Microtubule-severing proteins and their role in the development and degeneration of the central nervous system

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DEDICATION

My entire work would simply not possible without the continuous love and support that my parents gave and will always give to me unconditionally and continuously.

The blessing of my great and only love of my life Carlos guided me through this windy and difficult journey, relieving all the sacrifices that I had to make and I will keep making in name of science.

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ABSTRACT

Neuronal cells are among the most morphologically elaborate cells in the human body. The creation, maintenance and modification of such high complexity are dependent on the organization of neuronal cytoskeleton elements. Microtubules play an essential role in this regard, thanks to their ability to form a resistant and yet plastic cytoskeleton. The acquisition of a more rigid or a more dynamic configuration depends upon the coordination of a large number of regulatory proteins. The present work is on a poorly characterized but translationally promising family of such regulatory proteins called microtubule-severing proteins. The work focuses on two such proteins in two different scenarios: fidgetin in neurodevelopment and spastin in neurodegeneration.

Fidgetin was found to play a unique role in neurodevelopment by targeting the dynamic domain of the axonal microtubule as opposed to its stable domain, with fidgetin thus behaving as a microtubule plasticity suppressor. Its inhibition was found to boost the dynamic microtubule mass of the axon, thereby enhancing neuroplastic characteristics such as axon growth and the number of processes extended by an individual neuron. This profound effect on dynamic microtubules has interesting translational implications in neuropathology: from treating the injured central nervous system to better understanding neurodevelopmental disorders such as autism.

On the other side of the spectrum, there is spastin, mutations of which cause a selective slow degeneration of first order neurons from the cortico-spinal tracts. The prevailing idea of this neurodegeneration is that neurotoxic effect of mutated spastin occurs through a loss-of-function mechanism. The present study challenges this concept by demonstrating the existence of an alternative gain-of-function mechanism of action. The data suggest that mutated spastin could induce neurodegeneration by aberrantly activating kinases, in particular casein kinase II, which in turn, compromises vital neuronal processes such as intracellular transport. Continuation of the work will open the door to a completely new translational paradigm to find an efficient treatment to this incurable condition.
BACKGROUND AND LITERATURE SURVEY

Under the functional point of view, the nervous system can be considered as an exquisitely complex network in which information continuously circulates and modifies, allowing its rapid adaptation to the ever-changing external environment. This network is composed by the organized, selective connectivity between the functional elements, the neurons, whose function is not only to transmit signals from one point to another, but also to integrate, enrich or selectively remove information that will ultimate result in a change of a functionality of the target recipient cells [1]. The response that the nervous system can generate in correspondence to a specific stimulus can be very complex. This requires the activation of a selected subset of neurons in a very narrow, and at the same time, very precise spatial–temporal window. In order to express this complex function, every element of this network must respond adequately at every given time, conversely, the alteration of such function would result in pathological dysfunction of one or more process [1].

Neurons are extremely specialized cells that during the evolution perfected their capability to receive, integrate and ultimately transmit neuronal signals from one cell to another to carry growing information along the neural network [2]. In order to do this, the neuronal cells specialize different parts to a specific function [2-4]. Structures called dendrites are responsible for receiving information from other cells. At the cell body level, information is gathered and integrated, eventually to be transmitted toward the next cell target through another specialized structure called the axon. Considering the tight bond between the function and the structure, the neuronal morphology plays an essential role in the physiology of the entire nervous system. Cytoskeletal elements, such as actin filaments and microtubules play a pivotal role to keep this morphological-functional connection working [5, 6]. In this work I will particularly focus on microtubules and how the regulation of this cytoskeleton component has strong implication in the neuronal functionality during physiological processes such as neurodevelopment and during neuropathological events such as neurodegeneration.
Microtubule cytoskeleton is particularly important for the neuronal functionality because its capability to form a stable cellular structure that is resistant to physical stimulation is extremely important [7]. However, considering the intrinsic dynamic nature of microtubules, this solid, stable cytoskeleton is everything but static, it continuously goes through rearrangement, allowing the cells the possibility to easily readapt its shape whenever it is needed. These opposing properties are even more important in neurons in which the continuous environmental changes demand a plastic, malleable morphology. But the contribution of microtubules on neuronal cell physiology is far to be just restricted on structural organizer of neuronal cell shape. Microtubules participate in the neuronal functionality in several different aspects: by creating preferential tracks for intracellular transport [8, 9]; by setting up intracellular signals for specific organelle distribution [10]; by generating intracellular forces and act as signaling devices [6]. The overlap of one or more of these intracellular processes make the phenomenon such as neuronal migration possible [6, 11], neuronal polarization/differentiation [12], maintenance of neuronal connectivity and finally, rearrangement of synaptic contact with neuronal plasticity [13]. The ultimate goal of all these processes is to locate each single neuron in the nervous system in the right position and make it capable to be a functional network component, a phenomenon that simply would not be possible without the presence of a responsive microtubule cytoskeleton.

Evidences of the importance of microtubule cytoskeleton in neuronal physiology can be appreciated by taking a look on the correlation between altered microtubule regulation and neuropathology, from both neurodevelopmental disorders to neurodegenerative diseases [14-16]. Interestingly, considering the essential role of microtubule generally in the overall cell physiology (inside and outside the central nervous system), very limited number of pathologies can be directly connected to alterations occurring on the tubulin protein (so called tubulinopathies) [17, 18]. These apparent two can explain discrepancy major reasons: first the tubulin gene during the process of
speciation and evolution sustained several events of duplications, which gene product was conserved as microtubule elements. The presence of multiple functionally analogous genes makes the impact of a single gene mutation much less detrimental, and less prone to induce pathological consequences [18]. A second reason is the nature of mutation: in all the tubulinopathies the mutation causes minor changes of the tubulin structures, indicating that major alterations on tubulin function is simply incompatible with the most basic cellular processes, hence with life [18]. Many of the alterations pertaining to the microtubule cytoskeleton and neuronal functionality are instead the result of dysregulation on microtubule associated proteins (MAPs) [19-23], regulatory proteins (such as microtubule-severing proteins) or motor protein moving along microtubule and its regulators, and enzyme regulating microtubule post-translation modification (PTMs) such as acetylation [24].

**Microtubules**

Microtubules are the largest cytoskeleton structure in the cell. The basic constituents are the tubulin heterodimers, subunits composed by the constitutive association of α tubulin and β tubulin, forming a stable structural subunit [18]. The polarized assembly of these heterodimers (head to toe), forms a still polarized ultra-structural unit called a protofilament. Further assembly of protofilaments by using lateral interactions, creates a hollow, still polarized structure called microtubules. Maintenance of polarity has important biological consequence in many aspects of the microtubule physiology. The most important implication is the dynamic character of the two extremes (called tips) from each microtubule, in which the extreme subunit, α-tubulin, is much less dynamic, and for this reason called the minus tip, whereas the other extreme subunit, GTP-β tubulin, results in a more rapid turnover and for this reason is called the plus tip [25, 26]. In many cell types microtubules are specifically organized and ordered to maintain their polarity orientation coherently toward the same direction. Neurons is a classical example, where a specialized cellular compartment such as the axon has almost all the microtubules oriented with the plus-end facing the
axon tip and the minus-end facing the cell body [2, 27]. This very uniform polarization allows the fine regulation of important processes such as the unidirectional, vectorial transport of specific cellular organelles such as vesicles. Indeed, the two major molecular motor proteins responsible for organelles transport along the axon, Kinesin-1 for the anterograde transport and Dynein for the retrograde one, can perform the unidirectional transport so efficiently as a result of the homogeneous polarity of microtubule in the axon [2].

Microtubules continuously undergo to event of disassembly (called catastrophe) and re-assembly (called rescue) [28] allowing for the formation of a cytoskeleton that has a great degree of plasticity and at the same time conferring a strong structural support [7]. The fine-tuning of the polymerization versus depolymerization process can make the entire microtubule cytoskeleton more plastic or more stable. The regulation of these properties depend on the selective activation of a large series of microtubule- regulatory proteins, discussed later, generally called as microtubule- associated proteins (MAP). However, the processes of polymerization and depolymerization are directly determined by the biophysical properties of the tubulin subunits [26].

Each tubulin polypeptide can be divided functionally and structurally into three domains: the N-terminal domain, the intermediate domain and the C-terminal domain. The N-terminal domain is responsible for GTP binding, and slightly differs between α tubulin and β tubulin, where the binding site in the α tubulin is structurally more rigid, impairing the GTP hydrolysis capability. Because of this the binding site of the α subunit is called the N-site (not exchangeable site). The β-polypeptide instead has a GTP binding site more flexible, and hence competent for a fast GTP hydrolysis (E-site). The differences on this domain make a substantial difference between the strong intra-dimer interactions and the much weaker inter-dimer interactions, accounting for the disassembly of the microtubule polymer, the heterodimers remain the undividable elements. The intermediate loop is instead responsible for lateral proto-filament interactions. Finally, the C-terminal domain contains the C-terminal tail which is the hotspot for all the PTMs on microtubules,
except for acetylation. This region, conversely from the other part of the tubulin protein, is unstructured and does not participate in any tubulin-tubulin interaction on the structure. Not surprisingly, the different \( \alpha \) and \( \beta \) isoforms diverge the most on this region, indicating the possibility of a differential mechanism of regulation between the different tubulin isotypes\[29, 30\].

**Tubulin diversity I: tubulin isotypes**

A remarkable characteristic of all the tubulin isoforms across the species, is the highly conserved tridimensional structure, composed by a compact globular body and a relatively unstructured C-terminal tail. The highly conserved tridimensional structure does not correlate with a high degree of similarity in their primary sequence: indeed, across the species all the tubulins have primary sequence homology no greater than 40\%\[18, 30\]. Strikingly, within the same species several different tubulin isotypes exists and each one has subtle, but probably biological relevant differences in their primary sequence. Great part of the differences between the tubulin isotypes lay along the high variable C-terminal tail on both \( \alpha \) and \( \beta \) tubulin, indicating that this region is minimally involved in the critical regions for microtubule polymerization\[18, 30\]. Instead, that is the site where the majority of microtubule PTMs occur. In mammals, at least 9 \( \alpha \) and 9 \( \beta \) tubulin isotypes exist\[31\]. The expression of each isotype can be tissue specific examples are the \( \beta \text{III/} \beta \text{II} \) tubulin in nervous tissue where \( \beta \text{III} \) is almost exclusively neuronal\[32\], and \( \beta \text{I} \) in the marginal band on platelets\[33, 34\]) or developmentally regulated. The reason of this divergence in the primary sequence is not completely understood. In the past it has been proposed that the difference of primary sequence could reflect a difference in function from each isotype. But the discovery of the capability from all the different tubulin isoforms to a freely formed “mixed” tubulin polymer, disproves in great part this theory\[35\]. In reality from all the different isoforms just a few tubulin isotypes may play a unique cell-specific function. A classic example is \( \beta \text{I} \) tubulin, that is expressed in platelets and is responsible for the formation and contraction of the marginal band, is an essential
process for the dramatic morphological changes occurring during the activation of these cells [33, 34].

