, 40] that chemically block regeneration. Of note, the glial scar is important to create a scaffold for the formation of a new vascularization network and to separate the healthy tissue from the injured one, preventing damage progression [41].

3.2 Intrinsic Factors

Successful axonal regeneration after injury has to be preceded by successful assembly of a competent growth cone at the tip of a cut axon. After axotomy, local calcium influx appears to be a prerequisite for the assembly of a new competent growth cone and afterwards for axon growth [42]. Moreover, axotomy also triggers membrane depolarization that further promotes local calcium influx by activating voltage-gated calcium channels and calcium release from internal stores. Recently, it was reported, in mouse peripheral sensory neurons, that such enhanced calcium influx elicits a back-propagating calcium wave that reaches the cell body and causes epigenetics changes that modulates the switch from non-elongating to growth competent axons [43]. Importantly, epigenetic changes lead to an induction of a large repertoire of regeneration-associated genes (RAGs) promoting of

sensory axon regeneration [44]. In fact, in contrast to PNS lesions, following dorsal column lesions, no significant upregulation of RAGs is reported in CNS neurons [45, 46].

Additionally, the elevation of free intracellular calcium triggers intracellular signalling required for specific initial events, including membrane retrieval by sealing and accumulation of Golgi-derived anterogradely transported vesicles, local cytoskeleton restructuring characterized by depolymerization of microtubules and actin, activation of calcium dependent calpains which proteolytically cleaves submembrane spectrin cortex, activation of local protein translation and retrograde molecular signalling [42]. The cytoskeleton rearrangement is a critical step to transform the cut axon into a competent growth cone. Intriguingly, while PNS neurons are able to assemble a functional growth cone after injury, lesioned CNS axons form swellings at their tips known as retraction bulbs. Retraction bulbs are observed after spinal cord injury and characterized by disorganized microtubules and accumulated organelles anterogradely transported including *trans*-Golgi-derived vesicles and mitochondria [47]. Similar bulbous structures were already described in *Aplysia californica* axons after axotomy under conditions that limit calcium influx into the cut axonal end [48].

Moreover, the calcium transient activates calcium dependent adenylate cyclase leading to increased cAMP which is described to potentiate axonal regrowth, reconnection of axonal fragments and formation branching to postsynaptic targets [49]. In addition, increased levels of cAMP signal to dual leucine zipper kinase (DLK-1) promoting cytoskeleton remodelling needed for growth cone assembly. The calcium transient retrogradely propagates to the cell body eliciting nuclear export of histone deacetylase 5 (HDAC5) thus enhancing histone acetylation and priming chromatin for subsequent transcription events [43]. Following the calcium dependent early phase, specific injury signals including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and signal transducer and activator of transcription 3 (STAT3) are locally activated and retrogradly transported to the nucleus which leads to a robust alteration of the transcription profile needed to increase the neuronal growth capability (fig. 3). In fact, several RAGs expressed upon injury have been identified, for example the cytoskeleton-related genes tubulin and actin [50, 51]. Importantly, emerging evidence suggest that regulators of actin and microtubule dynamics might be attractive targets for controlling the speed and trajectory of regenerating axons. Although microtubules are important for the maintenance of the elongated axon and the speed of growth, it is accepted that the engine needed to generate the force required for axon extension is the actin cytoskeleton [52].

In contrast, CNS neurons have a limited regeneration ability as the consequence of decreased calcium changes upon injury, no increase in histone acetylation, lack of robust synthesis of RAGs, limited local protein synthesis and the presence of inhibitors of axon regrowth [33].

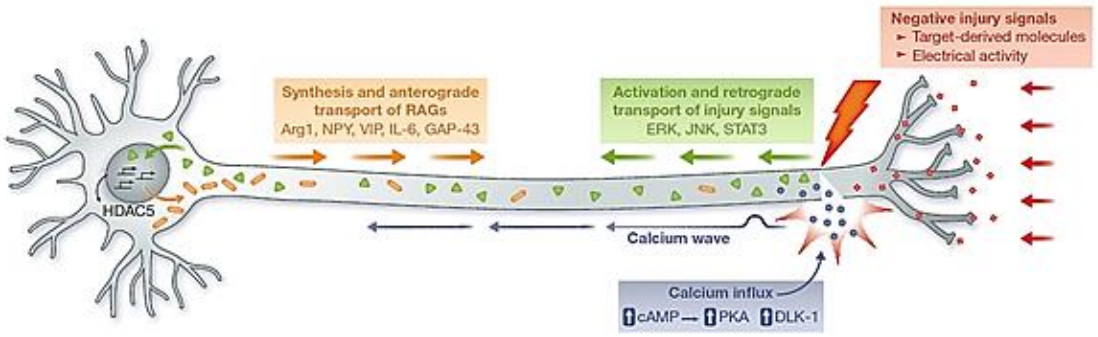


Fig. 3 **– Cell intrinsic mechanisms of axonal regeneration in PNS.** Once a neuron innervates its target, target-derived signals must repress the intrinsic neuronal growth capacity to allow proper synaptic development. This repression is relieved upon axotomy through the interruption of those negative injury signals. Upon axotomy, calcium influx into the axoplasm activates cAMP signaling to PKA and DLK-1. The calcium wave back-propagates to the cell body leading to nuclear export of HDAC5 and robustly altering the transcription profile, along with retrograde transport of positive injury signals including ERK, JNK, and STAT3. Thereafter, RAGs are expressed promoting axon regrowth. Arg1, arginase 1; NPY, neuropeptide Y; VIP, vasointestinal peptide; IL-6, interleukine 6; GAP-43, growth-associated protein-43. Taken from [33].

3.3 The conditioning lesion (CL) model in axonal regeneration

Despite the general inability of CNS axons to regenerate, regrowth may occur under specific conditions, particularly the conditioning lesion effect.

DRG neurons are pseudo-unipolar neurons that possess a peripheral axon branch that regenerates when injured, and a central axon branch that enters the spinal cord originating the dorsal column fibers and does not regenerate upon injury. However, when the peripheral branch is lesioned or conditioned prior to lesioning the central branch, the central axon is prompted to regenerate and can overcome the glial scar inhibitory effect [53] (fig. 4). The conditioning effect is probably due to the fact that the regenerative machinery is already activated and assembled prior to CNS lesion. It is possible that some proteins like cytoskeleton proteins made in the cell body after peripheral lesion are transported down the regenerating axon where they will be available to mount a competent growth cone upon re-lesion, enabling centrally injured neurons to regenerate [45]. In fact, studies have shown that increased regeneration ability of conditioned DRG neurons correlates with RAG upregulation [44] and changes in axonal transport [51].

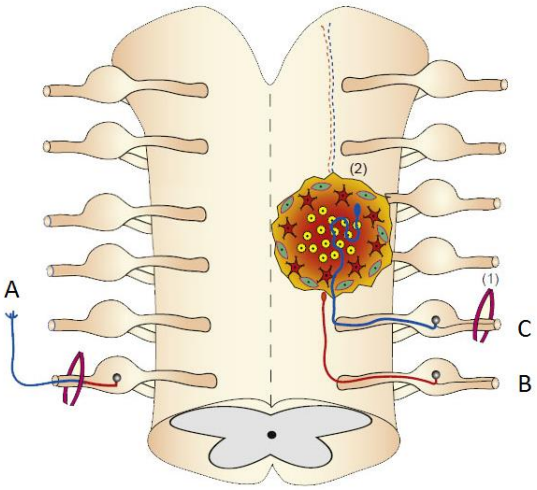


Fig. 4 **– The conditioning lesion effect.** After a peripheral lesion, the severed axons (blue) regenerate (***A***) whereas, after a central lesion, the regeneration of CNS axons (red) is minimal (***B***). However, when the peripheral branch is conditioned (1) prior to lesioning the central branch (2), the central axon is prompted to regenerate and cross glial scars (***C***). Adapted from [45].

3.2.1 Preliminary data

Interestingly, although increase of cAMP in DRG has been shown to prompt neurons to overcome inhibitory factors and to acquire regenerative function following conditioning lesions [54], none of the players identified so far in this effect was shown to be sufficient to mimic a conditioning lesion. Hence, our group used the paradigm of conditioning lesion in order to further identify mechanisms underlying enhanced growth capacity of DRG neurons. For that, we conducted a proteomic analysis of DRG from rats with either SCI or CL using two independent methods, a phosphoprotein antibody array (commercially available, Kinexus) and iTRAQ (isobaric tag for relative and absolute quantification-19; done in collaboration with Dr Bernd Wollscheid, Institute of Molecular Systems Biology, ETH, Zurich). In both cases, two independent experiments were performed and results were filtered so that only proteins with variations with $p<0.05$ were considered. This analysis allowed the comparison of the proteome of DRG neurons from rats with SCI and rats with a conditioning SCI.

As a result, several cytoskeleton-related proteins emerged as proteins differently expressed in conditioned neurons. Specifically, actin-binding proteins were present namely Pfn1, cofilin-1, adducins and spectrin emphasizing the importance of the modulation of actin dynamics to achieve proper axonal growth. In particular, Pfn1 a candidate identified by iTRAQ, was 4.5-fold increased in SCI site after CL when compared with animals with SCI alone. This result obtained by proteomics was later validated by western blot (fig. 5).

In the context of axonal regeneration, such increased levels of Pfn1 in the SCI site are in accordance to its function. Since Pfn1 acts as an essential polymerization enhancer in the actin treadmilling context, it is expected that increased levels of Pfn1 would provide the necessary force for growth cone motility and ultimately promote axonal elongation. Moreover, it is interesting to note that the inactive form of Pfn1 (phospho-Pfn1) was decreased in the SCI site after conditioning lesions (fig 5B). Our preliminary data further suggest that Pfn1 is essential in axon elongation as knocking down Pfn1 expression more than 90% in hippocampal neurons inhibits axon formation and growth (fig. 5D). In this sense, these findings support the notion that the organization of cytoskeleton namely through regulation of Pfn1 as well as through cofilin-1 and adducins is a pivotal downstream determinant for successful axonal regeneration. Therefore, understanding actin dynamics in the context of growth cone formation and axonal growth after injury, may allow us to manipulate the regeneration process and probably unravel the therapeutic potential of Pfn1 for spinal cord injury treatment. The next topic will summarize the current knowledge concerning Pfn1 function in nervous system.

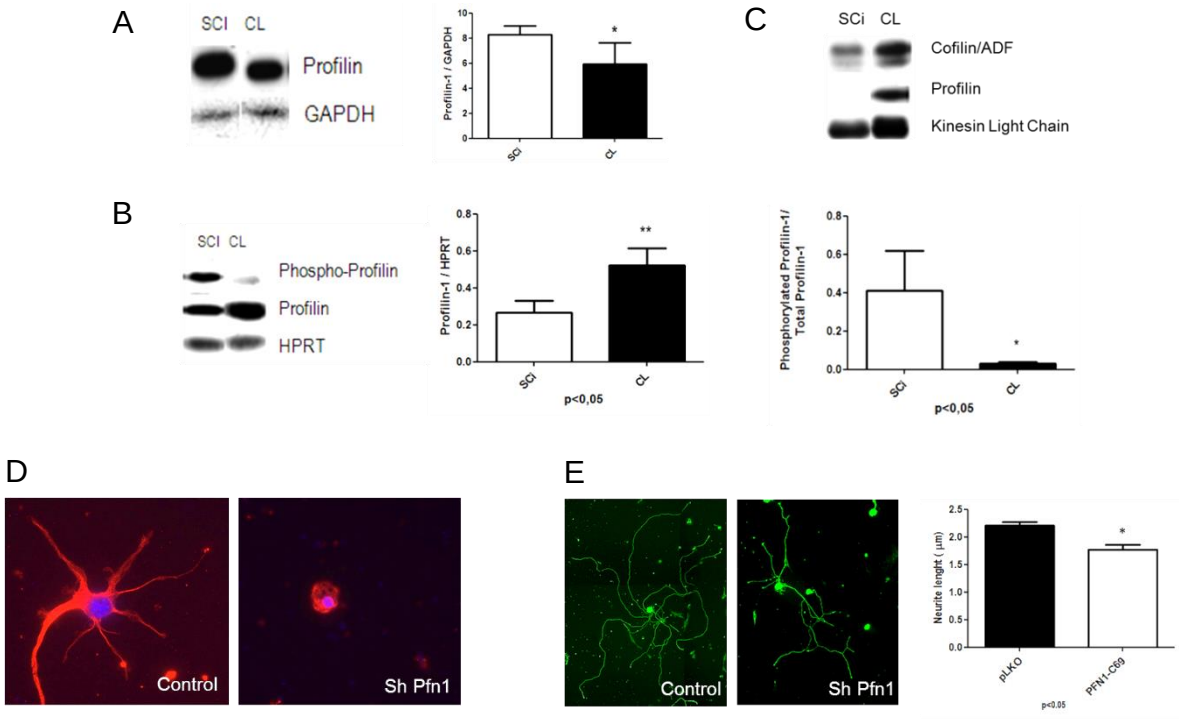


Fig. 5 – **Pfn1 has a critical role during early neuritogenesis and axonal regrowth following injury.** ***A.*** Representative western blots and quantification of the Pfn1 levels in DRG of animals either with spinal cord injury alone or conditioning before spinal cord injury. ***B.*** Representative western blots and quantification of the Pfn1 levels in spinal cord of animals either with spinal cord injury alone or conditioning before spinal cord injury. N=6 rats/condition; p-value was calculated using Student's t-test. ***C.*** Representative image of immunoprecipitation of Pfn1 and kinesin light chain in conditioned animals. ***D.*** βIII-tubulin staining (red) of hippocampal neurons transfected with a control ShRNA or a Pfn1 ShRNA. ***E.*** βIII-tubulin staining (green) of DRG neurons transfected with a control ShRNA or a Pfn1 ShRNA and neurite outgrowth quantification.

4. Profilin

4.1 Characteristics and functions

Profilin, first described by Carlsson and colleagues in 1976 [55-57], is one of the most abundant proteins within cells [58]. It is an evolutionarily conserved 15-kDa small actin-binding protein expressed in eukaryotes [57, 59, 60] and certain viruses [61]. Four distinct *Pfn* genes have been identified so far namely i) *Pfn1,* which codes for the ubiquitous isoform; ii) *Pfn2*, neuronal specific, that in mouse may be alternatively spliced producing a major Pfn2A isoform and a minor Pfn2B isoform which differs in the C-terminal 32 amino acids [62]; iii) *Pfn3*, kidney and testis specific [63]; and iv) *Pfn4*, testis specific [64]. Concerning testis-specific isoforms, it is suggested that Pfn3 and Pfn4 may regulate testicular actin cytoskeleton remodelling, having a particular role in acrosome formation and sperm morphogenesis [64].