Another example more relevant to this dissertation is the (almost) neuro-specific βIII tubulin, expressed only in neurons of the central nervous system, and outside from that, some other locations such as Sertoli cells and sperm cells [36], vestibular organ [37], nasal and colon epithelia[38]. In the brain, about one quarter of the overall tubulin is the βIII isotype. Considering the absence of βIII in glia, the relative amount of this tubulin isotype in neurons easily exceeds the 25% [39]. *In vitro* experiments have shown that this isoform of tubulin has a significant increase of dynamicity compared to the other isoform [40]. This higher dynamic behavior is particularly suitable for a young microtubule cytoskeleton of newly developing neurons where, phenomena like neuronal migration, cell division, axon extension and pathfinding require a particularly dynamic cytoskeleton. However, the higher dynamicity is just a part of the specific βIII function. Mature nervous system, composed by neurons with a required less dynamic cytoskeleton has about the same amount of βIII tubulin, making the question of its function at adult age puzzling, especially because during maturation the increased expression of microtubule stabilizing MAPs, such as MAP2 and Tau, is significantly increased [41, 42]. Another specific function of this isotype relies on its primary sequence: differently from any other tubulin, βIII isotype does not have a key cysteine residue in position 239 [43]. The presence of a serine instead of chemically more reactive cysteine, makes this tubulin isotype resistant to oxidative stress environments, typically present in nervous tissue. Indeed, any other tubulin isotype bearing in 239 position a cysteine residue are particularly sensitive to oxidation, and under oxidative stress the cysteine residue form an intra-molecular disulfide bridge that is incompatible with microtubule assembly. Moreover the presence of other cysteines in unusual positions (such as position 124, 127 and 129) in the βIII protein could help the clearance in the cytosol of reactive oxygen species without compromise the microtubule cytoskeleton[31].
**Tubulin diversity II: tubulin post-translational modifications**

Considering the large number of functions that microtubules perform at the same time in varying regions of the cells, an intriguing question is how does the cell use a particular subset of microtubules for a specific function, meanwhile using others for a different function. One partial answer could be the selective expression of specific tubulin isotypes as I previously illustrated. However, considering the high capability of different tubulin isotypes to copolymerize, the diversity of tubulin in the primary sequence remains a partial answer to the problem [35]. Another possibility is to create transient, differential, domains or interactive surface on microtubule in correspondence to a particular cellular event. Under the biological point of view, this domain can be created by PTMs [18, 44]. Indeed, microtubules are one of the most post-translationally modified structures in the entire cell. The PTMs occurring on microtubules can cause very different biochemical effects on the microtubule surface: some profoundly increase the total electrostatic charge (phosphorylation, poly/mono-glutamylation, poly-amination); others conversely can reduce it (for example, in the case of acetylation) [45]. However, the variety of PTMs does not stop on the change in electrostatic charge: proteolytic cleavage can alter the tubulin polypeptide irreversibly, like in the case of Δ3-tubulin [44]. In any case, all the modifications thus far have *per se* a limited or even nonexistent effect on processes related to microtubule polymerization. The numerous past *in vitro* studies and the elegant new *in vivo* approaches suggest that all these modifications have a profound effect on microtubules and its related function in virtue of their capability to recruit, activate or inhibit secondary effectors. In other words, microtubule PTMs may alter the capability of MAPs to bind/interact, altering microtubule related functions. Despite decades of dedicated work in the microtubule PTMs field, a lot of progress is still necessary to have a clear understanding as to what the function of this alteration is and what is the foundation of the so called Tubulin Code hypothesis [44].
For the purpose of this dissertation I will describe briefly the current idea of the best studied microtubules PTMs, giving a major emphasis to the acetylation, the PTM that has the greatest relevance from the work that I am going to illustrate.

**Tubulin Acetylation**

Tubulin is the most common acetylated substrate in the cytoplasm [46]. A growing number of PTM studies show that microtubules can be acetylated in different sites [47]. However due to the early nature of this discovery very little is known about the function of these modifications. Acetylation on α-tubulin at lysine 40 is the exception. This specific PTM has been studied since the middle 80’s [48] and several pieces of information can be extracted from this work, which has allowed us to have a better understanding of the complexity of the microtubule PTM world [44]. The acetylation on tubulin can be performed by several enzymes: ELP3, NAT10, ARD1, GCN5 just to mention a few [45]. However to date the major tubulin acetylase is considered α-TAT1 (α-tubulin acetyl transferase, MEC17 is the *C. elegans* ortholog) since the depletion of this protein causes the complete ablation of acetylation (on α-tubulin K60), meanwhile the depletion of the other tubulin acetylases causes only a partial effect on this PTM [49]. A clear interpretation of the biological function of this PTM to date is still challenging, but a series of experiments indicate the potential correlation, in a context dependent scenario, between acetylation and microtubule stability. This idea arises from a series of very elegant experiments performed in the 80s. First when a microtubule preparation was incubated with destabilizing agents such as nocodazole or colchicine, the acetylated fraction of microtubule localized in the more stable fraction [50, 51]. Conversely, treatment with taxol, which make microtubules resistant to strong microtubule-destabilizing agents such as cold or CaCl$_2$, caused a net increase of microtubule acetylation [16]. In a second set of studies, biotinylated brain tubulin was microinjected in human fibroblasts and the relative amount of newly incorporated tubulin was measured shortly after injection (1 hr.). In this experiment almost all the non-acetylated tubulin was biotinylated (corresponding to the fraction of
microtubules recently incorporated, hence labile), meanwhile only the 50% of the acetylated tubulin was biotinylated, indicating that the turnover of acetylation was much slower, indicating that PTM occurs in tubulin subunits polymerized in stable, longed lived microtubule [52, 53]. These two experiments and many others since that time demonstrated that acetylation was a consequence of microtubule stabilization. The slow catalytic activity of α-TAT1 merged with it required introduction and slow diffusion in the luminal space of microtubules, is in agreement with the reduced possibility of the enzyme to target newly assembled tubulin subunits [54].

The acetylation is a PTM on microtubules that pose several challenging question, starting from the enzymatic activity. The enzymatic acetylation targets specifically the Lys-40 on the α-tubulin, when the polypeptide is fully assembled in the microtubule structure (acetylation of cytosolic tubulin heterodimer is minimal, if not negligible) [55]. In this configuration, the Lys-40 on the α-tubulin is exposed on the lumen of the microtubule and not on the cytosol, making this site completely unavailable to any cytoplasmic enzyme. Several mechanisms have been proposed to explain this apparent contradiction: in one scenario, α-TAT1 would enter in the lumen of microtubule during the microtubule assembly, or on the opened (un-capped) luminal end in shrinking/kinetically stable microtubules. In this model the acetylation on the stable fraction of microtubules would occur because the slow enzymatic activity of the α-TAT1, which would happen when the region of the enzyme incorporation has already turned into a stable domain. A second scenario hypothesizes the presence of transient lateral opening of the microtubule lattice caused either by the transient exchange of tubulin subunit with the soluble pool (so called “breathing model”) [56]. This transient opening would behave as hot-spot for enzyme entry in the luminal space of microtubules, allowing the acetylation to occur also in capped microtubules in which the entrance of the enzyme trough the plus-end opening would be impossible [46, 57].
The acetylation process on microtubules, differs from other microtubule modifications (such as Δ2 tubulin) because the modification is reversible. Also in this case, several potential tubulin deacetylases enzymes can be described, but the two major are: 1) HDAC6 (histone deacetylase 6, a member of class II HDAC) [58] and 2) Sirt-2 (sirtuin 2) a member of the non-classical HDACs, a less effective tubulin acetylases [59]. Chemical inhibition of these enzymes by using tubacin or nicotinamide (respectively for HDAC-6 and Sirt2 [60, 61]) or silencing by using siRNA/ShRNA strategy are common tools to induce microtubule hyperacetylation [59, 60, 62-65].

The biological effects on acetylation is still a matter of intense debate, probably because of the effect of microtubule acetylation on microtubule stability has to be mediated by secondary effectors [66], making the correlation with any specific phenotype very challenging to interpret and fully understand [44]. However some in vivo studies have shown a light alteration on intracellular transport mediated by kinesin-1 and dynein [67, 68], in which increased microtubule acetylation via HDAC6 inhibition causes a slight enhancement of kinesin-1 to microtubules and a redistribution of kinesin cargo (such as BDNF filled vesicles). The absence of a similar effect in an in vitro purified preparation indicates the requirement of secondary factors to this complex modulation [69, 70].

The presence of a marginal biological effect on acetylation or any other microtubule PTMs can be interpreted in different ways. A simplistic interpretation would explain the lack of a strong phenotype after a robust alteration of one PTM as a marginally relevant biological phenomenon. Another interpretation considers the complexity of the biological system especially in overlapping/redundant mechanism such as PTMs in protein. This interpretation (proposed by Carsten Janke in his 2014 review [44]) states that the Tubulin code could follow a probabilistic signal theory, where a change of PTMs, would not result in an abrupt interruption of a biological process or the determination of it, but rather a bias toward of a specific phenomenon. In other words, a modification of a single microtubule PTM would cause the marginal modification of a
signal that would be in great part compensated by the expression of overlapping functionally/chemically similar PTMs. However the result of this perturbation would still have its effect: a loss of fine tuning for a series of process which would have important biological consequences, but that often can be only marginally appreciated experimentally [44, 71]. This would make sense especially in biologically essential processes such as organelle transport and other microtubule related functions in which total compromise would result in an incompatibility with life.

**Other microtubules post-translational modifications**

Analogously to microtubule acetylation, most of the microtubule PTMs take place in tubulin subunits already incorporated in the polymer. A typical example is tubulin detyrosination. This PTM occurs preferentially on the C-tail of α-tubulin that, except for few exceptions, has a key tyrosine residue. This residue is selectively removed by unknown putative carboxypeptidases when the subunit is incorporated in microtubules [72], and is re-tyrosinated by the tubulin tyrosine ligase (TTL) enzyme, where the subunit is made again available in the soluble pool [44, 73, 74]. The result of this differential tubulin target is the creation of a dynamic domain of microtubules that is rich in tyrosinated tubulin, and a stable portion that is rich in detyrosinated tubulin. However the presence of detyrosinated tubulin in the stable domain of microtubules is also possible because the convergence of other factors such as the inhibitory activity on microtubule depolymerizing agents, such as Kinesin 13 family members (MCAK or KIF2) [75, 76]. Another important interaction between detyrosinated tubulin and tubulin interacting proteins is kinesin-1. *In vivo* and *in vitro* studies showed the preferential binding (landing rate) and increased processivity of this molecular motor protein along detyrosinated microtubules [70, 77-79]. The opposite modification, the re-tyrosination, is catalyzed by a non-redundant TTL enzyme. The presence of newly assembled, virtually un-modified tubulin seems to be essential for different neuronal processes [44]. First tyrosine tubulin is the docking site recognized by a large complex of protein localizing on the plus
end of the microtubule tip (plus-end binding proteins). The binding of this complex on the plus tip is mediated by CLIP-170, which recognize tyrosine tubulin by exposing its CAP-Gly domain (cytoskeleton associate protein glycine rich domain) [80]. Some domains seem to be crucial for the recognition of the retrograde molecular motor protein complex (dynein complex) on the plus tip of microtubule. The docking of this complex on this location is probably mediated by p150<sup>Glued</sup>, a protein that forms part of the dynein complex and that bear a CAP-Gly domain similarly to CLIP170 [81]. Interestingly, ablation on TTL in mouse results in premature death of mouse pups [82]. The TTL-KO animals have several histological alterations in the developing brain [83]. The absence of dynamic microtubules indeed could cause an aberrant accumulation of stable microtubules [84], and an irreversible inhibition of numerous processes that require a great deal of dynamic microtubule such as neurogenic cell division, neuronal migration and axon pathfinding.

Polyglutamylation is another major microtubule PTM occurring on assembled tubulin polymers in long-lived microtubules [85]. A time course of microtubule polyglutamylation in differentiating neurons have shown that with the approaching of neuronal differentiation the microtubule polyglutamylation increases [85, 86]. This PTM is catalyzed by a group of enzymes related to TTL, and for this reason called Tyrosine Tubulin Ligase Like proteins (TTLs) [87, 88]. In mammals nine different members of this subgroup of enzyme exist, and each protein perform a slightly different action toward this PTM based upon the preference toward the α or β subunit or the capability to initiate or elongate glutamate chains [89]. The depletion of any one of these enzymes results in mice affected by ciliopathy, indicating the essential and not redundant role that these enzymes have toward polyglutamylation, and the pivotal role of this PTMs in the mobile cilia functioning [90].

The removal of these residues is mediated by a novel subgroup of carboxipeptidases (CCP) [91]. So far three separate CCP deglutamylases have been identified to reverse the action of polyglutamylases [91]. The depletion of this enzyme, with a resultant increase of polyglutamylation in mice, has drastic consequence in the central nervous system of these animals. In particular
Purkinje cells are the cellular target particularly sensitive to hyper-glutamylation, which will undergo premature degeneration in this animal model [92]. Under molecular mechanistic point of view, the specific role of polyglutamylation has not been extensively studied. In the past years it has been demonstrated that polyglutamylation could bind to microtubule lattice specific subsets of tubulin poly-glutamylated sensitive proteins such as spastin [93], a microtubule-severing protein whose function will be illustrated later in this dissertation.

**Microtubule stability in neurons**

The dynamic character of microtubules as an intrinsic property of this polymer can be easily reproduced in the most diverse experimental condition, sometime very far away from a biological environment. Incubation of just purified free tubulin, in the right environmental conditions (37°C, magnesium and GTP) results in the spontaneous formation of microtubules, which will sustain rapid cycles of assembly and disassembly, showing it to be a potentially extremely dynamic polymer, already in these simple conditions [7]. However the rapid bouts of microtubule assembly and disassembly are not uniform from the plus tip or minus tip of microtubule. The minus tip of microtubule has a much lower dynamicity (means going to a lower number of assembly and disassembly events per unit of time) compared to the plus tip more dynamic. Having a higher dynamicity also means that in order to observe an event of assembly the concentration of free tubulin necessary to carry out this process (called critical concentration) is lower than the one required in regions with a lower dynamicity (such minus tip). The mechanism that governs these constant bouts of assembly and disassembly has been described for the first time by the Mitchison and Kirsch group in 1984; proposing the so call model of *Dynamic Instability* [94]. In this model the existence at every given time of each microtubule is explained as the result of a combination of events of disassembly alternating to events of assembly, where the latter prevails to the first. This mechanism is regulated by the slow GTP hydrolysis activity of β tubulin. In order for free tubulin to be assembled in microtubules, the β-subunit has to be in the GTP bounding state. Briefly after
the polymer incorporation of tubulin, the subunits start to hydrolyze GTP to GDP. For structural reason, in order to be stable the microtubules must contain a region composed mostly by GTP-tubulin on the plus-end [26]. If this region is rapidly substituted by GDP-Tubulin depolymerization instead of assembly will occur. This process, in an \textit{in vitro} situation, where microtubules are all equal and are not subject to special stabilizing factors, is stochastic and therefore in a population of microtubules at any given time, are going to a polymerization phase, meanwhile some are rapidly depolymerizing.

However the high turnover that can be observed in this isolated system is far away from what actually happens in an intact biological scenario. In intact cells, microtubules have a very variable dynamic profile depending on the context and the compartment considered. In some regions, microtubules can have a higher turnover (frequency of polymerization and depolymerization events), and therefore are considered as more dynamic or called as \textit{labile}, meanwhile in other compartments microtubules have an evident reduction of the frequency of catastrophe and rescue, and therefore are considerate \textit{stable}. In specific regions of the cell, and at specific stages of its life (e.g. neuron in adult ages) a third classification appears evident: this class of microtubules can be considered “hyper-stable,” in virtue of their capability to stay in a polymerized state even in the most extreme microtubule-depolymerizing conditions, such as cold temperature [95]. This subset of microtubule differs from the labile and the stable because the absence of any dynamic behavior (remarkably different concept from the stable microtubule which have a lower, but still very well present dynamicity). In this work I will use the term as \textit{cold stable} or \textit{non-dynamic} microtubule to refer to this particular set of cytoskeleton elements [7]. The presence of one specific subset of microtubules (stable, labile, non-dynamic) does not preclude the co-existence of other subsets in the same cell, and actually the opposite is quite common, where the co-existence of all the three classes of microtubules shape the entire microtubule cytoskeleton. A classic example are developing neurons where microtubules in specialized regions such as the growth cone are very
dynamic (hence full of labile microtubule), meanwhile in the axon shaft a higher percentage of microtubules are stable and, although in small amount, even hyper-stable [7, 96]. A compelling question therefore is how microtubules can alter their dynamic profile? The answer, although to date not completely explained in details, relies on the combination of PTMs and binding with microtubule-associated proteins (MAPS) [97]. In neurons the non-dynamic/ hyper-stable fraction of microtubule is obtained by an unusual PTM (for microtubules) called poly-amination, where a great deal of positive charge is added to the polymer (mean while the majority of other PTMs are either neutral or negatively charged) [95, 98].

MAPs such as MAP-6, or Tau or MAP2 are responsible for creating stable microtubules. This variability in the dynamic character of microtubules is vital for a proper functioning of any biological system, because it gives the possibility of fine tuning and local changes in the microtubule cytoskeleton biophysical property to make the entire cell more adapt to a specific environmental variation [7, 99-101]. The division of these three different microtubule behaviors is not just theoretical and actually come from a large series of experiments performed in the early 90s from Baas and Black. The labile-stable fraction was described for the first time in neurons by incubating neurons with a medium concentrated with nocodazole, which in time-dependent manner, will selectively depolymerize labile microtubule, and then much slowly and not completely the stable domain. The most sensitive fraction of tubulin already depolymerized after 15 minutes of incubation had obviously a more dynamic property than the rest of microtubules, and for this reason is called labile. The hyper-stable/non-dynamic fraction was instead accidentally discovered by a cold treatment of tubulin, in which the low temperature is not capable to polymerize this fraction of purified microtubules [102].

**Microtubule-severing proteins**
Within the constantly growing list of microtubule binding/regulating proteins, the microtubule-severing proteins is a subfamily that has particularly strong implication in neuronal morphology [103-105]. Microtubule severing can be defined as the enzymatic reaction that causes the fragmentation of a longer microtubule into a shorter piece, by the generation of an internal breaks [106]. This subgroup of proteins belongs to the big AAA superfamily (ATPase associated with diverse cellular activity), based upon the strong similarity on the catalytic site [107, 108]. So far eight different homologs have been identified: Spastin, Katanin, Fidgetin, VPS4, Katanin-like 1/2, Fidgetin like 1/2. Within these, the first three (Spastin, Katanin and Fidgetin) have been demonstrated to sever microtubules both in cells and in vitro. VSP4 is a paralog in mammalian, that does not sever microtubule but rather participates in endosomal trafficking and endoplasmic reticulum remodeling [106, 109].

The effect on neuronal cytoskeleton of a microtubule-severing event can have very different outcomes depending on the cytoplasmic context and on the protein factors that are associated with those microtubule pieces. If the fragments are composed by labile microtubules, the severing will result inexorably in a rapid depolymerization. If the severed fragments were part of a stable microtubule, these can depolymerize like the labile fragments, or can act as “microtubule seeds” where the short fragments act as a mold and free tubulin can rapidly assemble to form a longer microtubule.

The first investigations on microtubule-severing proteins were performed in C. elegans, by the study of Mei-1 and MEI-2 proteins, the orthologs of p60 katanin-like and its regulatory subunit p80-katanin in vertebrates. In C. elegans oocyte knockouts for katanin, a significant decrease of total microtubule mass in the meiotic spindle together with a minimal loss of soluble tubulin was observed [110, 111]. In these experiments, the researchers speculated that the unusual phenotype was the result of an insufficient severing from a putative microtubule-severing enzyme, which
would cause a reduction of microtubule ‘‘seeds’’ that could grow and increase the overall microtubule mass in the oocytes.