Although highly conserved, the Pfn family exhibits a surprisingly low percentage of homology between isoforms and between profilins from different species. Regardless of Pfn1 and Pfn2 being only 62% identical in primary sequence, all profilins assume similar three-dimensional structures consisting of seven beta sheets and four helices (fig. 6A) [65, 66]. However, concerning Pfn1 and Pfn2, it is described a differential exposure of hydrophobic residues which leads to distinct surface features and consequently different affinities for their ligands [65]. Given the plethora of ligands, Pfn is considered a quite promiscuous protein. In order to execute its function, Pfn interacts with its binding partners through specific binding domains namely the actin-binding [67], the poly-L-proline (PLP)-binding [68] and the phosphatidylinositol lipids-binding domains [69, 70] (fig. 6A).

4.2 Profilin and actin dynamics

Actin dynamics is characterized by the transition between monomeric and filamentous actin with actin polymerization occurring at the barbed end and depolymerization occurring in the opposite site of the filament (fig. 6B) [23]. Changes in actin dynamics can be recognized when actin is under the influence of intracellular effectors such as actin binding proteins. Actin binding proteins carry out a wide range of functions, including actin filament nucleation, elongation, severing, capping, and crosslinking that support actin dynamics. Several studies have convincingly demonstrated that actin dynamics critically depends on Pfn activity. By interacting through its actin-binding domain, Pfn enhances the exchange of ADP for ATP on actin monomers and Pfn-bound ATP-actin provides a pool of polymerization-competent monomers that can be incorporated to free

barbed ends to support actin polymerization [66, 71]. Molecular dynamics simulations already demonstrated that Pfn stabilizes the open conformation of the actin nucleotide pocket resulting in a high nucleotide exchange rate [72]. Curiously, the affinity of Pfn1 for monomeric actin is about five times higher than the affinity of Pfn2 for actin [73]. Along the actin filament, ATP is slowly hydrolyzed by the intrinsic ATPase activity of actin, which generates ADP-actin in the older part of the filament where depolymerizing proteins such as cofilin can execute their function.

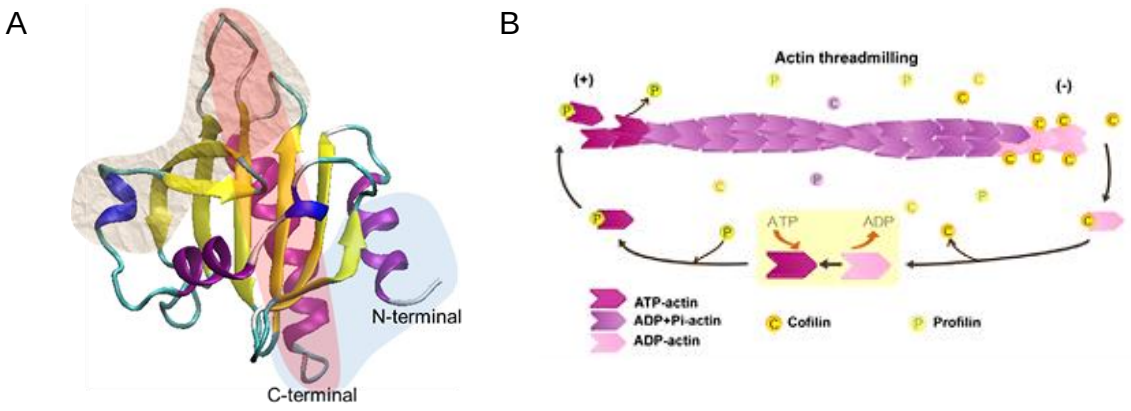


Fig. 6 – **Pfn structure and function.** ***A.*** Pfn1 structure showing actin (grey) and PLP (blue) binding domains overlapping with PtdIns(4, 5)P_2 binding sites (red); PDB database: 2BFT; coloured by secondary structure. ***B.*** Role of Pfn in actin treadmilling. Profilins replenish the pool of ATP–actin monomers by increasing the rate of nucleotide exchange by 1000-fold compared with the rate of nucleotide exchange based on simple diffusion; taken from [74].

It is well established that the cyclical polymerization and depolymerization of actin filaments is necessary to generate the force for growth cone protrusion and motility and ultimately for axon growth. Although actin dynamics is well recognized as a key player in axonal elongation, how different actin-binding proteins control this process is still not fully understood. Given the Pfn activity described earlier, it is conceivable that its loss would markedly impact actin dynamics and growth cone protrusion. Recently, it has been shown that Pfn1 depletion may cause substantial defects in membrane extension and slower velocity of protrusion in either neuronal or non-neuronal migrating cells [75-78]. Notably, membrane protrusion requires *de novo* actin nucleation or elongation of pre-existing filaments, catalyzed by at least three major classes of actin-binding proteins including N-WASP/WAVE, Ena/VASP, Arp2/3 complex and formins. These proteins share a common feature that is the presence of PLP-rich regions which allows them to interact with Pfn at the leading edges. Specifically, the Ena/VASP tetramers act as an actin filament elongation factor and tend to create unbranched, longer and crosslinked bundles of F-actin. Ena/VASP recognizes Pfn-actin complexes and recruits them in order to enhance F-actin bundle elongation [24]. Moreover, the WAVE-complex, activated by both PtdIns(4, 5)P_2 and Rac,

can recruit Arp2/3 complex [23] which together with Pfn2 [79] is important for site directed actin nucleation leading to a preferentially branching pattern. In addition, Pfn is required to formin-mediated barbed-end elongation [80]. These evidence provide the idea that Pfn can interact simultaneously with actin and PLP-rich sequences. In fact, poly-proline sequences bind preferentially profilin-actin complexes than Pfn alone [24]. In conclusion, Pfn1 interacts with promoters of actin nucleation and elongation at the leading edges facilitating actin polymerization and membrane protrusion during cell migration. In this sense, Pfn would play a critical role in neuronal cytoskeleton at different levels of neuronal differentiation and in the mature neuron.

4.3 Profilin and neuritogenesis

As mentioned above, in the early phase of establishing a neuronal phenotype, the neuronal sphere is broken by a local instability of actin at the cell cortex promoting neuritogenesis. Da Silva and colleagues, in 2003, reported that RhoA and its specific downstream effector ROCK are essential during neuritogenesis in mammalian hippocampal neurons by modulating actin dynamics via Pfn2 [29]. Previously, others have reported that Pfn specifically interacts with ROCK, in brain extracts [79]. Importantly, once activated, ROCK may inactivate both isoforms of Pfn through phosphorylation [29, 81] as well as it may activate LIMK-1 which, in turn, inhibits the depolymerizing actin-binding protein cofilin [82]. Thus, a model of the involvement of Rho/ROCK/Pfn2a cascade in neuritogenesis was proposed [29]. At the early differentiation stages, a positive stimuli has the capacity to inactivate RhoA [83] which locally dissociates from the membrane reducing the interaction between ROCK and Pfn2a and consequently Pfn2a phosphorylation. This is translated into a localized shift in the local F/G actin ratio, favoring the monomeric form leading to actin instability and sprout formation. Interestingly later on, the same authors revealed that Rho/ROCK/Pfn2a cascade can be inactivated by plasma membrane ganglioside sialidase (PMGS) that asymmetrically accumulates at the tip of the future axon in stage 2 neurons [84]. Such specification occurs by triggering PI3K- and Rac1-dependent inhibition of RhoA signalling that, in turn, cannot recruit ROCK/Pfn2 complexes culminating in a local actin instability and axon specification.

Besides the neuronal-specific Pfn2, the ubiquitous Pfn1 has been also implicated in neuritogenesis since it localizes at the leading edge of growth cones [85]. However, evidence suggest a functional divergence between Pfn1 and Pfn2. While Pfn1 overexpression has a mild effect in promoting filapodia formation [85], Pfn2 overexpression in hippocampal neurons strongly reduces neurite number and length [29], suggesting opposite roles in neuritogenesis. Curiously, in the cancer field, emerging evidence also

revealed differential actin remodelling by Pfn isoforms. In fact, in non-neuronal cells, Pfn2 suppresses protrusion activity and migratory behaviour by promoting Ena/VASP protein-mediated actin polymerization actin filaments that assemble into contractile bundles [2]. In contrast, Pfn1 promotes membrane protrusion, motility and invasion which could be explained by having more affinity to WAVE that, in turn, promotes branched polymerization by Arp2/3 complex activation. However, seemingly contrary to the view of Pfn1 promoting migration, invasive breast cancer cells present downregulation of Pfn1 [86]. Surprisingly, Pfn1 is considered to be a tumor suppressor protein as it negatively regulate PtdIns(4, 5)P_2 availability which recruits lamellipodin to the leading edge [87]. Indeed, Pfn binds to the membrane through its interaction with PtdIns(4, 5)P_2. In fact, Pfn contains two phosphatidylinositol lipids-binding sites of which one overlaps with the actin-binding site and the other with the PLP-binding site [70]. By competing with both actin and proline-rich containing proteins, PtdIns(4, 5)P_2 is considered to be a master regulator of Pfn function. Importantly, PtdIns(4, 5)P_2, bound to Pfn, can only be hydrolyzed into second messengers by phospholipase Cγ1 (PLCγ1) which becomes activated in response to external stimulus. Upon hydrolysis, Pfn is released from the membrane to the cytosol where it can interact with actin and other ligands. Thus, Pfn emerges as a link between intracellular signalling and cytoskeleton remodelling.

4.4 Profilin and neuronal plasticity

Pfn has also a relevant role in regulating actin dynamics in dendritic spines. Evidence indicates that Pfn, via its PLP-binding domain, is targeted to spine heads upon postsynaptic N-methyl-D-aspartate (NMDA) receptors activation inducing suppression of actin dynamics and long-term stabilization of spine morphology which is associated with memory formation [88]. In addition, it was reported that fear conditioning of rats drives Pfn into dendritic spines, which is associated with enlargement of postsynaptic densities, an important factor in synaptic plasticity [89]. Moreover, NMDA receptor activation leads to nuclear accumulation of Pfn by involving actin rearrangements [90]. Furthermore, as Pfn can simply diffuse towards the nucleus, specific export mechanisms exist such as exportin6 that recognizes Pfn-actin complex and transports it out of the nucleus [91]. The presence of Pfn in the nucleus suggests that it could alter the transcription profile. In fact, it is known that Pfn interacts via its PLP-binding domain with the Myb-related transcriptional factor p42POP which is widely expressed in mouse tissues [92]. In the nucleus, Pfn1 has also been localized in Cajal bodies, pointing to a role of Pfn in mRNA processing [93].

Additionally, Pfn1 may also play a role at inhibitory synapses since it interacts with the postsynaptic scaffolding protein gephyrin allowing cytoskeleton-dependent clustering of receptors in the post-synaptic membrane [94].

Furthermore, current knowledge indicates that Pfn is involved in membrane trafficking since its attachment to the Golgi is required for the formation of constitutive transport vesicles at the *trans*-Golgi network probably via interaction with dynamin-2 [95]. Pioneering proteomic experiments performed in mouse brain unravelled the involvement of Pfn also in endocytosis and synaptic recycling. Noticeably, it was documented the intimate relation between Pfn2 and dynamin-1 leading to the idea that Pfn actively brings together cytoskeleton elements and proteins that belong to the endocytic machinery [79]. Taken together, these evidences implicate Pfn as an important regulator of the neuronal cytoskeleton during differentiation and plasticity processes.

4.5 Implications of profilin *in vivo*

The promiscuity of Pfn for a plethora of signalling molecules explains the relationship between several cellular processes and actin remodelling but it also complicates the interpretation of Pfn function *in vivo*. Profilins have been implicated to play a role in many pathological conditions such as muscular atrophy, dementia, cancer, allergies, cardiovascular diseases and diabetes. Intriguingly, in what concerns to the nervous system, neurons have a second Pfn isoform to provide additional functions besides modulation of actin dynamics.

Genetic approaches demonstrated that Pfn1 deletion results in a preimplantation embryonic lethal phenotype, pointing to an indispensable role of this isoform in the earliest stages of mouse development [96]. In fact, Pfn1 is expressed through all of developmental stages. Moreover, although adult heterozygous mice are viable even presenting alterations in brain morphology (displacement of the hippocampus), deletion of a single Pfn1 allele leads to reduced survival during embryogenesis. In this drastic scenario, it was further observed that Pfn2, albeit weakly expressed in early embryos, does not compensate for the lack of Pfn1. In contrast, mice lacking Pfn2 are viable, express normal levels of Pfn1 and show no gross brain abnormalities [25]. Concerning such evidences, it is convincingly understood that Pfn2 is not essential for embryonic development. Indeed, this isoform is not required for actin-dependent neurite outgrowth that underlies neuronal polarization or migration processes. Deletion of Pfn2 leads to alterations in neurotransmitter release which correlates with the hyperactivity and enhanced novelty-seeking behaviour of Pfn2 mutant mice. In summary, while Pfn1 seems to regulate cytoskeleton architecture, Pfn2 may

regulate membrane trafficking, having an inhibitory role in vesicle exocytosis/presynaptic excitability.

Other studies contributed to further understand Pfn function *in vivo*. For example, cartilage-specific deletion of Pfn1 culminates in defective abscission during late cytokinesis of chondrocytes and consequently progressive chondrodysplasia [97]. In addition, Pfn1 deletion during development impairs brain morphology and radial migration of CGN [98] due to glial cell adhesion defects [99]. Such Pfn1 mutant mice show ectopic cerebellar granule neurons, cerebellar hypoplasia and aberrant cytoarchitecture of the cerebellar cortex layers which were later associated with impaired motor coordination [100]. Besides its importance in the CNS, it is already documented that Pfn1 is a pivotal requirement for Schwann cells lamellipodia formation, radial sorting and myelination during PNS development [101].