The effect of microtubule severing in living cells was further elucidated by studies on katanin in a plant system (A. thaliana). In this system, the microtubule-severing activity was necessary to allow the parallel orientation of cortical microtubules for the directional deposition of cellulose fibers to form the vegetal cellular wall. Reduction of katanin levels in A. thaliana cells resulted in a striking phenotype where the deposition of these fibers was very disorganized [112]. Again, it was also speculated the existence of an enzyme capable to sever microtubules to allow their uniformly orientation in another system.

The following studies in Drosophila larvae and cell lines definitively demonstrated the existence and the effect of microtubule-severing activity of spastin at the beginning, katanin immediately after and finally with Fidgetin [113]. The biological actions of each of these three proteins were in some cases overlapped, in other divergent. For example in the Drosophila S2 cell line, Spastin and Fidgetin localize around the centrosome and perform severing on the minus-end and perform minus-end depolymerization. Conversely katanin’s enzymatic activity performs a plus-end depolymerization [113].

The crystallographic structure of katanin and spastin has been already resolved [114-116]. These two enzymes, different from almost all the other AAA protein family members, can oligomerize to form very complex quaternary structures [114]. These enzymes utilize ATP to perform the enzymatic activity. Because of this, they can be present in two different forms: the ADP-bound form, which keeps the polypeptide in a monomeric form, and the ATP bound form that would cause its oligomerization in a hexameric form. In a highly isolated in vitro environment the oligomerization of spastin requires a relatively high concentration of the enzyme, probably too high in a physiological scenario. However, the critical concentration for spastin or katanin to oligomerize
can be dramatically lowered if in the reaction mix there are microtubules. The presence of tubulin polymers allows the enzyme to dock on the surface and induce a fast hexamerization [114, 116]. The enzyme hexamer shows particular structural characteristics that are very important for its catalytic activity: the six subunits form a central channel composed by helices with several positively charged amino acids, whose function is to specifically recognize the un-structured C-terminal tail of tubulin, usually negatively charged by the presence of several glutamate residues. The interaction of the enzyme’s internal channel with the (negative) C-terminal tail of tubulin, merged with the conformational changes occurring at the enzyme hexamer after ATP hydrolysis, causes the continuous tuck of the tubulin subunit outside the microtubule lattice. This in turn results in an overall reduction of the lateral protofilament interactions, causing the formation of structural defects in the microtubule lattice. The following extraction of several tubulin subunits around the same microtubule region forms a breakage point that will resolve in a microtubule-severing event [106, 114].

Despite the fact that experiments on microtubule-severing protein are still in a phase of developing, many exciting discoveries have been made, especially in the developing central nervous system, where all of the three recognized microtubule-severing proteins, katanin, spastin and fidgetin are expressed at the highest level (comparatively to the value in other systems) [117].

**Katanin**

The neuronal function of Katanin (the catalytic subunit, p60 katanin) has been demonstrated in the early phase of neuronal development, regulating processes such as axonal outgrowth [109, 118]. The depletion of katanin in developing neuron reduces the capability of a developing axon to properly extend, and the lack of its microtubule-severing activity around the centrosome, causing the accumulation of long microtubules anchored to this structure. The explanation of this complex
phenotype could lie on the relation between the newly formed microtubules in the centrosome and their capability to act as seeds in the axon to boost the local microtubule mass. In a physiological condition, microtubules originated \textit{de novo} in the centrosome are periodically severed around the minus-end to be transported in the axon to increase the microtubule mass. The absence of this abscission from the centrosome results in a much lower number of microtubules transported in the axon, which in turn will negatively affect processes such as axon growth that needs the continuous supply of microtubules to properly grow [118]. Just recently, a series of interesting work has started to unravel the regulation of its catalytic activity. Katanin in neurons seems to have a higher preference toward acetylated microtubules [119]. Its catalytic activity is limited by the steric inhibition of specific MAPs which they intercalate between the severing enzyme and the microtubules, protecting the cytoskeleton polymer from being severed. The most remarkable MAP acting with this protective mechanism is tau, preferentially localized in the axon, where microtubules acetylation is particularly high [120].

**Spastin**

Study on spastin started much earlier than the discovery of its microtubule-severing activity, since mutation on the \textit{SPAST} gene (encoding for spastin) is the chief cause of hereditary spastic paraplegia, a non-progressive neurodegenerative condition causing the selective impairment of lower limb mobility[121].

Pioneering studies on \textit{Drosophila} unravel the molecular and cellular action of this enzyme in eukaryotic cells. Spastin depletion in fruit flies cause aberrant increases of synaptic and satellite boutons in neuromuscular junctions in third instar larvae. In zebrafish, reduced level of spastin causes severe diminution of axonal outgrowth, loss of connectivity and extended neuronal apoptosis [122-124]. The data relative to the effect in invertebrate and in zebrafish suggest a possible mechanism of action of spastin mutation in HSP, where the lack of sufficient severing
would cause detrimental effects on axons from neurons of the corticospinal tract [125]. This speculation will be of interest in my second part of my research activity and extensively evaluated.
CHAPTER 1: VERTEBRATE FIDGETIN RESTRAINTS AXONAL GROWTH BY SEVERING LABILE DOMAINS OF MICROTBULES
**Introduction**

In the axon, each microtubule consists of a stable domain from which can elongate a labile domain [126, 127]. The stable and labile domains are situated toward the minus and plus ends of the microtubule respectively, with the labile portion of the microtubule directed toward the tip of the axon. For axons to grow in a robust fashion, there must be an expansion of the microtubule array, and especially of the labile domains. Also important for axonal growth is a process whereby long microtubules are severed into shorter ones by proteins that tug at the microtubule lattice to induce breakage [114]. If the breakage occurs in the stable domain of a microtubule, the result is two new microtubules, both capable of growing longer. This allows for an increase in microtubule number, locally within the axon, for example at sites of branch formation [104, 128]. If this breakage were to occur in a labile domain, the expected result would be just one microtubule that is shorter than the original, as a microtubule with no stable domain would depolymerize completely. Traditional, microtubule-severing proteins like katanin and spastin, preferentially sever microtubules in the stable domain [129, 130]. In theory, a microtubule-severing protein that targets the labile domain could be important for limiting the expansion of the microtubule array of the axon to modulate its growth. While *Drosophila* fidgetin is a traditional microtubule-severing protein [113], its vertebrate ortholog diverges in sequence and structure [131], and yet is still able to sever microtubules [132]. Results of the present studies indicate that vertebrate fidgetin regulates neuronal development by targeting labile domains of microtubules.
Results

Fidgetin depletion affects axonal development and microtubules in Drosophila. The effects of knocking down Drosophila fidgetin from fly neurons in the neuromuscular junction (NMJ) were first investigated. The Gal4/UAS system was used to express an RNAi hairpin targeting the Drosophila fidgetin gene (CG3326, or Fgn) under UAS control (UAS:Fgn-RNAi). Expression was limited to post-mitotic neurons using the Elav-gal4 driver [133-135]. The total number of synaptic contacts (boutons) was significantly higher in fidgetin -knockdown animals (fig 1B, 1E) compared to outcrossed control (Ctl) animals (fig 1A, 1E). The number of satellite boutons (which are small growths of presynaptic membranes that extend out from axonal terminal arbors) in fidgetin-knockdown animals (arrows in fig 1D, 1E) was greater than Ctls (fig 1C, 1E). Increased number of satellite boutons has been observed in flies bearing mutations to other microtubule-severing proteins [122] [136]. A significant increase in the ratio of acetylated to total tubulin (measured by quantifying fluorescence intensity) was observed in the axonal shaft and distal synapse, compared to two independent control RNAi lines (Ctl1, Ctl2) (fig 1F). This was due to a significant increase in microtubule acetylation in these areas, as total tubulin levels remained relatively unchanged (fig 1F). A similar result has been reported in Drosophila neurons with compromised katanin [136]. These results are consistent with Drosophila fidgetin behaving similarly to traditional microtubule-severing proteins in the neurons of the fly.

Fidgetin expression in developing neurons.

Fidgetin was discovered in vertebrates as a gene spontaneously mutated in a mouse strain that displayed a fidgeting phenotype [131, 137]. As shown in fig 2A, vertebrate fidgetin is larger than Drosophila fidgetin, with a region of over 300 amino acids toward the N-terminus that is absent from the fly ortholog. The Walker A motif in the AAA region is the same as in fly, but the Walker B has unusual amino acid substitutions. Multiple attempts at developing fidgetin antibodies in the past have failed for unknown reasons [131]. For this reason, a line of mice that knocks out fidgetin
by replacing most of the fidgetin gene for LacZ was purchased, so that fidgetin’s expression pattern could be observed by staining for β-galactosidase. Fidgetin expression was observed in various tissues, but was especially high in developing nervous tissue (fig 2B, 2b).

Like cultures of fetal rat hippocampal neurons used in relevant previous studies [104, 120, 128, 138], cortical neurons undergo stereotyped developmental stages in which a lamellipodium (stage 1) becomes multiple minor processes (stage 2), one of which then becomes the axon (stage 3) after which the rest become dendrites (stage 4). Consistent with previous studies with mouse GFP-fidgetin [131], ectopically expressed rat GFP-fidgetin was found to reside in the nucleus, but is also cytoplasmic, distributing throughout the neuron. The morphological effects of fidgetin overexpression were a shorter axon and fewer immature processes (fig 2C and 2c, quantification fig 2E). There was no evidence of short fragmented microtubules as a result of fidgetin expression. For example, in fig 2D and 2d, long microtubules appear in the growth cone in GFP- fidgetin-expressing neurons as well as Ctl GFP-expressing neurons, without any obvious short microtubule fragments. Per unit length of axon, there was no difference in microtubule levels in Ctl and fidgetin-depleted axons (fig 2E). Whether the construct was GFP- fidgetin or fidgetin-GFP or whether a flag tag was used, no fragmentation of microtubules was observed in neurons or in rat fibroblasts (Sup fig 1).

Effects of fidgetin depletion on cultured vertebrate neurons. siRNA was introduced just prior to plating. After 2 days of protein depletion, dense cultures were re-plated at a lower density to quantify differences in neuronal morphology when processes were permitted to grow anew. A day after re-plating, total number of minor processes per cell body was roughly doubled in fidgetin-depleted neurons (fig 2G and 2g) compared to Ctl (fig 2F and 2f), and fidgetin-depleted neurons had significantly longer axons (see also fig 2H and 2h, which respectively show tracings of additional Ctl and fidgetin-depleted neurons). Differentiation was accelerated as a result of fidgetin depletion, with fidgetin-depleted cultures at 24 hours (hrs) having a significantly higher percentage
of neurons in stage 3 compared to Ctl siRNA, and a corresponding reduction in the percentage of neurons in stage 1. Morphological effects of fidgetin depletion were essentially the inverse of the effects resulting from overexpression. Data are shown in fig 2I. For confirmation of knockdown, GFP-fidgetin was expressed in fibroblasts or neurons together with the siRNA for 24 hrs. In Western blot analyses, the GFP-fidgetin band, detected with a GFP antibody, was reduced by over 70% in the cultures transfected with fidgetin siRNA compared to those transfected with Ctl siRNA (fig 2J). Similar results were obtained on cultures in which each of the siRNA sequences was used individually (Sup fig 2).

Fidgetin depletion increases labile microtubule mass in axons.

In neurons depleted of fidgetin, there was a 53±8.9% increase in microtubule mass relative to Ctls as assessed by Western blotting (fig 3C), and a 62±8.63% increase in microtubule mass per unit area of axon as assessed by quantitative immunofluorescence (IF) (fig 3d). Nocodazole sensitivity was used to discern the stable and labile fractions of the microtubule mass [126]. After 30 min of drug treatment, the microtubule levels were indistinguishable from the corresponding drug-treated Ctls, as assessed with either Western blotting or IF (fig 3C, 3c, and 3d), indicating that the microtubule mass added as a result of fidgetin depletion is entirely labile.

GFP-EB3 (which tracks dynamic plus ends of microtubules) was expressed at the time of re-plating in neurons that had been transfected 2 days earlier with either fidgetin or Ctl siRNA. No difference in the number of the GFP-EB3 “comets” was found between fidgetin -depleted and Ctl axons, nor were there any differences in the rate or duration of the comets (fig 3A). These results, in conjunction with the results of the nocodazole study, suggest that fidgetin depletion does not increase microtubule number and therefore must lengthen the labile domains of microtubules (fig 3B). By contrast, knockdown of Drosophila fidgetin did not increase microtubule levels, but resulted in a higher proportion of the microtubules being acetylated (fig 3B).
Fidgetin depletion decreases ratio of acetylated to total tubulin in the axon. Cultures (Ctl-siRNA or fidgetin -siRNA) double-labeled for IF visualization of total or acetylated tubulin in microtubules revealed that total microtubule levels were elevated in the fidgetin-depleted cultures but acetylated microtubule levels remained roughly the same as in Ctl neurons (fig 4A and 4a). This same reduction in the ratio of acetylated to total tubulin relative to Ctls was observed by Western blotting of the cultured neurons (fig 4B and 4b), and by immunohistochemistry on E18.5 brain of the fidgetin knockout/reporter mouse (fig 4C-F).

To investigate whether fidgetin could be specifically targeting unacetylated tubulin, the same morphological experiments described earlier were conducted in the presence of tubacin, an inhibitor of HDAC6, the principal tubulin deacetylase in vertebrate cells [119]. Higher concentrations of tubacin can alter neuronal morphology, which is not unexpected given that the acetylation status of the microtubule affects how it interacts with a variety of different microtubule-related proteins. Chosen here was a lower concentration of the drug that had no statistically significant effect on axon length or process number, relative to DMSO (vehicle)-treated Ctls. However, even at this low dosage, there was an increase in tubulin acetylation, as assessed by IF (ratio-imaging of acetylated to total tubulin; fig 4G and 4g) and by Western blotting (fig 4H). Treatment of neurons with this dose of tubacin prevented fidgetin siRNA from increasing axon length or minor process number relative to Ctl siRNA (fig 4I, upper graphs), and prevented overexpression of fidgetin from reducing axon length and process number (fig 4J).

Because acetyl transferases and deacetylases do not exclusively affect tubulin and may have other effects on microtubules apart from their acetylation status, another approach was taken to further investigate. The chief tubulin acetyl transferase in vertebrate cells, namely α-TAT1 [139], was overexpressed. A mutant form of α-TAT1 (called α-TAT1 D157N) that cannot acetylate tubulin
but presumably can do anything else α-TAT1 may do was used as Ctl. Validation of the wild-type construct but not the mutant to increase microtubule acetylation is shown in fig 4h. These constructs were transfected as GFP fusions into fetal cortical neurons just prior to re-plating neurons that had been transfected with fidgetin siRNA 2 days prior. Two days later, the lengths of the axons and the number of minor processes from neurons expressing the constructs were quantified. Consistent with the tubacin results, both the increase in axon length and minor process number resulting from fidgetin depletion were preserved in the presence of α-TAT1 D157N, but were obliterated by the wild-type α-TAT1. Quantification of these results is shown in fig 4I (lower graphs). Together, these and the tubacin results indicate that morphological changes resulting from increasing or decreasing the levels of fidgetin depend on the presence of poorly acetylated microtubule domains.
Discussion

The present results indicate that vertebrate fidgetin functions during development to tamp back the elongation of the labile domains of microtubules so that they assemble in a regulated fashion. Overexpression of vertebrate fidgetin does not result in obvious loss of microtubule mass from cells in which the labile domains are not expanding, but does suppress normal axonal elongation, a process that depends on the expansion of labile domains. Accordingly, depletion of vertebrate fidgetin results in longer labile microtubule domains and longer axons. Changes in process number resulting from alterations in fidgetin levels can also be explained this way. The situation is different in *Drosophila*, where the data suggest that fidgetin functions as a traditional microtubule-severing protein, preferentially severing stable microtubule domains. Unlike flies, which only have one fidgetin gene, vertebrates have two fidgetin-like genes [137], which may have allowed fidgetin itself to diverge. Vertebrate axons are much longer than *Drosophila* axons, which may be why vertebrates evolved machinery dissimilar from flies for regulating the growth capacity of the axon.

As to mechanism, an initial idea was that vertebrate fidgetin might co-hexamerize or co-dimerize with spastin, and thereby act as a dominant-negative to spastin’s function. This idea came to mind because the phenotype of fidgetin knockdown is similar to that of spastin overexpression, especially with regard to higher numbers of minor processes [104]. Another idea is that the unique properties of vertebrate fidgetin may result from the N-terminal domain of vertebrate fidgetin absent from invertebrate orthologs acting in a regulatory fashion, similar to the situation with certain kinesins, where an inhibitory domain can fold over to suppress the activity of the functional domain [140]. However, neither of these ideas easily explains how fidgetin preferentially severs labile domains of microtubules. To this point, perhaps the amino acid substitutions in the Walker B domain weaken the ATPase activity such that vertebrate fidgetin is only strong enough to break the lattice of the microtubule where it is weakest. This could result in a preference for the region of the microtubule...
toward its plus end, where much of the lattice has not yet fully closed. However, this would contrast with \textit{in vitro} data suggesting that vertebrate fidgetin has a preference for minus ends of microtubules [132]. The present results support another possibility, namely that vertebrate fidgetin targets labile domains of microtubules through a preference for unacetylated tubulin. This idea is appealing because it would make fidgetin the functional inverse of katanin, which targets stable domains through a preference for acetylated tubulin [119, 136].

Finally, there is the potential for therapeutic application. Inhibition of fidgetin might be useful for treating neurodegenerative diseases as well as nerve injury, as a boost in labile microtubule mass may restore lost microtubule mass and/or enable a regenerating axon to grow with more vitality [16].
Material and Methods

Fidgetin conditional knockdown flies. Transgenic fly lines UAS: CG3326-RNAi (abbreviated as UAS:Fgn-RNAi here) were obtained from the Vienna Drosophila Resource Center (stock #24746). RNAi expression was restricted to neurons by using the Elav^{c155}-Gal4 driver. Abdominal segment A3 from muscle 6/7 of third instar larvae was used for NMJ analysis. Immunohistochemistry was performed as previously described [141]. Primary antibodies were: anti-βIII-tubulin (Biolegend) specific for neurons, and anti-acetylated tubulin (Sigma). Fluorescently conjugated α-HRP (1:125, Jackson Laboratories) was used to visualize neuronal membranes. NMJ images were obtained using an Olympus Fluoview 1000 laser scanning confocal microscope.

Fidgetin knockout/reporter mouse. A fidgetin knockout/reporter mouse (Strain Name: B6.129-Fign^{tm1Frk/Frk}) in which a beta-galactosidase reporter gene was inserted into the fidgetin gene, replacing its catalytic domain [137], was purchased from Jackson Laboratory. Heterozygotes are fertile, while homozygotes die prenatally. X-gal staining of homozygote fetuses was conducted at E12.5. Brain immunohistochemistry for βIII-tubulin and acetylated tubulin was performed on homozygotes fetuses at E18.5 (sagittal 20 μm sections) with the same antibodies used for the fly studies. Images were acquired with the confocal Leica TCS SP2 VIS/405.

DNA constructs and siRNA. mEmerald-Fidgetin (termed GFP- fidgetin, as mEmerald is a modified GFP) and FLAG- fidgetin were generated from rat fidgetin (NM_001106484.1). Fidgetin -GFP and EB3-GFP were provided by W. Frankel and N. Galjart, respectively. EB3-GFP was obtained as described previously [128] α-TAT1 (pEF5B-FRT-GFP-α-TAT1) and α-TAT1 D157N (pEF5B-FRT-GFP-α-TAT1 [D157N] were from Addgene. For siRNA experiments, cells were transfected with a pool of 4 rat fidgetin sequences (Sigma-Aldrich, accession numbers: 3086 XM_229979,
Experimental procedures on cultured neurons. For microtubule quantification studies, cultures were pre-extracted in a microtubule-stabilizing buffer to release free tubulin as described previously, except that the detergent was 0.1% TritonX100, prior to further processing for IF or Western blotting [126]. Analyses were performed after 0 min, 30 min or 4 hrs in 2 μg/ml nocodazole or DMSO control [102]. For all other IF preparation, cells were fixed in 4% PFA and then post-extracted with 0.1% Triton. Images were acquired using identical exposure and other settings, thus allowing the comparisons between experimental groups. Ratio images of cultured neurons were obtained using the Zeiss Axiovert LSM 5 Pascal system with a HeNe laser microscope with no optical sectioning during imaging acquisition. Western blotting was performed by standard procedures, except that for the microtubule quantification experiments, the internal control was histone H3 level because GAPDH was lost during the pre-extraction step required to release free tubulin. Axon length was measured using Axiovision 4.6 software, considering as an axon any process longer than 50 μm. Minor processes were considered as any process longer than 6 μm but shorter than 50 μm. Developmental stages were defined as described in Results. For fidgetin depletion studies, more than 100 cells per condition per trial were analyzed. For overexpression studies, 30 cells per condition were analyzed in each trial. EB3 imaging was as previously described [128]. Three independent trials were performed for each analysis. For microtubule acetylation studies, tubacin was used at 2.5 μM and the α-TAT1 constructs were used as previously described [139]. Three independent trials were performed in each study, and an average of at least 100
neurons per condition per trial was taken during the quantification. For some experiments, RFL-6, a rat fibroblast cell line, was used in addition to the primary cortical neurons.