Moreover, neurodegenerative diseases have been linked to Pfn1. For example, in Huntington's disease, Pfn1 is considered to act as an anti-aggregation factor counteracting the toxicity of polyglutamine protein aggregates [81]. Pfn1 may also play a significant role in muscular atrophies. Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by the loss of alpha motor neurons in brain stem and spinal cord caused by reduced levels of functional survival of motoneuron (SMN) protein [102]. SMN protein, which modulates RNA splicing and nucleocytoplasmic transport of small nuclear ribonucleic particles (snRNPs) in neuronal processes [103, 104], is capable of interacting with Pfn through PLP stretches [105]. Alterations in SMN protein may compromise the formation of SMN-Pfn complexes promoting Pfn hyperphosphorylation by ROCK and establishing the link between ROCK pathway and SMA pathogenesis [106].

Missense mutations in the human *PFN1* gene were recently linked to familial amyotrophic lateral sclerosis (ALS) [107]. ALS is an adult-onset disorder, characterized by progressive degeneration of upper (in the cortex) and lower (in spinal cord) motor neurons leading to progressive muscular weakness and atrophy followed by muscular paralysis. Thus, respiratory insufficiency occurs in patients with ALS being a major cause of mortality, generally within 1-5 years of disease onset. In addition, ALS patients may present degeneration in the frontotemporal regions of the brain and cognitive impairment [108] as well as degeneration of corticalspinal tract [109]. In ALS, approximately 10% of the cases are of familial origin, with a dominant inheritance mode and from these, 1-2% might be caused by *PFN1* mutations. Such mutations may contribute to ALS pathogenicity by inducing aggregation of TAR DNA-binding protein of 43 kDa (TDP-43) and inhibiting axon dynamics. Moreover, it was reported that Pfn1 associates with stress granules and that ALS-associated *PFN1* mutations might alter stress granule dynamics [110]. Additionally, all

ALS-linked *PFN1* mutations interfere with actin-binding residues of Pfn1 which clearly links [107] actin cytoskeletal alterations to ALS pathogenesis. However, the mechanism by which Pfn1 acts in the growth cone, in axon growth and degeneration and for example in the formation and maintenance of the NMJs remains to be elucidated.

Together the body of knowledge gathered supports the notion that Pfn exhibits characteristics of a protein interaction platform which assists convergence of signals. As a multifunctional platform, alterations on Pfn lead to numerous pathological phenotypes.

Objectives

This project aims at dissecting the Pfn function in neurons. In particular, this work aims at:

i) unravelling the role of Pfn1 in axon growth and regeneration using the Thy1cre$^{+/-}$ Pfn1$^{fl/fl}$ mice model;

ii) evaluating the impact of Pfn1 in axon formation and synapse maintenance in the DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice model;

iii) understanding the molecular mechanisms that underlie Pfn1 function.

Methods and Materials

Animals

Mice were handled according European Union and National rules. The protocols described in this work have been approved by the IBMC Ethical Committee and by the Portuguese Veterinarian Board. Mice were bred in the animal housing facility of the IBMC, had *ad libitum* access to water and standard rodent food, and were kept on a 12 hour light and dark cycle. Genotyping was performed by IBMC CCGen facility.

Floxed Pfn1 mice ($Pfn1^{fl/fl}$; a kind gift from Prof R Fassler, Max Planck Institute of Biochemistry) and Slick-H mice (a kind gift from Dr Guoping Feng, Duke University Medical Center; mice that coexpress inducible-$CreER^{T2}$ and YFP under the control of the Thy1 promoter [111]) were used to generate neuronal-specific conditional Pfn1 knockout mice ($Thy1cre^{+/-}Pfn1^{fl/fl}$). For that, $Pfn1^{fl/fl}$ were crossed with Slick-H mice. $Thy1cre^{+/-}Pfn1^{fl/wt}$ were selected and then crossed with $Pfn1^{fl/fl}$ mice and $Pfn1^{wt/wt}$ mice in order to generate $Thy1cre^{+/-}Pfn1^{fl/fl}$ mice and $Thy1cre^{+/-}Pfn1^{wt/wt}$. Given the neuroprotective effects of tamoxifen [112], tamoxifen-treated $Thy1cre^{+/-}$ $Pfn1^{fl/fl}$ animals were compared to tamoxifen-treated $Thy1cre^{+/-}Pfn1^{wt/wt}$ animals. Induction of cre expression was performed by tamoxifen injection at weaning (3-4 weeks of age).

$Pfn1^{fl/fl}$ mice and doublecortin (DCX)-cre mice (a kinf gift from Dr Ulrich Mueller, The Scripps Research Institute) were used to generate $DCX\text{-}cre^{+/-}Pfn1^{fl/fl}$ mice. $DCX\text{-}cre^{+/-}$ $Pfn1^{wt/fl}$ were selected and then crossed with $Pfn1^{fl/fl}$ mice in order to generate $DCX\text{-}cre^{+/-}$ $Pfn1^{fl/fl}$ mice and $DCX\text{-}cre^{-/-}Pfn1^{fl/fl}$ mice.

In all experiments animals of either sex were used.

Surgical procedures

Sciatic nerve injury

7-weeks-old animals were anesthetized with 5% isoflurane in a closed chamber for the initial induction, followed by delivery of 2-3% of isofluorane using a mask, which was maintained through the surgery procedure. In order to expose the sciatic nerve, the skin was shaved along the femur and the fascia linking the two muscles was ripped with a scissor, leading to the sciatic nerve exposure. The exposed sciatic nerve was injured at the mid-thigh level, one week prior to sacrifice. Specifically, for the assessment of peripheral regenerative capacity, it was performed a unilateral sciatic nerve crush in $Thy1cre^{+/-}Pfn1^{fl/fl}$

(n= 6) and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ (n= 3) mice. For the *in vitro* neurite outgrowth assays, both sciatic nerves from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ (n= 2) and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ (n= 2) were transected. Postsurgical analgesia was performed twice a day for 3 days with subcutaneous injection of buprenorphine (0.08 mg/Kg).

Spinal cord hemisection

8-weeks-old Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ (n=4) and Thy1cre$^{+/-}$Pfn1$^{wt/wt}$ (n=4) mice were deeply anesthetized with a medetomidine/ketamine mixture (medetomidine 1mg/Kg; ketamine 75mg/Kg) via intraperitoneal injection. In order to compensate for the diuresis caused by anaesthesia and for the blood loss, a fluid supplement (5% glucose saline solution) was subcutaneously injected. The skin was shaved along the vertebral column and one incision was performed through the thoracic vertebras 6 to 11 (T6-T11). Under a stereo microscope, the vertebral column was exposed and a laminectomy was performed at the T8 level. A 30-gauss needle was used to disrupt the dura mater followed by dorsal hemisection using a micro feather ophthalmic scalpel. The muscle and skin were sutured and the animals were allowed to recover. Postsurgical analgesia was performed twice a day for 3 days with subcutaneous injection of buprenorphine (0.08mg/kg) as well as a fluid therapy with Duphalyte®. Wet food was provided in the cage floor and manual voidance of the bladder was performed twice a day during the period of the experiment.

Conditioning lesion

Animals were subjected to a sciatic nerve transection 1 week prior spinal cord injury.

Injection of cholera toxin B into the sciatic nerve

Animals with conditioning dorsal hemisection were allowed to recover for 4 weeks. Four days prior euthanasia, 2µl of 1% cholera toxin B (CT-B; List Biologicals) were injected in the left sciatic nerve. For that, animals were anesthetized with isofluorane, as described above, and a tight knot was placed below the exposed left sciatic nerve notch in order to stabilize the nerve. Thereafter, cholera toxin B injection was performed with a 10µl syringe (Hamilton, USA). The muscle and skin were sutured and the animals were allowed to recover. Postsurgical analgesia was performed twice a day for 3 days with subcutaneous injection of buprenorphine (0.08mg/kg).

Analysis of regeneration of dorsal column fibers

The conditioned animals were perfused with PBS followed by formalin, tissues were post fixed at 4°C for 1 week, cryopreserved in 30% sucrose and secctioned subsequently at 50µm (cryostact; Leica). Consecutive spinal cord sagittal sections were collected for free floating immunohistochemistry with goat anti-CT-B (1:30 000; List Biologicals). Briefly, sections were blocked and permeabilized by incubation for 1 hour at room temperature (RT) in blocking buffer (5% FBS, 0.3% Triton X-100 in PB) and then incubated overnight at 4 ºC with anti-CT-B. Antigen detection was performed following incubation with biotinylated horse anti-goat (1:200; Vector) for 2 hours at RT, sections were washed (TBS, 1% TritonX-100) and then incubated with Alexa Fluor® 568 streptavidin (1:1000, Invitrogen) in blocking buffer (TBS, 1% TritonX-100) for 1 hour at RT. After washing, coverslides were dried and mounted with Vectashield containing DAPI (Vector Labs). Dorsal column fiber images were adquired by confocal microscopy on a Leica TCS SP5 II equipped with Leica Application Suite Advanced Fluorescence (LAS AF) software and image analysis were performed with Fiji software. Dorsal column fibers were quantified by counting the total number of CT-B labeled axons/YFP^+ axons within the glial scar and by measuring the distance of each CT-B labeled axon/YFP^+ axon, found rostrally to the injury site, to a vertical line (used as origin) placed at the rostral end of the dorsal column tract (i.e., where CT-B/YFP labeling accumulates). All quantifications of axonal regeneration were performed with the researcher blinded for genotype and experimental group.

Histological and morphological analyses

Sciatic nerves were dissected and fixed by immersion on 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 week. After postfixation with 1% OsO4 in 0.1 M cacodylate buffer (pH 7.4) for 2 hours, nerves were dehydrated and embedded in Epon (Electron Microscopy Sciences). Sections (1-µm-thick) were stained for 10 minutes with 1% p-phenylenediamine (PPD) in absolute methanol, dried, and mounted on a drop of DPX (Merk). The entire area of the nerve was photographed on an Olympus optical microscope equipped with an Olympus DP 25 camera and Cell B software, and images were imported into Photoshop (Adobe). For the determination of the density of myelinated fibers (MFs), the total number of MFs was divided by the area of the entire cross section of the sciatic nerve.

Immunohistochemistry

DRGs were collected from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ and post fixed in carnoys and processed for imunnofluorescence in 4 µm paraffin sections.

Sections from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRGs were incubated for 1 hour at RT with blocking buffer (5% fetal bovine serum [FBS], in TBS-T). Primary antibodies, mouse anti-GFP (Clontech) dilution, and rabbit anti-Pfn1 (Abcam) dilution, were diluted in blocking buffer and incubated overnight at 4°C. After washing with TBT-T, secondary antibodies, Alexa Fluor® 488 donkey anti-mouse IgG H+L and Alexa Fluor® 568 donkey anti-rabbit IgG H+L (1:1000, Invitrogen) were diluted in blocking buffer and incubated for 1 hour at RT.

Sections from DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ DRGs were incubated with 0.1% sodium borohydrade in Tris:EDTA pH 9.0 for 10 minutes, washed and treated with NH_4Cl for 10 minutes to reduce autofluorescence. Subsequently, sections were incubated for 1 hour at 37°C with blocking buffer (5% normal donkey serum, in PBS). Primary antibody, mouse anti-cre recombinase (Covance) dilution, was diluted in blocking buffer and incubated overnight at 4°C. After washing with PBS, secondary antibody, Alexa Fluor® 568 donkey anti-mouse IgG H+L (1:500, Invitrogen) were diluted in blocking buffer and incubated for 1 hour at RT.

All sections were washed, mounted with Vectashield containing DAPI (Vector Labs) and analysed by epifluorescence on a Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axiovision 4.7 software.

Primary neuron cultures

Dorsal root ganglia neuron culture

DRG were dissected aseptically from slickH-Pfn1 mice, freed of roots and treated with 0.125% collagenase-IV-S (Sigma) for 90 minutes at 37°C with 5% CO_2. After enzyme treatment, a single-cell suspension was obtained by trituration with a fire-polished Pasteur pipette. The cell suspension was centrifuged into a 15% BSA (NZYTech) gradient for 10 min at 200g. The obtained pellet was resuspended in DMEM/F12 medium (Sigma) supplemented with 1% penicillin-streptomicin (Invitrogen), 1× B27 (Invitrogen), 2mM L-glutamine (Sigma), 50 ng/ml NGF (Milipore) and plated in poly-L-lysine (PLL, 20µg/mL, Sigma)/laminin (5µg/mL, Sigma) coated 13 mm coverslips and maintained at 37°C with 5% CO_2 for 12 hours.

Hippocampal neuron culture

The hippocampi were dissected aseptically from E17.5 NMRI embryos and treated with 0.06% trypsin (Sigma) in HBSS for 15 minutes at 37°C with 5% CO_2. After enzyme treatment and washing, the hippocampi were ressuspended in Neurobasal medium (Invitrogen) and a single-cell suspension was obtained by passing the solution up and down in a micropipette and through a 70µm cell strainer to remove chunks or indissociated tissue. The single-cell suspension was ressuspended at the correct dilution in Neurobasal medium supplemented with 1% penicillin-streptomicin (Invitrogen) and 1× B27 (Invitrogen), and plated in poly-L-lysine (20µg/mL, Sigma) coated 13 mm coverslips and maintained at 37°C with 5% CO_2 for 4 days.

Neurite outgrowth evaluation

To measure neurite outgrowth, immunocytochemistry for βIII-tubulin was performed. Briefly, primary neurons were fixed for 30 minutes with 4% PFA, permeabilized (0.4% TritonX-100) for 5 minutes, blocked (3% BSA, 0.2% TritonX-100) for 1 hour at RT and incubated with primary antibody, mouse anti-βIII-tubulin (1:1000, Promega) in blocking buffer, overnight at 4 ºC. After washing with PBS, secondary antibody, Alexa Fluor® 568 donkey anti-mouse IgG (1:1000, Invitrogen) was diluted in blocking buffer and incubated for 1 hour at RT. The coverslides were washed and mounted on microscope slides with Vectashield containing DAPI (Vector Labs). Slides were analyzed and the images were acquired on an inverted epifluorescence microscope, Leica DMI 6000B equipped with an ORCA-Flash4.0 V2 C11440-22CU digital camera and LAS AF software. The length of the longest neurite was traced manually with NeuronJ plugin for ImageJ. The number of neurons traced was 39-48 for conditioned and 91-167 for naïve DRGs from Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice.