**Statistical analyses.** All of the data analyses, statistical comparison and graph were obtained by using SPSS 20 (IBM) and Excel (Microsoft). Data represent, if not otherwise specified, mean ± SD. Mean difference was considered to be significant at the 0.05 level (*p≤ 0.05). Multiple group comparison was performed by one-way ANOVA followed by Bonferroni post hoc, and for pair comparison, Student's *t* test.
Fig 1: *Drosophila* fidgetin knockdown increases synaptic connections *in vivo*. A-B show confocal images of third instar larval NMJs, muscles 6 and 7, labeled with α-HRP (white) to detect presynaptic neuronal membranes. A shows a Ctl NMJ. B shows an NMJ from a fidgetin knockdown animal (*Dcr2; UAS:Fgn-RNAi; elav-Gal4*). Note the significant increase in bouton number in fidgetin knockdown animals compared to Ctls. C shows a representative individual bouton from control animals. D shows a bouton with associated satellite boutons from a fidgetin knockdown animal.
**Fig 1 (continued)**: Arrows show the bouton. Arrowheads show satellites. Scale bar, **A** and **B**, 10 μm. Scale bar, **C** and **D**, 10 μm. **E** shows quantification of the number of total boutons and satellite boutons in fidgetin knockdown animals compared to Ctls. **F** shows quantification of immunofluorescence intensity of acetylated tubulin [24], total tubulin (middle) and ratio of acetylated to total tubulin (bottom) between fidgetin knockdown animals (*Dcr2; UAS:Fgn-RNAi; elav-Gal4*) and outcrossed Ctls (*Dcr2 ;; elav-Gal4* and *UAS:Fgn-RNAii/+*) at both the axonal shaft and distal synapse near the bouton. Note the significant increase of the ratio of acetylated tubulin to total tubulin. Error bars represent standard error. *=p<0.05, ***=p<0.001.
**Fig 2: Studies on vertebrate fidgetin expression in rodent neurons.**

A amino acid alignment of fidgetin (in figure abbreviated as Fgn) orthologs from rat (759 aa), mouse (759 aa) and *Drosophila* (523 aa). The two vertebrate orthologs are 99% identical but notably different from *Drosophila* fidgetin (see Results). B shows X-gal staining of fidgetin knockout/reporter mouse fetus at E12.5. Fidgetin is highly expressed in central nervous system regions such as brain and eye (B) and spinal cord (b). C-d show microtubule immunostaining (anti-βIII-tubulin antibody) in cortical neurons expressing either GFP or GFP-fidgetin. Fidgetin-overexpressing neurons have statistically shorter axons (70.04±6.95 μm) and fewer minor
Fig 2(continued): processes (3.33±0.163) compared to GFP-expressing Ctl neurons (axon length: 99.44±8.0 μm; minor process number: 5.22±0.35). Quantification is shown in E. Student's t test p ≤0.05. No difference in growth cone morphology or relative microtubule mass was observed as a result of fidgetin overexpression (D, d). Similarly, there was no significant difference in microtubule mass in the axon (Student's t test ≥0.05, (E) third graph from left) between Ctl GFP (484±69.42 AFU) and fidgetin-expressing neurons (552±46.95). Scale bars: (B): 0.5 mm; (b) 2 mm; (c): 10 μm; (d): 5 μm. F-J shows the effect on cortical neurons of fidgetin siRNA pool. F-h show immunostains for βIII-tubulin. F and G are stage 2 neurons from Ctl and fidgetin siRNA groups, respectively; while f and g stage 3 neurons from the same experimental groups. FIDGETIN depletion increases axon length (158.59±14.90 μm (g;I) right), minor process number (10±0.183 (G;g;I) left) and rate of polarization (stage 1: 30%±5.6; Stage 2: 40±3.5; Stage 3: 31±2.5 H;I) compared to Ctl siRNA neurons (F,f,H) (axon length: 102.77±5.95; process number 6 ±0.47; polarization stage 1: 45 ±4.4; stage 2: 42 ±3.5; stage 3: 14±1.7) (Student's t test p ≤0.05). Quantification is shown in (I). Validation of fidgetin siRNA is shown in (J) where RFL-6 cells were co-transfected GFP- fidgetin and either Ctl siRNA (left) or fidgetin siRNA [142]. FIDGETIN siRNA reduced (Student's t test ≥0.05) by more than 70% the GFP-fidgetin expression observed with Ctl siRNA, as evaluated by Western blotting (Fold change: Ctl siRNA: 1±0.104; fidgetin siRNA: 0.28±0.194, J) and immunocytochemistry (data not shown). Scale bars: (f): 20 μm; (h): 50 μm.
Fig 3: Vertebrate fidgetin depletion increases labile microtubule mass in the axon but does not alter microtubule number. A Cortical neurons that had been treated for 48 hrs with fidgetin or Ctl siRNA and then re-plated were treated with nocodazole (0.2 µg/ml) for 0 hrs, 30 min, or 4 hrs. Cultures were pre-extracted to release of free tubulin, and then prepared for IF or Western blotting. Fidgetin -depleted cultures had 52.75±8.9% greater microtubule mass compared to Ctl siRNA (one-way ANOVA p≤0.05). At 30 min nocodazole, microtubule mass decreased to roughly half (one-way ANOVA p≤0.05) compared to the nocodazole-free time-point (Ctl siRNA: 45.21±1.21% decrease; fidgetin siRNA: 54.71±15.92% decrease), with no difference in microtubule levels between the Ctl siRNA and fidgetin siRNA groups (one-way ANOVA p≥0.05). Fidgetin-depleted cultures and Ctl cultures also did not differ in microtubule mass (one-way ANOVA p≥0.05) after 4 hrs in nocodazole (Ctl siRNA: 31.97±5.46%; fidgetin siRNA: 34.78±10.27%). Quantification of microtubule mass in the axon by IF (B) indicates that fidgetin depletion increases the overall microtubule mass in the axon by 62%.
Fig 3 Continue (Ctl siRNA: 327.09±28.26; fidgetin siRNA: 530.55±34.88; one-way ANOVA test p≤0.05).

No differences were observed (one-way ANOVA p≥0.05) between fidgetin siRNA and Ctl siRNA after treatment with nocodazole (0.2 µg/ml) for 30 min (Ctl siRNA: 150.34±11.84; fidgetin siRNA: 167.36±17.66). C Neurons were transfected at day 0 with fidgetin or Ctl siRNA and re-transfected with EB3-GFP at the time of re-plating, so that dynamic plus ends of microtubules could be visualized as fluorescent comets (tracked as 1 frame per sec) during bouts of microtubule assembly. The 2 experimental groups did not differ (Student’s t test p≥0.05) in terms of comet number (Ctl siRNA: 44.70±14.43; fidgetin siRNA: 47.40±14.39), duration (Ctl siRNA: 10.35±0.46 sec; fidgetin siRNA: 10.82±0.33 sec) or speed (Ctl siRNA: 0.25±0.0073 µm/sec; fidgetin siRNA: 0.23 ±0.0098 µm/sec). D Schematic shows interpretation of data. *Drosophila* fidgetin behaves similarly to spastin or katanin, targeting the stable domain of axonal microtubules. Knockdown causes an increase in the proportion of the microtubule that is stable, without altering the length of the microtubule. Vertebrate fidgetin targets the labile domain of axonal microtubules, such that knocking down vertebrate fidgetin increases the length of the labile domain.
Fig 4: Fidgetin is sensitive to acetylation status of microtubules in the axon. A and a show ratio images of acetylated tubulin to total βIII-tubulin displayed in fire-scale pseudo-color (white being the highest intensity and red being the lowest). Fidgetin siRNA neurons (a) display decrease of acetylated to total tubulin ratio compared to Ctl siRNA. (A) same result, by Western blot analysis, with (b) quantification indicating that Ctl siRNA cultures have higher ratio of acetylated to total tubulin compared to fidgetin siRNA (fold change: Ctl siRNA: 2.1±0.29; fidgetin siRNA: 1; student t test p≤0.05). C-e Brain immunohistochemistry of acetylated tubulin (C and c) and total βIII-tubulin (D and d) Fig 4 (continue): from E18.5 wild-type mouse and fidgetin knockout/reporter mouse respectively. Knockout brain has a decreased ratio of acetylated to total...
tubulin as shown in figure (e) compared to wild-type (E). Quantification is shown in (F) as fold-change normalized to the value of fidgetin knockout ratio value as 1. Such difference was observed in all brain areas analyzed (the ratio value for the WT mouse are: olfactory bulb: 2.2; cortex: 2.2; septum 1.31; thalamus: 2.6; pretectum: 1.9). G and g show ratio images in fire-scale pseudo-color of neurons treated with 2.5 μM tubacin or DMSO vehicle (Ctl), and immunostained for acetylated tubulin and total tubulin. The ratio of acetylated to total tubulin is higher with tubacin treatment. H Western blotting of parallel cultures, also showing increase in acetylated tubulin. h RFL-6 fibroblasts prepared for IF visualization of acetylated tubulin after overnight expression of an enzymatically inactive mutant of αTAT-1 (called αTAT-1 D157N) or the wild-type αTAT-1. Wild-type αTAT-1 shows higher staining intensity of acetylated microtubules than the mutant. Similar results were obtained on neurons (data not shown). I morphological quantification of experiments similar to those from fig 2, except that microtubule acetylation was manipulated by either tubacin treatment or αTAT-1 overexpression. Ctl for the former and latter were DMSO vehicle and the αTAT-1 D157N, respectively. Neurons depleted of fidgetin treated with vehicle (DMSO) have significantly longer axons (175±11.04 μm; p≤0.05, one-way ANOVA), higher number of minor processes (10±0.91 p≤0.05, one-way ANOVA) compared to DMSO Ctl siRNA neurons (average axon length: 105±8.29 μm; process number: 5±0.49). With tubacin treatment, there were no morphological differences between Ctl (average axon length: 117±6.80 μm; process number: 4.85±0.37) and fidgetin siRNA (average axon length: 117±9.00 μm; process number: 6.26±0.64; p>0.05 one-way ANOVA for both measurements). Neurons depleted of fidgetin expressing αTAT-1 D157N have longer axons (146±9.78 μm; p≤0.05, one-way ANOVA) and higher number of minor processes (10±0.86; p≤0.05, one-way ANOVA) compared to αTAT-1 D157N Ctl siRNA neurons (average axon length: 96±8.62 μm; process number: 6±0.63). With αTAT-1 expression, there were no morphological differences between Ctl (average axon length: 98±8.80 μm; process number: 6±0.71) and fidgetin siRNA (average axon length: 110±7.21 μm; process number: 7±0.79 p>0.05; one-way ANOVA for both measurements). J shows the effects on morphology when GFP-fidgetin overexpressing neurons when treated with tubacin (2.5 μM). Neurons ectopically expressing GFP- fidgetin and treated with vehicle (DMSO) show reduced minor process number (3.4±0.27) and axon length (81±6.04 μm) compared to neurons expressing GFP in presence or absence of tubacin (minor process number: 5.1± 0.38 and 4.8± 0.38 respectively; axon
length 111±6.32 and 115±7.64 respectively p<0.05; one-way ANOVA). Scale bar (A), (g): 10 μm.; B: 100 μm. *indicates p value≤0.05, value expressed as average ± S.E.M.
Figure 5: Overexpression of fidgetin in fibroblasts does not result in the appearance of short microtubule fragments, regardless of tag on fidgetin construct. A rat fibroblast cell line (RFL-6) was transfected with plasmids coding for: (B) fidgetin-GFP (C-terminal GFP tagging); (C) GFP-fidgetin (N-terminal GFP tagging); (D) FLAG-fidgetin (N-terminal FLAG tagging); (A) empty vector; or (E) GFP-spastin. Cells were fixed and immunostained to reveal microtubules. As expected, overexpressed spastin resulted in severing of the microtubule array into short microtubule fragments. This effect was not evident in the case of any of the fidgetin constructs. Scale Bar, 10 µm
Figure 6: Individual siRNA sequences produce the same phenotype as when they are pooled. Morphological analyses (axon length and minor process number) were performed on primary cortical neurons transfected with the Ctl siRNA or the same amount of each single sequence as the total amount of the pool used for studies shown in the main article. The neurons transfected with Ctl siRNA have statistically lower numbers of minor process and shorter axons (3.79±0.195 and 109.82±4.89 respectively, p<0.05 one way ANOVA) compared to the neurons transfected with a single fidgetin siRNA sequence (fidgetin siRNA seq.1: 6.57±0.33 and 149.61±6.10 respectively; siRNA seq.2: 6.94±0.35 and 173.23±6.10 respectively; siRNA seq.3: 7.97±0.46 and 173.35±8.05 respectively; siRNA seq.4: 8.10±0.38 and 164.62±6.91 respectively). The sign * indicates a statistical p value ≤ 0.05; all values are expressed as average ± S.E.M.
Chapter II: Spastin mutations associated with hereditary spastic paraplegia promote axonal transport deficits through an isoform-specific mechanism that involves aberrant casein kinase II activation.
Introduction