Virus production

HEK293T cells were maintained in DMEM:F12 medium supplemented with 1% penicillin-streptomicin (Invitrogen) and 10% FBS (Invitrogen). For viral production, HEK293T cells were seeded at 90% conlfuence in T75cm^2 flasks and transfected using Lipofectamine (Invitrogen) with packaging plasmids pPAX (6µg) and VSVG (3µg) (a kinf gift from Dr. Relvas, IBMC) and a lentiviral vector coding for puromycin resistance and expressing the shRNA of interest (6µg, Sigma). Forty-eight hours post transfection, the supernatants were collected, centrifuged at 1000 rpm for 5 minutes at RT and filtered

through a 0.45 µm filter. Viral titration was done by infecting HEK 293T cells with serial dilutions of viral stock and using puromycin selection.

Gene transduction

DRGs from slickH-Pfn1 mice were isolated as mentioned previously. One day after plating, cells were infected with lentivirus (5000 IU per well), produced as described above, for 12–16 hours, and 24 hours later treated with puromycin (5 µg/ml) for 48 h. For neurite outgrowth, cells were trypsinized, replated for 12 h, and βIII-tubulin immunocytochemistry (1:1000; Promega) was done. The number of neurons traced was 41-103.

Overexpression of Pfn1 mutants in hippocampal neurons

Residues of Pfn1 were mutated to generate an actin-binding deficient Pfn1 ($Pfn1^{R74E}$), a PLP-binding deficient Pfn1 ($Pfn1^{H133S}$) and a phospho-resistant ($Pfn1^{S137A}$) or phospho-mimetic mutants ($Pfn1^{S137D}$). Mutations were generated by PCR-based site-directed mutagenesis using QuickChange II XL (Agilent Technologies). Hippocampal neurons were cultured, as described above, and co-transfected before plating with 200 ng pmaxGFP™ (Lonza) and 600 ng pCMV-SPORT6 vector coding the full length Pfn1 open reading frame (Addgene, clone IRATp970C034D) or each of the mutant plasmids mentioned. Transfected cells were allowed to grow for 4 days and afterwards fixed by incubation with 4% PFA (30 minutes). Thereafter, immuhistochemistry was done for βIII-tubulin and GFP^{+}/βIII-$tubulin^{+}$ neurons were slected for neurite outgrowth evaluation. For the analysis of the relative expression of wild-type Pfn1 or each mutant, the neuronal cell line CAD was used [113].

Overexpression of Pfn1 in CAD cells

CAD cells were maintained in DMEM medium supplemented with 1% penicillin-streptomicin (Invitrogen) and 8% FBS (Invitrogen). For overexpression of wild-type Pfn1, CAD cells were seeded at 80% confluence in a 24-well plate and transfected using Lipofectamine (Invitrogen) with 500 ng pCMV-SPORT6 vector coding the full length Pfn1 open reading frame (Addgene, clone IRATp970C034D) and 500 ng pmaxGFP™ (Lonza). Forty-eight hours post transfection, protein lysates were prepared for biochemical analyses.

Analysis of actin retrograde flow

In order to evalute defects on actin cycle at the growth cone level in slickH-Pfn1 mice model, Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice were bred and used to perform actin retrograde flow analysis. DRG neurons from 8-weeks-old animals, dissected and cultured as mentioned previously, were nucleofected (4D Nucleofector Amaxa system, Lonza, CU137 program) with either 750 ng LifeAct-mCherry plasmid (in the case of Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice DRG neurons) or 750 ng LifeAct-mCherry plasmid plus 150 ng pmaxGFP™ (Lonza) (in the case of Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice DRG neurons). After transfection, cells were left in suspension overnight and then plated for 12 hours in a PLL (20µg/mL)/laminin (5µg/mL) coated µ-Dish 35 mm (Ibidi) previously treated with 2M HCl. Twelve hours after plating, time-lapse recordings were performed, in phenol-free DMEM/F12 (Invitrogen) supplemented as mentioned above, for 40 frames every 5 seconds at 37ºC on Spinning Disk Confocal System Andor Revolution XD (ANDOR Technology, UK) equipped with an iXonEM+ DU-897 camera and with IQ 1.10.1 software (all from ANDOR Technology, UK). Kymographs were made using Kymograph plugin for ImageJ for retrograde flow quantification. The number of growth cones recorded was 4-6.

Western blotting

Protein lysates from CAD cells overexpressing Pfn1 (as described above) were prepared by sonication in PBS containing 0.3% Triton X-100, protease inhibitors cocktail (cOmplete, Mini; Roche), and 2 mM orthovanadate. Protein lysates (20 µg per lane) were separated on 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk (Sigma-Aldrich) in TBS containing 0.01% Tween20 (w/v) and probed with the following antibodies: rabbit anti-Ser475-Akt (1:1000; Cell Signaling), rabbit anti-Thr308-Akt (1:1000; Cell Signaling), rabbit anti-total-Akt (1:1000; Cell Signaling), rabbit anti-Ser9-GSK3β (1:500; Cell Signaling), rabbit anti-Tyr216-GSK3β (1:500; Santa Cruz), mouse anti-GSK3α/β (1:1000; Santa Cruz), rabbit anti-Ser241-PDK1 (1:1000; Cell Signaling), rabbit anti-total-PDK1 (1:1000; Cell Signaling), rabbit anti-Ser380-PTEN (1:1000; Cell Signaling), rabbit anti-total-PTEN (1:1000; Cell Signaling), mouse anti-total-Pfn1 (1:1000; Santa Cruz Biotechnology), rabbit anti-Ser137-Pfn1 (a kind gift from Dr Jieya Shao, University of California, San Francisco) and mouse anti-β-actin (1:5000; Sigma). Blots were developed with Luminata Forte Western HRP (Milipore) and exposed to Hyperfilm (Amersham). Films were scanned on a Molecular Imager GS800, and Quantity One (Bio-Rad) was used for quantifications.

Morphometric analysis of the NMJs and assessment of the muscle innervation pattern

Postnatal day 15 and 7-months-old DCX-cre Pfn1 mice were used to address potential defects in the morphology of the NMJs as well as in muscle innervation pattern. For that, hemidiaphragms were dissected and fixed in formalin. The collected tissues were incubated overnight in 0.1M glycine (Promega); permeabilized for 30 minutes with 0.2% TritonX-100 and incubated with 0.2M of ammonium chloride (Merck) for 30 minutes to reduce autofluorescence. For the labelling of the endplates, muscles were incubated with tetramethylrhodamine-conjugated anti-bungarotoxin (1:200; Invitrogen) in blocking buffer (1mg/mL BSA, 0.2% TritonX-100 in PBS) for 1 hour at RT. Washed hemidiaphragms were mounted on coverslides with PBS and slides were analyzed by epifluorescence on a Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axiovision 4.7 software. Images were merged using Photoshop (Adobe) and recording measurement tool was used to determine the innervation area and count the number of endplates. For morphometric characterization of endplates, 20x magnification z-stacks were acquired using a z-step of 1µm. Z-stacks were deconvolved and 3D-rendered using 3D Huygens Deconvolution Software, and their length, surface, volume and number fragments were quantified.

Statistical analysis

Results were expressed as the mean ± SEM, and data were analysed with Prism software (GraphPad Software). To compare 2 groups, two-tailed Student's t test was used. For comparison of more than 2 groups, 1-way ANOVA tests were performed, followed by Tukey's multiple comparison tests.

Results

Part I – Dissecting the role of Pfn1 in axon growth and regeneration

As previously mentioned, intriguing preliminary data (fig. 5) revealed that *in vitro* Pfn1 deletion inhibits axon formation and growth. Moreover, the fact that Pfn1 was increased in the SCI site after CL supported the notion that the organization of the actin cytoskeleton is a pivotal downstream determinant for axon growth capacity. In order to further address the involvement of Pfn1 in axonal regeneration *in vivo*, we generated Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice that co-express both tamoxifen inducible CreER^{T2} and YFP under the control of the Thy1 promoter. This genetic approach allowed neuron-specific deletion of Pfn1 avoiding confounding effects of Pfn1-depleted glia. Moreover, the inducible gene excision allows not to compromise the development of any component of the nervous system. Such deletion was qualitatively confirmed in DRG by immunohistochemistry (fig. 7A), where in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice one can observe that YFP^{+} axons (green) are Pfn (red) deficient. A possible Pfn2 compensation in this model should be further documented in the future.

Given the role of Pfn1 in promoting actin dynamics and F-actin growth in the axonal growth cone, our hypothesis was that neurite outgrowth would be impaired in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice. To confirm this hypothesis, DRG neuron cultures from naïve and conditioned Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice were performed. Conditioned animals that underwent a peripheral lesion, specifically, a sciatic nerve transection one week prior to DRG dissection and culture, were used to increase the intrinsic growth capacity and possibly observe a more striking effect after Pfn1 deletion. As depicted in fig. 7, pre-injured DRG neurons when cultured extend long and unbranched neurites while naïve DRG neurons extend small and highly ramified neurites. Although in naïve neurons no effect was observed resulting from Pfn1 deletion (fig. 7B, D-I), preinjured Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRG neurons lacking Pfn1 exhibited a slight decrease in neurite outgrowth capacity when cultured (fig. 7C, J-O). Interestingly, our preliminary data demonstrated that knocking down Pfn1 expression in DRG lead to a slight decrease in neurite extension capacity. This discrepancy might be related to the fact that, whereas in a Sh knockdown there is an acute downregulation of expression, in a KO system, adaptation in a chronic scenario may occur.

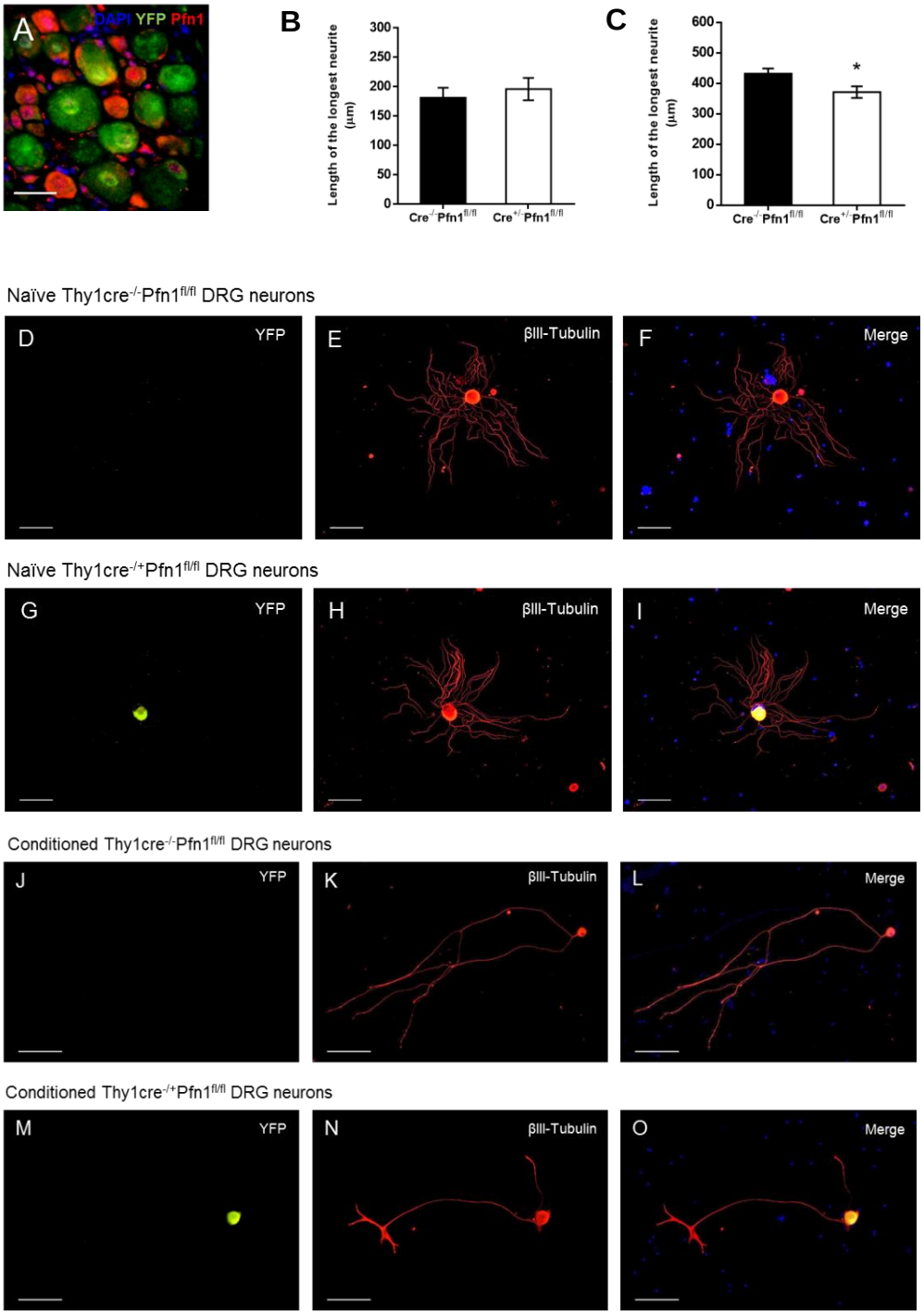


Fig. 7 – **Neurite outgrowth of Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRG neurons.** ***A.*** Representative image of Pfn1 and YFP immunohistochemistry in DRG neurons from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice; scale bar: 30 µm. ***B.*** Neurite outgrowth of naïve DRG neurons from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice. ***C.*** Neurite outgrowth of conditioned DRG neurons from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice. Two-tailed Student's t test with $p < 0.05$ (*). All error bars are SEM. Representative images of naïve (***D – I***) and conditioned (***J – O***) mouse DRG neurons stained for βIII-tubulin; scale bar: 100 µm.

Deletion of both Pfn isoforms impairs axonal growth

As it is possible that Pfn2 might compensate for Pfn1 function, ShRNA for Pfn2 was delivered through lentiviral infection into DRG neurons from both Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{+/-}$Pfn1$^{wt/wt}$ mice. In order to mimic the conditioning effect, DRG neurons were later trypsinized and replated. ShRNA knockdown of Pfn2 significantly decreased almost to half neurite outgrowth (fig. 8) whereas in control DRG neurons ablation of Pfn2, in the presence of Pfn1, did not decrease the elongation capacity (data not shown). In summary, when both profilin isoforms are deleted, the neurite outgrowth capacity was strongly impaired.