Hereditary spastic paraplegia (HSP) is a debilitating disease of the central nervous system (CNS) in which patients suffer from spasticity and gait deficiencies that are usually adult-onset. Axons of the adult corticospinal tracts selectively degenerate in a dying-back manner. Mutations in SPAST (previously called SPG4), the gene that encodes a protein called spastin, are the most common cause of HSP, accounting for more than 40% of the cases [143]. Spastin is a member of a family of AAA enzymes that physiologically sever microtubules [144]. This is important because long microtubules in the axon must be severed into shorter pieces to undergo mobile events such as their ongoing transport down the axon during the life of the neuron [145, 146]. At least one isoform of spastin, called M1, has a hydrophobic domain capable of interacting with membrane, thus potentially enabling spastin to participate in membrane remodeling as well as microtubule severing.

Based on the autosomal dominant characteristics of SPAST-based HSP (termed SPG4-HSP) as well as the abundance of over 200 different mutations that give rise to the disease, most workers have favored a pathological mechanism based on haploinsufficiency [147]. In this view, there is insufficient spastin to carry out its normal functions. However, there are reasons to be skeptical that haploinsufficiency is an adequate explanation for the disease. There are no detectable flaws in neuronal development associated with spastin mutations [148], despite the fact that the demands for microtubule severing are greater during development than in the adult [149]. In addition, haploinsufficiency does not satisfactorily explain why degeneration occurs almost exclusively in the corticospinal tracts, nor does it account for the fact that a small number of pathogenic mutations are not function blocking [150].
A gain-of-function mechanism may better explain the pathology. In this mechanism, the mutated protein misfolds, and negatively impacts biochemical pathways relevant to the functioning of other proteins. \textit{SPAST} has two start codons, the first of which is less efficient [151]. The vast majority of spastin appearing throughout the body at all stages of development and in the adult is the shorter isoform, termed M87 in humans or M85 in rodents. Because of its hydrophobic domain (not shared by M87), M1 is especially prone to misfolding. In studies on squid axoplasm and cultured mammalian neurons, truncated or mutated M1 inhibited axonal transport and perturbed axonal outgrowth, whereas the corresponding truncated or mutated M87 did not [117, 152].

There is precedent for misfolded proteins exerting their toxicity by hyper-activating kinases, and for molecular motor proteins being among the most responsive targets for abnormal phosphorylation [153-155]. Here, we sought to ascertain whether the gain-of-function toxicity of mutant M1 is due to such a mechanism, and if so, to identify the relevant kinase.
Results

Mutated M1 spastin impairs fast axonal transport in squid axoplasm. Ours previous work [117] and other independent works recently published indicates how the expression of mutated spastin in neurons impairs organelle transport along the axon [156, 157]. In particular, we previously showed that the M1 specific isoform causes the most notable effect in fast axonal transport (FAT), meanwhile the shorter mutated isoform has a negligible effect. In this work we wanted to understand whether the impairment of axonal transport previously shown is phenomenon that can be caused by any pathologically relevant mutation of spastin, if there is an isoform-specific toxicity and which is the eventual underpinning mechanism of action.

We initiate to evaluate alteration on axonal transport by using the fast axonal transport paradigm in squid giant axon. We artificially translated several human M1 mutation of spastin: four missense mutation (M1E442Q, M1E112Q, M1L195, M1C448Y) and one truncation mutation (M1-STOP) and we infused nano-molar concentration of each single mutation in a separate axoplasm. In all the case, the infusion of mutated spastin causes drastic reduction on anterograde and retrograde transport in squid axoplasm in matter of minutes (M1E442Q impairment of 12% anterogradely and 15% retrogradely; M1-STOP impairment of 14.7% anterogradely, and 7.7% retrogradely; M1 C448Y impairment of 17% anterogradely and 15% retrogradely). When we infuse the correspondent mutation located this time in the shorter M87 isoform, no one mutation was capable to compromise FAT (M87E442Q impairment of 0.63 % anterogradely and 0.8-0% retrogradely; M87C448Y impairment of 0.2% anterogradely, and 8% retrogradely; M87-STOP impairment of 1.17% anterogradely and 0% retrogradely). These data indicate that the impairment on FAT induced by mutated spastin is isoform specific (occur in all the M1 isoform but not in the M87 isoform) and independently from the mutations (all the mutation equally affect FAT).
The alteration on axonal transport under the mechanistic point of view could be caused by a direct interaction of the mutated protein to the transport machinery or by secondary mechanisms caused by the activation of protein effectors, such as aberrant activation of protein kinases. To understand which one is the prominent phenomenon we choose two different mutations M1 we inhibit Casein Kinase 2 (CK2) in the perfusion buffer containing the mutated M1 mutation. For this purpose we chose two missense mutations: M1E112Q and M1C448Y and the truncated M1-STOP mutation. Treatment with relatively high concentration of CK2 inhibitor peptide reinstate the FAT to control level independently from the mutation (M1-STOP+ inhibitor peptide impairment 0% anterogradely and 0.83% retrogradely). To demonstrate the specificity on FAT by CK2 activation, and the absence of non-specific effect caused by the peptide, similar experiments were performed by using this time a different strategy of inhibition (5 µM DMAT, 2 µM TBCA chemical inhibitors). Also in this case the chemical inhibition of CK2 result in a prevention of FAT impairment in presence of mutated M1 isoforms (M1E112Q + TBCA impairment of 1.9% anterogradely and 0.8% retrogradel; M1-STOP + TBCA impairment of 6.25% anterogradely and 2.5% retrogradely).

**Expression of mutated M1 spastin increases CK2 activity.** Under our hypothesis, the presence of mutated M1 spastin would cause a large series of detrimental effects in neurons, one of which is the impairment of FAT, by over-activating endogenous CK2. To demonstrate the connection between these two elements we evaluate the expression level and the activity level of CK2 in cells stably expressing M1-STOP or M87-STOP spastin. We initially evaluate at the gene and protein level the expression of CK2, by using PCR and western blotting. Neuroblastoma cells expressing either M1-STOP or M87-STOP have equivalent level of expression of CK2 either at mRNA level (expression level normalized to cyclophilin: M1-STOP = 0.95; M87-STOP= 0.8), and western blotting level. However, the two cells line differs considerably in terms of activity of the enzyme. By performing a luminescence assay coupled to the CK2 activity from M1-STOP and M87-STOP
cell lysate, we observed a significant increase of activity in M1-STOP expressing cells (activity after 32 min: 2800 AFU) compared to cells expressing M87-STOP spastin or control (activity value of both after 32 min: 900 AFU). The absence of differences in terms of enzymatic activity between the control cell and the cells expressing M87-STOP indicate that the mutated M87-STOP protein does not trigger any inhibitory effect on CK2 activity. We also wanted to evaluate whether the regimen of CK2 inhibition used in many of our experiment, was sufficient to lower the enzymatic activity of CK2 in cells that express M1-STOP spastin. The use of 2 µM concentration of inhibitor was sufficient to significantly lower the CK2 level of M1-STOP expressing cells (2800 AFU and 300 AFU respectively).