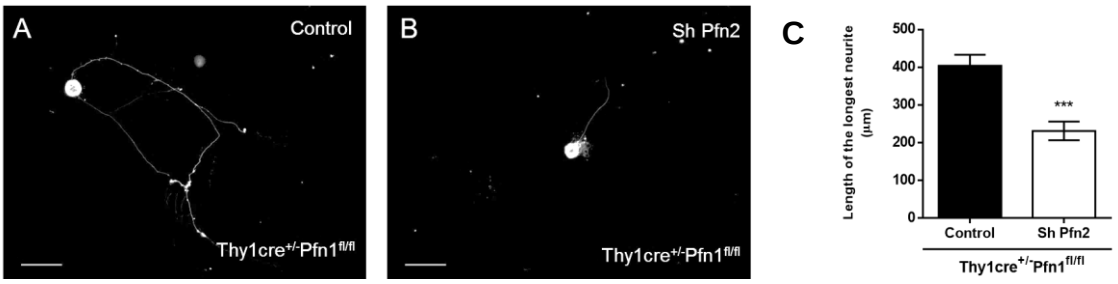


Fig. 8 – **Depletion of Pfn1 and Pfn2 impairs axon extension. *A*.** Representative images of Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRG neurons transduced with lentivirus carrying the ShRNA for Pfn2; scale bar: 50 µm. One-way ANOVA, Tukey's multiple comparisons test, $p < 0.001$ (***). Error bars are SEM. ***B.*** Quantification of neurite outgrowth of DRG neurons representatively depicted in ***A***.

Loss of Pfn1 impairs actin dynamics

Given Pfn function in F-actin polymerization, it is conceivable that its loss would markedly impact actin dynamics causing defects in membrane extension and growth cone protrusion. Besides, localization studies in mouse fibroblasts have shown that Pfn is enriched in areas of high actin dynamics, such as leading lamellae and ruffles [114, 115]. Specifically, actin dynamics at the leading edge is guaranteed by the actin treadmilling and by actin retrograde flow activity levels. To test if deletion of Pfn1, a polymerization enhancer, would affect the actin cycle in growth cones, the retrograde flow of F-actin was measured in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ growth cones, lacking Pfn1, and compared with Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ growth cones. In the absence of Pfn1, actin retrograde flow was significantly lower in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ growth cones (fig. 9). Future experiments should assess other parameters in Pfn1 KO neurons such as the protrusion frequency and distance. In summary, Pfn1 impairs actin turnover at the leading edge of growth cones.

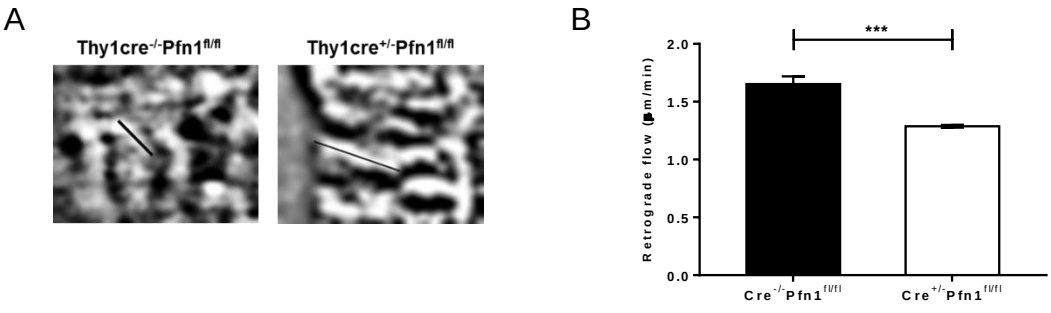


Fig. 9 – **Depletion of Pfn1 impairs actin dynamics in growth cone.** ***A***. Representative kymographs in respect to Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ growth cones used for actin dynamics quantification. ***B.*** Quantification of actin retrograde flow. Two-tailed Student's t test with $p < 0.001$ (***). All error bars are SEM.

Analysis of the role of Pfn1 in axonal regeneration

Given the previous *in vitro* results, the potential role of Pfn1 in promoting the regeneration process emerges since its absence impairs the actin cycle at the growth cone and consequently might compromise the elongation capacity of neurons following injury. To evaluate the regenerative capacity of both peripheral and central axons upon a lesion in the absence of Pfn1, *in vivo* approaches were performed.

The conditioning lesion model (sciatic nerve injury prior to spinal cord injury) was performed in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice and control Thy1cre$^{+/-}$Pfn1$^{wt/wt}$ mice (n=4, per group). After CL, the regenerating dorsal column fibers were traced with CT-B and the CT-B^{+}/YFP^{+} axons that were capable of entering in the glial scar were quantified (fig. 10). Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice showed a tendency to present less axons within the glial scar when compared with control ones, albeit that difference was not statistical probably given the low number of animals analysed (fig. 10). Further surgeries need to be performed in order to increase the number of samples per experimental group. No significant differences were observed in the distance of regenerating axons of Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice to the rostral end of the glial scar when compared with control mice (data not shown). These results point to an intriguing role of Pfn1 in central regeneration since its absence may lead to a decreased number of regenerating axons capable of protruding within a non-permissive environment without impairing their elongation capacity.

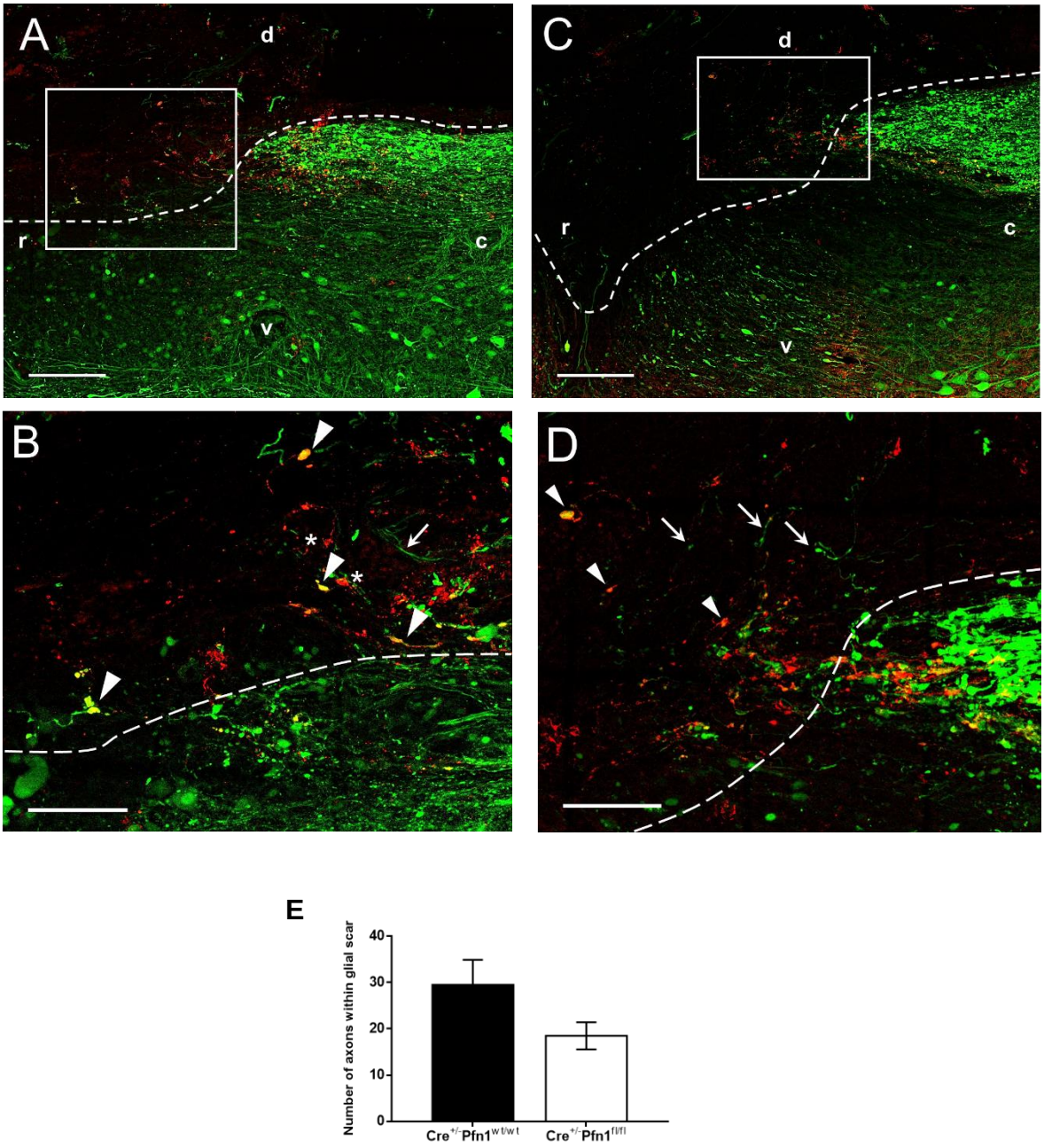


Fig. 10 – **Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice tend to present less axons within the glial scar.** Representative images of CT-B^{+} fibers in sagittal spinal cord sections following CL in Thy1cre$^{+/-}$Pfn1$^{wt/wt}$ (***A, B***) and Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice (***C, D***). YFP^{+} axons are shown in green and dorsal column fibers traced with CT-B are labeled in red; scale bar: 200 µm. Higher magnifications of Thy1cre$^{+/-}$Pfn1$^{wt/wt}$ and Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ spinal cords showing the YFP^{+}/CT-B^{+} axons (arrowheads) within the glial scar are shown on the right; CT-B^{+}/YFP^{-} (asterisks) and CT-B^{-}/YFP^{+} axons (arrows) are also pointed; scale bar: 100 µm. R: rostral; C: caudal; D: dorsal; V: ventral; Dashed lines label the border of the glial scar. ***E.*** Quantification of the number of CT-B^{+}/YFP^{+} dorsal column fibers that are able to enter in the glial scar (n= 4 animals/per condition). All error bars are SEM.

Besides the assessment of central axon regeneration ability, peripheral regenerative capacity was also addressed in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice. For that, a unilateral sciatic nerve crush, the most commonly studied nerve injury paradigm that partially recapitulates the embryonic processes of axon elongation, was performed in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice one week prior to sacrifice. We observed that all sciatic nerve fibers from slickH mice express YFP (fig. 11A) meaning that, in Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ sciatic nerves, all fibers virtually lack Pfn1. Although the density of myelinated fibers of Pfn1 KO animals, one week upon injury, was almost half of that observed in WT animals, the differences were not statistical (fig. 11B, C). Given the high variability between animals from each experimental group, this experiment should be repeated in the future to further demonstrate that Pfn1 deletion impairs axonal regeneration in the sciatic nerve. Nevertheless, these data further reinforce that the absence of Pfn1 interferes with axonal regeneration. In summary, Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice exhibit signs of defective regeneration both the in central and peripheral nervous system.

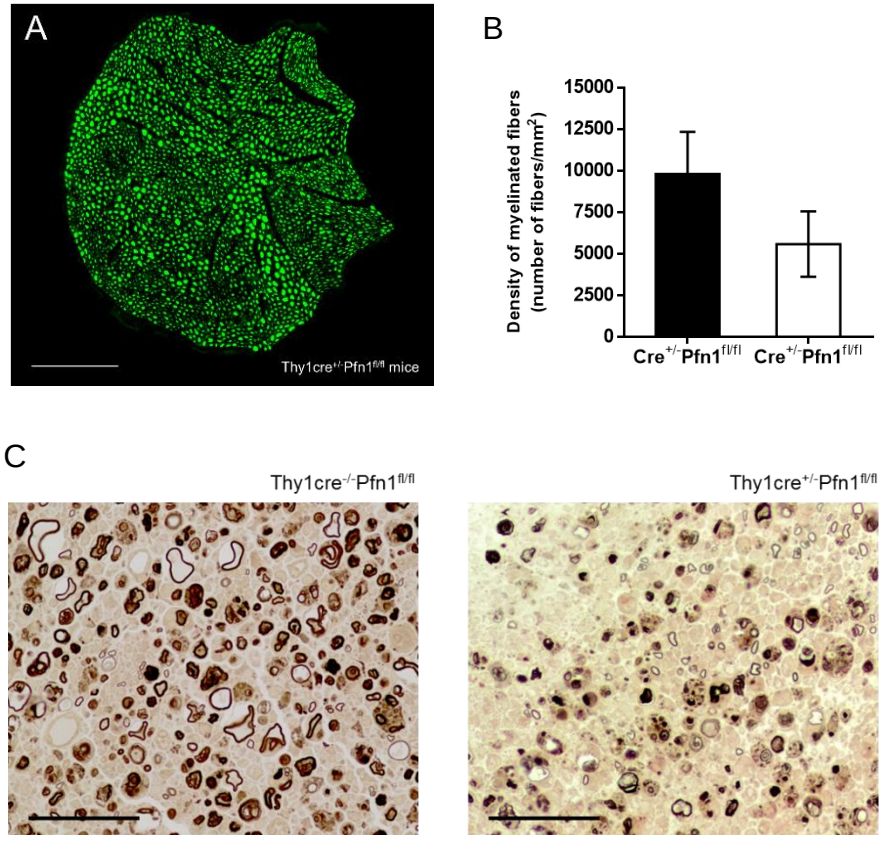


Fig. 11 – **Impact of Pfn1 deletion in axon regeneration in sciatic nerve of Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice.** ***A.*** Representative image of a sciatic nerve from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ mice expressing YFP all over the nerve; scale bar: 100 µm. ***B.*** Quantification of myelinated axon density in sciatic nerves from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice. Error bars are SEM. ***C.*** Representative images of sciatic nerves from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ and Thy1cre$^{-/-}$Pfn1$^{fl/fl}$ mice following sciatic nerve crush; scale bar: 50 µm.

Part II – Dissecting the role of Pfn1 in axon formation and NMJ maintenance using the DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mouse model

Given that neuritogenesis during development requires actin turnover and that Pfn participates in actin dynamics, we developed the DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice expressing cre recombinase under the control of the doublecortin promoter. Doublecortin is a marker protein of migrating neuronal precursor cells meaning that, in this model, Pfn1 deletion is expected to occur in neurons early in development in such a way that the axon-dendrite polarity might be affected. In this model, Pfn1 does not impair a gross normal brain development (data not shown) as previously demonstrated using nestin-cre promoter [98]. This animal tool is however important to evaluate synapse formation in the PNS, i.e. to evaluate NMJ formation and maintenance. In these animals, we verified Pfn1 deletion by genomic PCR (fig. 12A) and cre recombinase expression by immunohistochemistry in DRG tissue (fig. 12B, C). Further validation of our model should be done at younger ages by quantifying the percentage of cre^{+} neurons and by showing Pfn1 deletion and evaluating possible Pfn2 compensation in several tissues, including DRG and spinal cord.

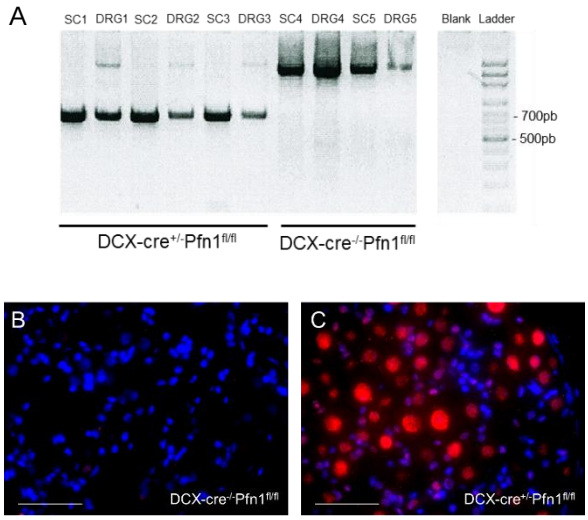


Fig. 12 – **Validation of DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mouse model.** ***A.*** Genomic PCR showing Pfn1 deletion (700pb band) in spinal cord (SC) and DRG from DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ (mice number 1, 2 and 3) DCX-cre$^{-/-}$Pfn1$^{fl/fl}$ (mice number 4 and 5). The blank and the ladder are shown on the left. ***B-C.*** Representative forty times magnification images of cre recombinase immunohistochemistry in DRGs from 6-weeks-old DCX-cre$^{-/-}$Pfn1$^{fl/fl}$ (***A***) and DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ (***B***) mice, confirming the expression of cre recombinase; scale bar: 50 µm.

Reports relating mutations in Pfn1 to familial ALS have been recently published [107]. Such ALS-linked mutations may contribute at least in part for ALS pathogenicity by inhibiting axon dynamics. However, the biological function of Pfn1 in motor neurons, that could underlie the development of ALS, remains to be elucidated. Some reports claim that

the first pathogenic event in this disease is the destruction of the NMJ, more specifically of the postsynaptic apparatus, followed by axonal degeneration and late-onset degeneration of motor neuron cell body [116]. Therefore, given the importance of actin dynamics in the leading edge of the axon and the fact that Pfn1 mutations are related to ALS, we evaluated the establishment and maintenance of NMJs in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice model. For that, acetylcholine receptor (AChR) clusters at the motor endplate of diaphragm muscle in post-natal day (P) 15 and 7-months-old animals were fluorescent-labeled with the postsynaptic marker bungarotoxin allowing both the morphometric characterization and the assessment of the innervation pattern. For the morphology assessment, we performed a detailed analysis of NMJs in 4 different regions of the right hemi-diaphragm (fig. 13A). Analysis of the shape and size of individual AChR clusters did not reveal differences between P15 DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ animals and control mice (fig. 13B-D). This analysis included the evaluation of several parameters such as the fragmentation level, volume, surface and length of the NMJs. In respect to the innervation pattern, the absence of Pfn1 did not reflect alterations either in the percentage of innervated area or in the number of NMJs per evaluated area (fig. 14). However, as a phenotype may only arise with age, the evaluation of NMJs degeneration was also performed at later timepoints, specifically at 7 months of age. Older animals did not show abnormalities in AChR distribution (fig. 14). An in depth analysis of NMJ morphology should now be performed.

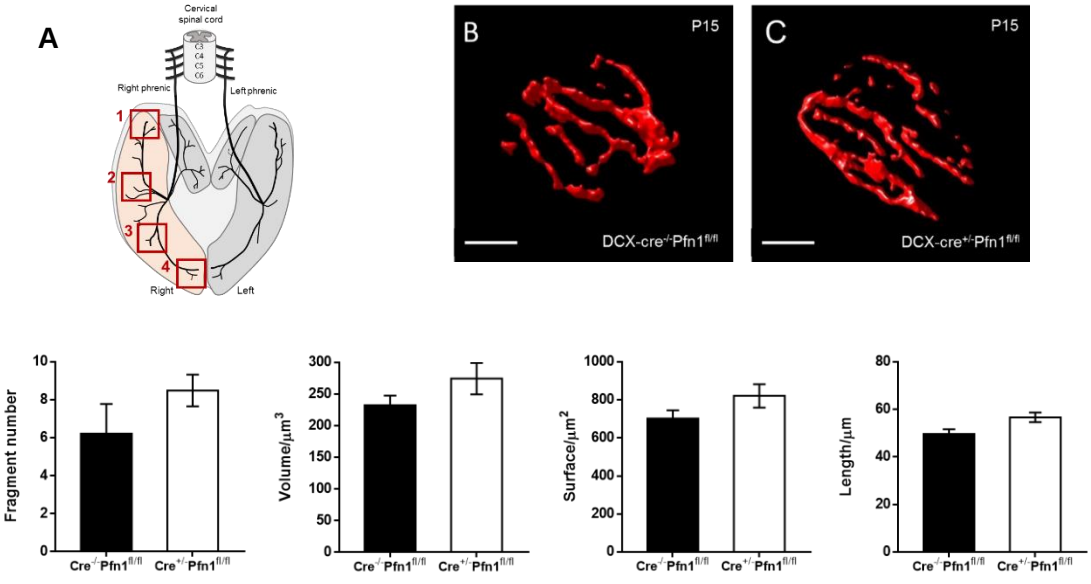


Fig. 13 – Morphometric evaluation of neuromuscular junctions at P15 in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice. ***A.*** Schematic representation showing the right hemi-diaphragm and the regions analysed (boxes 1-4). Representative 3D reconstructed images of NMJs from either DCX-cre$^{-/-}$Pfn1$^{fl/fl}$ (***B***) or DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ (***C***) mice; scale bar: 5 µm ***D.*** Morphometric quantification of NMJs at P15 in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ animals including the assessment of the fragmentation level, volume, surface and length. All error bars are SEM.

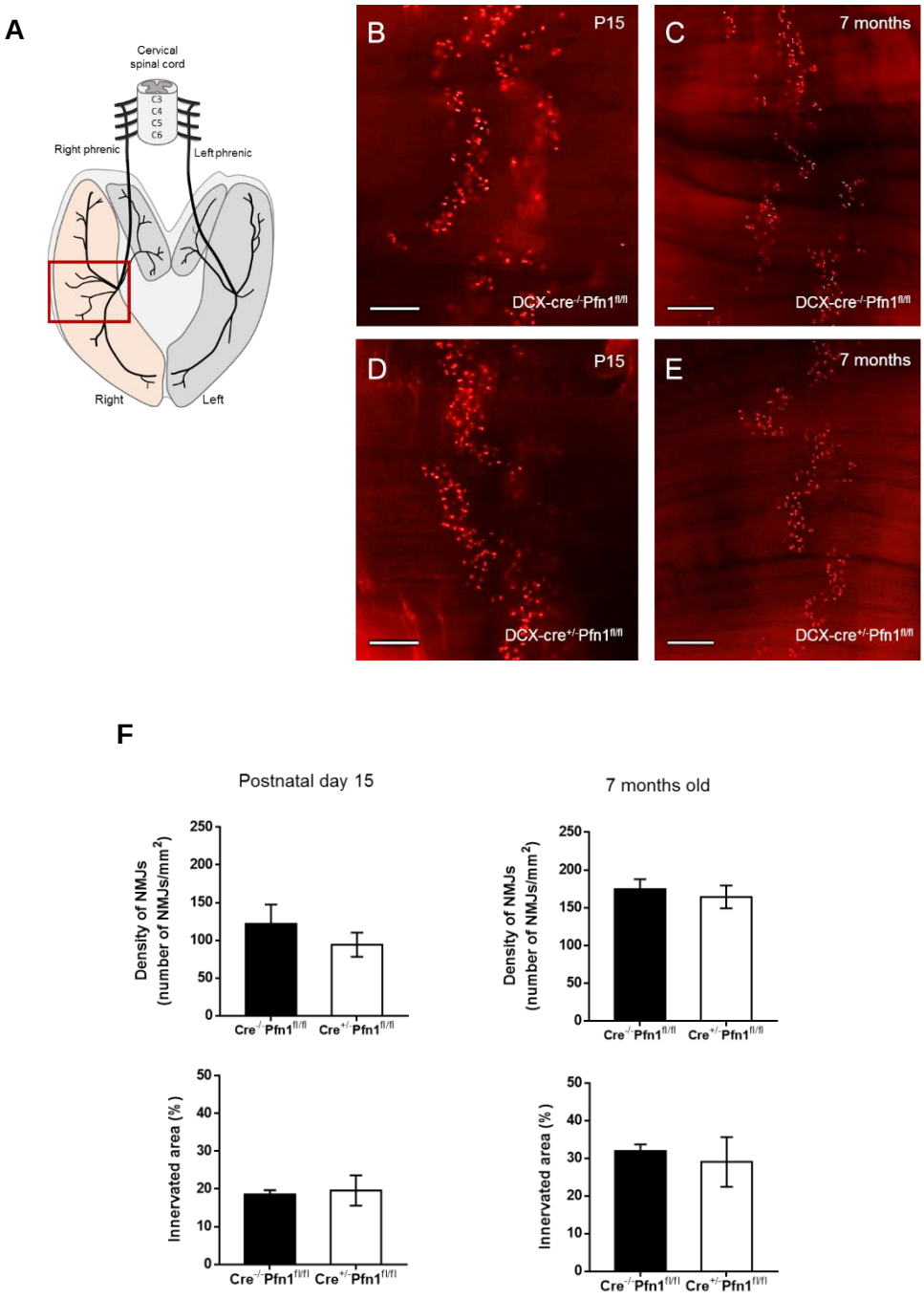


Fig. 14 – Assessment of the innervation pattern in diaphragm muscle at P15 and 7-months-old in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice. ***A.*** Schematic representation showing the right hemi-diaphragm and the region analysed (red box). Representative images of acetylcholine receptor clusters at the motor endplate of diaphragm muscle stained with bungarotoxin at P15 (***B, D***) and 7-months-old in DCX-cre$^{-/-}$Pfn1$^{fl/fl}$ and DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ (***C, E***) animals; scale bar: 50 µm. ***F.*** Quantification of the number of NMJs per evaluated area and the percentage of innervated area in diaphragm muscle at P15 and 7-months-old in DCX-cre$^{-/-}$Pfn1$^{fl/fl}$ and DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice. All error bars are SEM.

Part III – Dissecting the molecular mechanisms of Pfn1 action

The third aim of this project intended to evaluate molecularly the action of Pfn1 and how the disruption of one or more ligand interactions affects axonal formation and growth. For that, we generated different Pfn1 mutants that target either actin- ($Pfn1^{R74E}$), PLP- ($Pfn1^{H133S}$) binding domains or regulatory regions of the protein ($Pfn1^{S137A}$ and $Pfn1^{S137D}$). These mutants were overexpressed in primary hippocampal neurons and the neurite outgrowth was quantified (fig. 15). The relative expression of WT and that of mutant forms of Pfn1 was similar in all cases (fig. 15B). Only the phospho-resistant version of Pfn1 ($Pfn1^{S137A}$) i.e., the constitutively active form of the protein, increased specifically axon length in a robust fashion (fig. 15C-F). Since Pfn1 is negatively regulated by phosphorylation by ROCK [29], that impairs its actin binding activity, this result goes along our initial hypothesis that active Pfn1 is required for effective axon growth. A phosphomimetic Pfn1 mutant ($Pfn1^{S137D}$) was without effect which probably results from its inability to fully mimic phosphorylation. In contrast, mutations in actin ($Pfn1^{R74E}$) or PLP ($Pfn1^{H133S}$) binding domains on Pfn1 as well as WT Pfn1 did not alter neuronal extension capacity when overexpressed possible suggesting that these mutations are unable to completely block ligand binding. These data emphasize the importance of Pfn1 activity, specifically modulated by phosphorylation, in actin dynamics and axonal elongation context. Future experiments using the $Pfn1^{S137A}$ mutant *in vivo*, by transduction of DRG neurons should be performed to further demonstrate its capacity to enhance axon regeneration after injury.

In addition to its role as an actin binder, Pfn1 interacts with phosphatidylinositol lipids, with great preference for PtdIns(4, 5)P_2 pointing to a role of Pfn1 in modulation of signaling cascades including the PI3K/AKT pathway. In order to address whether this cytoskeleton-related protein may interfere with intracellular signaling, WT Pfn1 was overexpressed in CAD cells and the levels of several signaling molecules were evaluated 48 hours later (fig. 16). Increased levels of Pfn1 were detected after transfection (fig. 16A). It is however noteworthy that the levels of inactive (phosphorylated) form of the protein were also increased after overexpression which may introduce some complexicity in the evaluation of the subsequent results. We consistently observe a 2.5-fold increase in AKT phosphorylation at serine 473 and a similar trend (not statistic) at threonine 308 (fig. 16B). In accordance to a non-significant increase of Thr308 phosphorylation, active phosphorylated PDK1, the kinase responsible for Thr308-AKT phosphorylation, was also not affected (fig. 16C). No clear variation was detected in either phosphorylated (inactive) or total form of PTEN (fig. 16D). Interestingly, Pfn1 overexpression lead to a 2-fold increase in GSK3β phosphorylation at serine 9 (fig. 16E). Of note, AKT-mediated phosphorylation at

serine 9 negatively regulates GSK3 activity. Phosphorylation at tyrosine 216, which positively modulates GSK3, was not altered. In summary, overexpression of Pfn1 lead to activation of AKT which, in turn, seems to be capable of inactivating GSK3β via phosphorylation of Ser-9. In conclusion, these results support the notion that Pfn1 is capable of modulating intracellular signaling cascades (fig. 17).

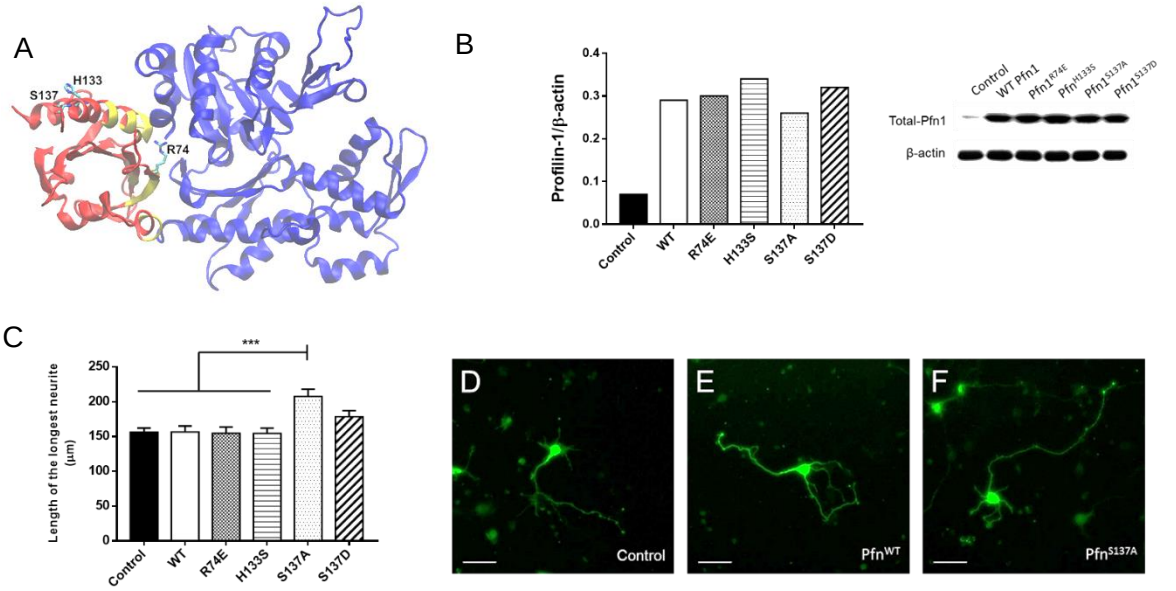
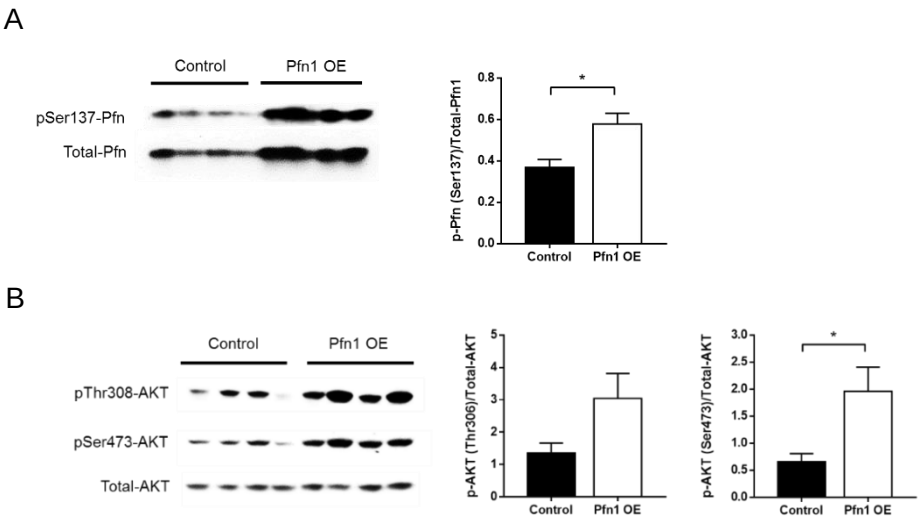


Fig. 15 – **Overexpression of a phospho-resistant mutant of Pfn1, the constitutively active form of the protein, increases axon growth.** ***A.*** Mutated resides on Pfn (red) bound to actin (blue); actin binding domain is shown in yellow. ***B.*** Western blot showing the relative expression levels of WT Pfn1 or Pfn1 mutants in CAD cells. ***C.*** Neurite outgrowth of GFP^{+}/$\beta III\text{-tubulin}^{+}$ hippocampal neurons following co-transfection with plasmids that code for GFP and for WT or mutant Pfn1. One-way ANOVA, Tukey's multiple comparisons test, $p < 0.001$ (***). Error bars are SEM. ***D – E.*** Representative images of ***C***, specifically neurons co-transfected with GFP (***D***), WT Pfn1 (***E***) and $Pfn1^{S137A}$ (***F***) plasmids; scale bar: 50 μm.



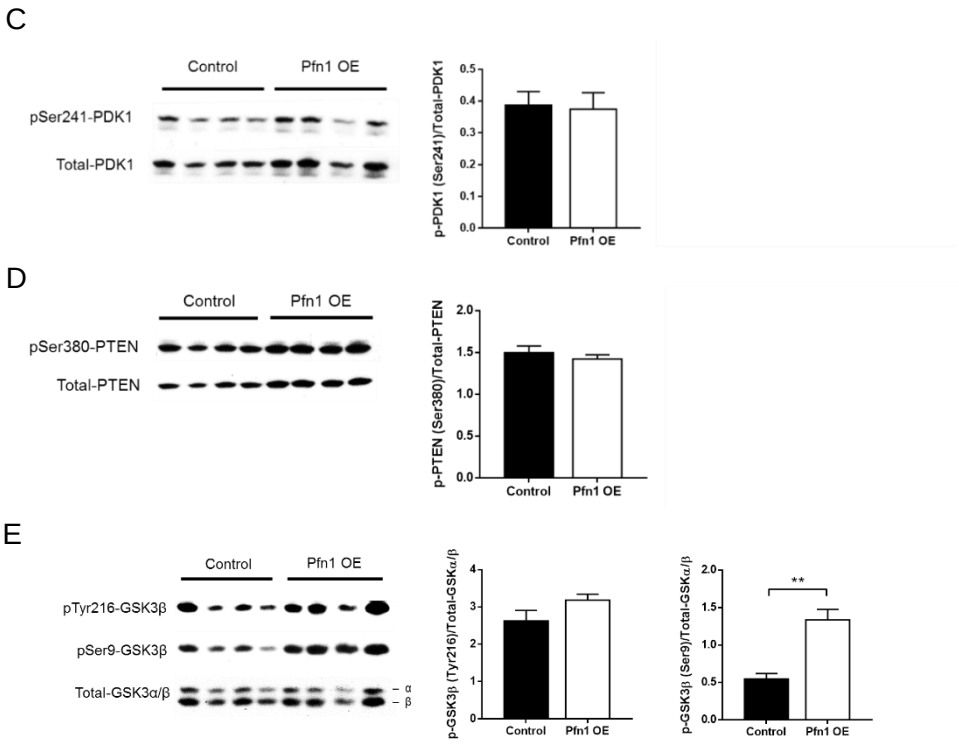


Fig. 16 – **Pfn1 is capable of modulating microtubule-associated signaling pathways.** Western blots presented on the left showing the relative expression levels of pSer137-Pfn1 and total-Pfn1 (***A***), pSer473-AKT, pThr308-AKT and total-AKT (***B***), pSer421-PDK1 and total-PDK1 (***C***), pSer380-PTEN and total-PTEN (***D)*** pTyr216-GSK3β, pSer9-GSK3β and total-GSK3α/β (***E)***, in CAD cells 48 hours upon Pfn1 overexpression. Quantification is shown on the right. Two-tailed Student's t test with $p < 0.05$ (*) and $p < 0.01$ (**). All error bars are SEM.

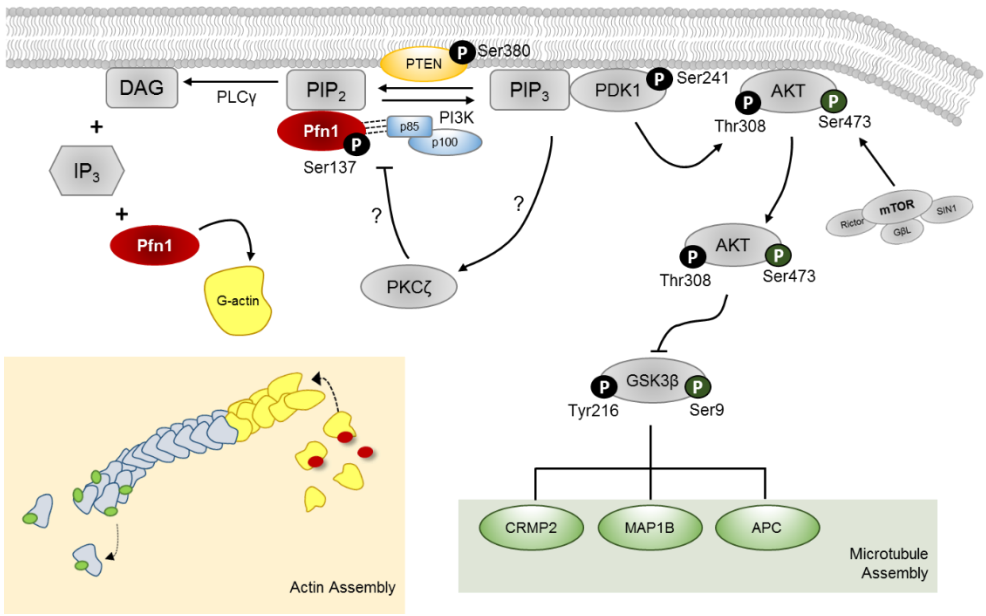


Fig. 17 – **Pfn1 is capable of modulating intracellular signaling cascades.**

Discussion

Part I – Dissecting the role of Pfn1 in axon regeneration

The inability of CNS axons to re-establish functional connections is a major obstacle in the treatment of CNS injury. Current knowledge supports the view that even counteracting or removing the extracellular inhibitory molecules, limited axon regeneration is observed meaning that a better understanding of cell-intrinsic mechanisms that underlie axonal regrowth is needed. It is known that axon growth is driven by the movement of the growth cone where specific cytoskeleton changes are crucial for regenerative capability. Although microtubules are important for the maintenance of the elongated axon and the speed of growth, it is accepted that actin dynamics plays a major role in generating the force required for axon extension. As such, interventions aimed at promoting axonal regeneration may converge on actin cytoskeleton manipulation. Although actin is well recognized as a key player in axonal growth, how different actin-binding proteins control its dynamics is still not fully elucidated. Previous data from our lab indicate that the actin-binding protein Pfn1 is 4.5-fold increased in regenerating axons upon a conditioning lesion and that its inactive phosphorylated form is decreased at the site of the lesion. Moreover, although Pfn1 levels upon CL are decreased in dorsal root ganglia, immunoprecipitation assays revealed the interaction of Pfn1 with the transport machinery occuring in conditioned animals which points to Pfn1 transport to the central branch where it would enhance the regenerative process. These evidence together with preliminary *in vitro* data emphasize the importance of the modulation of actin dynamics by Pfn1 to achieve proper axonal growth. In fact, actin dynamics is critically dependent on Pfn activity as profilins provide the pool of ATP-actin monomers that can be added to free actin ends to support their polymerization and growth. As such, this project aimed at dissecting the involvement of Pfn1 in axonal formation, growth and regeneration.

To unravel the importance of Pfn1 during axonal regeneration, we generated mice with an inducible neuronal deletion of Pfn1 using cre-lox technology. Naïve cultured DRG neurons from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$, lacking Pfn1, did not show defects in neurite outgrowth while, as depicted previously, acute depletion of Pfn1 by ShRNA in WT DRG neurons results in a significant decrease in axonal extension (fig. 5). Compensatory effects by Pfn2 could explain why chronical depletion of Pfn1 is not powerful enough to limit axonal growth. However, upon a conditioning lesion that recapitulates embryonic elongation capacity, Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRG neurons are sensitive to the lack of Pfn1, presenting a mild decrease of axon elongation capacity when compared to WT neurons. Additionally, this work revealed

that WT neurons are able to grow in the absence of Pfn2 but when such ablation is performed in chronically depleted Pfn1 KO neurons the outgrowth is severely affected. Growth cones from Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ DRG neurons also present an altered actin dynamics. Since microtubules extend in bundles out of the neuronal sphere to form the backbone of neurite, a theoretical F-actin disorganization caused by the lack of Pfn1 would preclude neuritogenesis. In summary, this apparent phenotype gave rise to the idea that Pfn1 might play an essential role in actin turnover at the leading edge of growth cones, a process that underlies neurite regrowth and extension capacity following injury. Moreover, the impact of Pfn1 function in actin dynamics at the growth cone could ultimately be correlated with the emergence of neurites out of the neuronal sphere. Accordingly, reports already demonstrated that ADF/cofilin, another major regulator of actin turnover that promotes F-actin depolymerization, are vital for actin organization and retrograde flow driving neurite formation [117].

In fact, *in vivo*, peripheral and central Pfn1 KO axons tend to display a decreased ability to regenerate upon injury. Therefore, Pfn1 emerges as a potential determinant for the axonal regeneration competence. Exploring the mechanisms by which Pfn1 acts in neurons upon injury would enable the design of strategies aiming at controlling the speed and trajectory of regenerating axons. Thus, these experiments will ultimately set the foundations for drug screens in order to unveil modulators of Pfn1 activity with therapeutic potential for SCI treatment.

Part II – Dissecting the role of Pfn1 in axon formation and NMJ maintenance using the DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice model

In this work, we also developed another mouse model, the DCX-cre$^{+/-}$Pfn1$^{fl/fl}$, which in the future will enable us to assess the importance of Pfn1 during axon formation and degeneration. In DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice, as Pfn1 is deleted in neurons early in development, it was expected that the neuronal cytoskeleton would be defective. Preliminary data in hippocampal neurons showed that after acute knockdown of Pfn1 by ShRNA axon formation and growth is impaired which points to a role of Pfn in the initial steps of neurite formation. In this model however neuronal deletion of Pfn1 during development does not impair a gross normal brain formation, as Kullmann and colleagues previously demonstrated using nestin-cre promoter [98], which suggests a possible Pfn2 compensation.

Mutations in the actin-binding protein Pfn1 have been recently linked to familial cases of ALS [107]. In fact, several studies have highlighted the involvement of cytoskeleton in ALS pathogenesis since the early signs of neuropathology in presymptomatic mouse

spinal cord and sciatic nerve are accompanied by alterations in the expression of cytoskeleton-related genes [118, 119]. Moreover, crystal structure of Pfn1-actin complex revealed that all ALS-linked mutations lied in close proximity to actin-binding residues of Pfn1 [107]. These observations clearly document that cytoskeletal pathway alterations contribute to ALS pathogenesis and that Pfn1 is central for the cytoskeleton dynamics required for normal axon growth. However, the biological function of Pfn1 in neurons, that could underlie the development of this disorder, and the mechanism by which Pfn1 acts in the axon remain obscure. Undoubtedly, such reports indicate that alterations in a gene that regulates actin cytoskeleton dynamics can affect motor neuron integrity. Indeed, because of their highly polarized axons, motor neurons might be particularly susceptible to disturbance in proper cytoskeleton function. In fact, distal cytoskeleton impairments in ALS might account for axonal swellings, composed of neurofilaments and altered neuronal organelles [120], aggregation of proteins, mitochondrial defects and dysfunctional axonal transport. Curiously, defects in Pfn1 have been shown to cause growth cone arrest and reduced axon outgrowth in embryonic motor neurons of Drosophila [121].

Remarkably, early pathogenic events of ALS are characterized by axonal retraction and motor neuron disconnection from neuromuscular joints [116, 122]. Taking into consideration that the dynamic actin cytoskeleton is the driving force behind neurite growth and axon pathfinding and that Pfn1 mutations are correlated with familial cases of ALS, we raised the hypothesis that the neuronal absence of Pfn1 impairs either the formation or the maintenance of specialized synapses as NMJs.

The NMJ is a tripartite structure composed of the skeletal muscle fiber, the motor neuron terminal and the non-myelinating terminal Schwann cells which collectively allow the neurotransmitter release and the generation action potentials across the muscle's surface, ultimately causing the muscle to contract. Regarding the already reported NMJ destruction in presymptomatic ages of ALS mouse model [122], we went to explore if a similar pathological denervation occurs in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice. However, in this work, deleting Pfn1 early in mouse development did not interfere with the proper formation or, even at the later timepoints, with the maintenance of NMJs. In the future, detailed histological and morphometric evaluation of NMJs in the diaphragm and other muscles from adult DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice is needed to further clarify if such deletion affects the shape or size of the NMJs. Additionally, it would be interesting to monitor the development of ALS-like phenotypes in DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ animals at multiple ages assessing, for example, the motor performance, muscle strength or gait abnormalities. Curiously, studies have showed in well-established ALS mouse models that, at postnatal day 30, NMJ innervation is reduced by 40% [123] which is accompanied by muscle weakness. Moreover, signs of axonopathy

and axon degeneration should be explored in aged DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ animals as well as the regenerative ability of Pfn1-deficient axons promoting re-innervation of target muscles.

Nevertheless, it is necessary to keep in mind that the mechanisms that underlie neurodegeneration in ALS seem multifactorial taking place both in neurons and non-neuronal cells. In fact, although pathological features at the NMJs can occur due to intrinsic factors within the motor neuron, recent evidence indicates that changes occurring in the skeletal muscle or in the terminal Schwann cells may also culminate in NMJ destruction. Specifically, axon guidance molecules such as semaphorin 3A [124] secreted by specific terminal Schwann cells, Nogo-A [125], expressed in muscle fibers and EphrinA4 expressed by motor neurons are upregulated in ALS mice contributing for the destabilization of NMJ integrity which might lead to retraction of the nerve terminal and consequently to a selective denervation. Moreover, the expression of trophic factors, which declines over the course of the disease, might preserve the NMJ by modifying the cytoskeleton of the motor neuron to a highly active state, affecting the course of motor neuron degeneration and NMJ denervation [126].

Future characterization of DCX-cre$^{+/-}$Pfn1$^{fl/fl}$ mice will provide new insights on understanding the role of cytoskeletal abnormalities in this neurodegenerative disorder. The body of knowledge generated may contribute to a better understanding of Pfn1-related ALS etiology and may thus set the foundations for drug screenings aimed at unraveling therapeutic modulators of Pfn1 activity.

Part III – Dissecting the molecular mechanisms of Pfn1 action

This work also aimed at unravelling the molecular mechanisms which underlie Pfn1 function during axonal formation and growth. As referred above, preliminary data showed that inactive phosphorylated Pfn1 levels are decreased following CL which indicates that probably the phosphorylation status of Pfn1 is relevant for axonal regeneration and elongation upon injury (fig. 5). Hence, determining the importance of different binding domains and regulatory regions of Pfn1 would provide a useful knowledge to unveil whether this cytoskeleton-related protein acts intracellularly promoting actin dynamics, axon growth, NMJ formation and cell signaling.

It is known that profilins bind to actin and PLP stretches using distinct domains and that phosphorylation at Ser-137 inhibits actin polymerization as well as interaction of Pfn with PLP-containing proteins [81, 127]. However, which regulatory regions of Pfn1 modulates its activity remains to be elucidated. In this work, the overexpression level of WT Pfn1 in hippocampal neurons did not increase the extent of axon growth over the growth that results from endogenous Pfn1. Probably, higher concentrations of Pfn1 could bind to

actin filaments counteracting the association of new actin monomers [128]. Curiously, high-level overexpression of WT Pfn1 in PC12 [85] and NIE-115 cells [129] does not significantly change the rate of neurite extension while low levels of Pfn1 originate more filapodia. Overexpression of WT Pfn1 in CAD cells also increases filapodia and F-actin-rich spots in the soma [85]. Additionally, hippocampal neurons that overexpress the phospho-resistant mutant of Pfn1 ($Pfn1^{S137A}$), constitutively active form of the protein, were able to extend efficiently longer axons. Indeed it could be hypothesized that, by blocking phosphorylation susceptibility, increased amount of active Pfn1 would be in close contact either with G-actin, enhancing ADP/ATP exchanges, or with actin nucleators containing PLP stretches such as Arp2/3 complex or formins. In our setup, Pfn1 with defective actin ($Pfn1^{R74E}$) and PLP ($Pfn1^{H133S}$) binding domains did not promote changes in the axonal elongation capability of hippocampal neurons. In contrast, in PC12 cells, by transfection of the mutant $Pfn1^{R74E}$, decreased neurite length as well as filapodia formation was observed [85]. The reason underlying the lack of effect of this mutant in hippocampal neurons needs to be explored. Collectively, these evidence further support the idea that phosphorylation of Pfn1 negatively modulates axonal growth probably by barring actin cycle. Complementary analysis should be performed in neurons transfected with constitutively active phospho-resistant Pfn1 mutant in order to verify changes in actin dynamics that could affect axon growth capacity. If Pfn1 phosphorylation actually precludes axon growth, it would be interesting to perform viral delivery of the phospho-resistant mutant Pfn1 to DRG neurons in animals that underwent spinal cord hemisection followed by the evaluation of regeneration of dorsal column tract axons. If by increasing the active form of Pfn1 truly leads to increased axonal regeneration following spinal cord injury, the modulation of Pfn1 activity may be a target of future therapies to enhance axon regeneration. Given that Pfn1 mutations are related with ALS and that defects in cytoskeleton dynamics might result in the destruction of specialized synapses as NMJs, it would also be interesting to overexpress the phospho-resistant mutant of Pfn1 in Pfn1 KO motor neurons co-cultured with glial cells and myoblasts using microfluidic chambers, and evaluate the impact of the phosphorylation of Pfn1 in the formation of AChR clusters [130].

Pfn1 also links signaling pathways to actin cytoskeleton organization by binding to phosphoinositides, with preference for PtdIns(4, 5)P_2. The interaction of Pfn with PtdIns(4, 5)P_2 is based on the binding of the negatively charged headgroup of the phosphoinositides to basic amino acids and thus the more basic mammalian Pfn1 isoform has more affinity for PtdIns(4, 5)P_2 than Pfn2 [131]. Specifically, Pfn1 binding to PtdIns(4, 5)P_2 precludes complex formation with actin and PLP-containing proteins due to overlapping binding sites [70]. On the other hand, it may compromise PtdIns(4, 5)P_2 membrane availability and thus

has the potential to interfere with intracellular signaling cascades, including pathways that are central for axon growth and survival such as PI3K/AKT/GSK3β and PI3K/PTEN/AKT/mTOR pathways, as recently reviewed [33]. In this work, overexpression of WT Pfn1 in a neuronal cell line lead to a significant increased phosphorylation of AKT only within the carboxy terminus at Ser473. This may represent a partial activation of AKT since this kinase also needs to be phosphorylated at Thr308 to achieve its complete activation. Pioneering work already proposed a model in which PI3K phosphorylates Pfn-bound PtdIns(4, 5)P_2 resulting in formation of PtdIns(3, 4, 5)P_3 which, in turn, activates PKC-ζ capable of phosphorylating Pfn1 [132]. Thus, Pfn phosphorylation is thought to promote dissociation from either regulatory subunit of PI3K or PtdIns(4, 5)P_2, acting as an autoregulatory mechanism. Indeed, present data show an increased phosphorylation of Pfn1 although active PDK1 levels that, jointly with PtdIns(4, 5)P_2 or PtdIns(3, 4, 5)P_3 can activate PKC-ζ via phosphorylation, are not affected when Pfn1 is overexpressed.

The apparent AKT activation is enough to strongly inactivate GSK3 by phosphorylating GSK3β at Ser-9. This evidence gives rise to the idea that higher levels of Pfn1 would interfere with microtubule-related pathways in a way that is thought to increase axon specification and growth. In fact, current knowledge suggests that during neuronal polarization, local accumulation of PtdIns(3, 4, 5)P_3 and activation of PI3K-AKT pathway at the tip of the nascent axon leads to the downstream inactivation of GSK3β by inducing Ser9 phosphorylation [133]. Local inhibition of GSK3 signaling at the growth cone enhances microtubule assembly and consequently axonal outgrowth. Once inhibited, GSK3 cannot phosphorylate (inactivate) collapsin response mediator protein 2 (CRMP2) and adenomatous polyposis coli protein (APC) which remain active and bind microtubules, thereby promoting microtubule polymerization and stability at distal ends of growing axons. These results support the notion that Pfn1 by extensively docking to the membrane via PtdIns(4, 5)P_2 binding may locally modulate PI3K/AKT signaling pathway and downstream GSK3β activity. Decreased GSK3 activity would allow microtubules dynamics in such a way that axonal growth is promoted. Hence, it could be speculated that an actin-binding protein might affect microtubule remodelling creating a novel linking bridge between actin and microtubule cytoskeleton.

Fascinatingly, there are evidences from the cancer field that confirm that PI3K/AKT pathway is sensitive to Pfn1 perturbations. In such studies it was observed that Pfn1 overexpression alters membrane recruitment of AKT and consequently its activation by upregulating PTEN levels [134]. In fact, seemingly contrary to the conventional model of the promigratory function of Pfn1, Pfn1 is downregulated in several types of adenocarcinoma as breast, pancreatic and hepatic. Pfn1 is actually considered to be a tumor-supressor by

counteracting aberrant activation of AKT pathway seen in malignant phenotypes through its phosphoinositides lipids binding domain. On the other hand, it is also described that Pfn1 negatively modulates PtdIns(4, 5)P_2 membrane availability with subsequent reduction in targeting of the PtdIns(4, 5)P_2-binding protein lamellipodin to the leading edge [87] which recruits Ena/VASP to the membrane in order to promote the assembly of actin filaments [135]. In addition to the physiological importance of Pfn1/PtdIns(4, 5)P_2 interaction, studies also reveal that a functional actin binding domain is required to reduce the tumerogenicity of cancer cells [136].

The Pfn/PtdIns(4, 5)P_2 interaction has been considered to be of major importance in the regulation of early neuritogenesis and models for such regulation have been proposed [29, 85]. It is hypothesized that an external stimuli activates phospholipase Cγ1 (PLCγ1) causing PtdIns(4, 5)P_2 hydrolysis into second messengers and disrupting Pfn/PtdIns(4, 5)P_2 interaction. Hence, Pfn1 is released from membrane to the cytosol where it can interact with actin and other ligands. Hydrolysis of PtdIns(4, 5)P_2 also facilitates neurite sprouting by dropping local membrane tension. Moreover, Rac and PI3K-depedent inhibition of RhoA decreases Pfn phosphorylation by ROCK supporting Pfn-mediated actin dynamics and subsequently the neurite formation and growth. In this sense, it could be pointed that PtdIns(4, 5)P_2 levels may change Pfn function downstream of ROCK during neuritogenesis.

Final remarks and future work

This project aimed at dissecting the role of Pfn1 in neurons. The body of knowledge gathered supports the notion that actin cytoskeleton dynamics in the growth cone is the engine needed to generate the force required for axon extension, a process that underlies the establishment of neuronal polarity and the regeneration following injury. Given its relevance, promising therapeutic approaches rely on modulators of this mechanism.

Importantly, by creating KO mouse models, this project emphasizes the importance of Pfn1 as a regeneration enhancer both in CNS and PNS context. Specifically, modulation of Pfn1 activity, namely by phosphorylation, is crucial for the actin treadmilling process at the growth cone level which in turn prompts neurons to a regenerative status. Moreover, this work highlights the physiological relevance of Pfn1 in cell signaling besides its role as a cytoskeleton modulator. Pfn1 is capable of regulating microtubule-regulated pathways in such a way that axonal growth is enhanced. In the future, it would be interesting to dissect in detail how modulators of Pfn1 activity, jointly with others therapeutic approaches, might promote a functional recovery following spinal cord injury. As such, this research will bridge scientific knowledge with its applicability to enhance therapeutic strategies for CNS regeneration.

Besides the potential impact in future strategies for SCI treatment, understanding the mechanisms that underlie modulation of Pfn1 activity would contribute to dissect several aspects of ALS pathogenesis as mutations on Pfn1 were recently linked to this neurodegenerative disease. As such, long-term goal of this work is to understand the biology of Pfn1 in motor neurons such that the knowledge generated may be exploited in future therapeutic applications.

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