Mutation on M1 spastin isoform causes a selective impairment of membrane bound transport via a CK2 dependent mechanism. The FAT data obtained in the squid axoplasm experiments shown that the expression of mutated M1 form of spastin causes an impairment of anterograde and retrograde transport of organelles along the squid axon. However the use of an invertebrate system limits our interpretation of the pathological mechanisms occurring in human pathology. For this reason we measure the kinetic of membrane bound organelle traffic in a eukaryotic system by using SH-SY5Y human neuroblastoma cell line. In particular, we measured possible alteration of membrane bound organelle trafficking by fluorescently labeling vesicles by the ectopic expression of Synaptophisin-RFP, and performing live cell imaging. We hypothesized that the stable expression of the M1-STOP isoform would result in a conspicuous impairment of membrane trafficking compared to our control cell line or cells expressing the less toxic M87 isoform. Coherently to our hypothesis, the expression of the M1-STOP isoform results in a diminution of the fraction of mobile vesicles (30 % of the total) compared to the fraction calculated in cell expressing M87 STOP isoform of spastin or control which mobile fraction was relatively higher (69.3% and 72.3% respectively). This evident M1-STOP specific impairment could be the result of M1 isoform-specific induction of CK2 activity, as suggested by our previously described data.
in invertebrate axoplasm. We evaluate the influence of CK2 in this process by treating the three different neuroblastoma cell lines with TBCA a potent CK2 inhibitor. The measurement by live cell imaging of membrane trafficking, indicates that the impairment of transport M1 dependent can be prevented by reducing the CK2 activity (fraction of mobile vesicles is 60% in M1 STOP expressing cells treated with TBCA, and 30% in the same cells treated with DMSO vehicle). Interestingly the treatment of TBCA alone does not directly affect membrane trafficking since the CK2 inhibition does not causes significant changes in M87 STOP expressing cells or control cell line (number of mobile vesicles in M87 + TBCA cell line 76% ; M87 + DMSO : 69.3%; Control + TBCA : 72%; Control + DMSO 72.3%).

Mutation of spastin causes mitochondria re-distribution in RFL-6 cells and primary cortical neurons. The data on vesicle trafficking observed in stably transfected neuroblastoma indicate that mutated M1 could impair membrane bound organelle transport via CK2 over-activation. A consequence of this impairment, is the altered distribution of several different organelles such as mitochondria, which itself could have important pathological implication. To investigate this particular aspect, we analyze the mitochondria distribution in RFL-6 cells transiently expressing one of the two separate isoforms of mutated spastin M1C448Y or M87C448Y. Mitochondria are organelles which distribution in the cells is heavily dependent on transport mechanism occurring along the microtubule array. In a physiological context, a typical fibroblast has great part of its mitochondria asymmetrically clustered in one pole of the cells and a smaller fraction widely dispersed in the remaining regions. By labeling the mitochondria with a fluorescent probe (orange Mitotraker ®) we observed that high percentage of cells have mitochondria that follow this distribution pattern (control, non-transfected cells 85% not dispersed). Transient expression of RFP or mutated M87 causes a slight diminution of this percentage (65% and 59% respectively), with no statistical difference between these groups, indicating that the general expression of an ectopic protein causes a slight impairment of membrane transport, independently from the kind of
protein expressed. However, the temporarily expression of the mutated M1 isoform causes a drastic alteration of mitochondria dispersion, reducing the number of cells that have a control-like mitochondria distribution to a much lower level (40\% of the total). These data indicate that the expression of the mutated M1 isoform influence the transport of mitochondria with a specific mechanism, not shared by the M87 counterpart. After we tested the possible involvement of CK2 over-activation also in this phenomenon, by treating the transiently transfected cells with vehicle or TBCA. Treatment of TBCA does not influence mitochondria distribution in our RFP transfected or non-transfected control cells (75\% and 14 \% cells with dispersed mitochondria respectively). Analogously CK2 inhibition did not affect the mitochondria distribution in cell expressing the mutated M87 spastin isoform (60\% dispersed cells in M87 C448YTBCA treated cells compared to 59\% in M87 vehicle treated). The action of CK2 on mitochondria distribution was instead evident in M1C448Y, in which CK2 inhibition causes a significant reversion of the mitochondria distribution phenotype, having a higher number of cells with a non-dispersed mitochondria (60\%), similar to the M87C448Y or RFP expressing cells.

A similar experimental paradigm was also used in primary cortical neurons, a better cellular model to study HSP. As observed in rat fibroblast the transient expression of the M1 C448Y mutated isoform of spastin causes mitochondria re-distribution in primary cortical neurons (35 \% cells with clustered mitochondria), compared to RFP transfected cells or M87C448Y transfected cells (36\% and 34\% of the total). Again, treatment with TBCA reduces the number of cells with dispersed mitochondria in M1C448Y over-expressing cells (35\%) and does not have significant effect in other over-expressing cells (M87C448Y + TBCA: 33\%; RFP + TBCA: 32\%). A general decrease of number of dispersed mitochondria was also observed in non-transfected neurons (non-transfected DMSO: 13\%, non-transfected +TBCA: 18\%).

Expression of Mutated M1 spastin causes Golgi dispersion as a sign of dynein inhibition. As in many other cell systems the organelle distribution and sorting depend upon the fine equilibrium
of their anterograde and retrograde transport, which is mainly orchestrated by conventional kinesin and dynein. As corollary, an imbalance of these transport mechanisms would result in altered distribution of organelles. One of the best-characterized examples of this re-distribution is the Golgi apparatus that appear disperse in correspondence of dynein inhibition [158, 159]. By using immunocytochemistry technique we investigated the possible alteration of dynein activity by performing a morphological analysis of Golgi distribution in stably transfected M1-STOP, M87-STOP and control SHS5Y cells. The cells expressing the M1-STOP isoform of spastin have a significant dispersion of Golgi apparatus (5.6% of total cellular area) compared to M87-STOP and GFP control (3.8% and 3.9% of total cellular area). To evaluate the dependence of this phenomena with an increase of CK2 activity we treated for 24 hrs the cells with TBCA. The inhibition of CK2 is capable to re-induce the physiological clustering of Golgi apparatus in M1-STOP cells (M1-STOP +TBCA: 3.8% total area), but does not have a significant effect in cells expressing M87-STOP spastin or GFP (3.8% total area, 3.8% total area respectively).
Discussion

Haploinsufficiency remains the most popular explanation for SPG4-HSP, and not without good reason. Over 200 different SPAST mutations that include missense, nonsense and single amino acid substitutions, all give rise to the same constellation of symptoms. Abundant evidence suggests that the functions of spastin are regulated by the amount of spastin expression, which is consistent with reductions in functional spastin levels being detrimental to neurons [157, 160]. Certain mutations would be expected to cause unstable mRNA and hence result in little or no mutant spastin protein, and in some patients the entire SPAST gene is deleted [161, 162]. Despite all of this, there are persistent reasons to question that haploinsufficiency is a sufficient explanation for the disease. For example, there is no correlation between the degree to which a mutation disables spastin function and the severity of the disease [163]. As for the predicted near absence of the protein with mutations expected to produce unstable mRNA, there is precedent for very low levels of mutant proteins, too low to be detected by conventional techniques, still being sufficient to cause neurodegeneration [164, 165]. Moreover, the only evidence on the lack of detectable mutant spastin in patients comes from cells that are not corticospinal neurons [129]. In fact, the one SPG4-HSP patient whose post-mortem spinal cord tissue has been investigated showed a major band on Western blots corresponding to mutant M1 [166]. As for patients with SPAST deletions, such deletions can also result in deletions of neighboring genes [167], and hence the situation in these patients is not straightforward to interpret.

Despite the controversy, most model systems in the HSP community have been SPAST knockouts or knockdowns, and hence when using these models, the logic becomes circular as to whether haploinsufficiency is the mechanism of the disease. For example, two different SPAST knockout mouse models have been generated [168, 169], as well as knockout or knockdown flies [122, 124, 170]. In a recent paper aimed at pharmacological rescue of HSP phenotypes, various different
models were used including *C. elegans*, *Drosophila* and zebrafish, and in all cases, the animals were *SPAST* knockouts (Julien 2016). The question becomes whether these lines of research are really modeling the disease or whether they are only modeling the phenotype of spastin knockout. In our own studies on *Drosophila*, expression of human mutant spastin caused a stronger HSP-like phenotype than knocking out endogenous spastin [152]. The same is true in cultured mammalian neurons, comparing spastin knockdown with expression of the pathogenic mutant spastin [117, 128, 150, 152]. The two different *SPAST* knockout mouse models mentioned above show almost no phenotype [168, 169], whereas a mild HSP-like phenotype has recently been shown in homozygotes of a new mutant mouse into which an HSP-like mutation was introduced into an endogenous mouse *SPAST* gene [171]. If haploinsufficiency were the more logical conclusion on the basis of the mouse models, the knockout mouse should display a stronger phenotype than the animal with half-normal and half-mutant spastin, and yet the opposite is true.

To date, mechanism-based therapeutic ideas for treatment of *SPG4*-HSP have centered on the use of microtubule-active drugs such as vinblastine, used at very low concentrations [170, 172, 173]. The premise has been that neurons with too little spastin suffer from hyper-stabilized microtubule arrays, because spastin normally pairs back the stable domains of axonal microtubules. Indeed, experimental knockdown of spastin in animal and cell culture models results in higher levels of stable microtubules [129]. Vinblastine used at nanomolar concentrations is a “kinetic stabilizer” of microtubules, meaning that it permits slower than normal subunit exchange of the microtubule with the free tubulin pool, but does not permit net elongation of the microtubule [16]. Thus the reported therapeutic benefit of vinblastine on spastin haploinsufficient cells is confusing, as the drug would not be expected to destabilize the microtubules as other workers have suggested, but rather to kinetically stabilize them. In our recent study, mutant spastin that contain the microtubule-binding domain were shown to bind to microtubules, especially in the perikaryal region of cells, and stabilize the microtubules [152]. However, the unbound mutant spastin (both
M1 and M87) had the inverse effect, namely to destabilize microtubules. Our present studies on our new neuroblastoma cell lines provide a hint of microtubule destabilization as a result of mutant spastin expression, as there is a minor elevation in the proportion of tubulin that is tyrosinated. Given that the microtubule destabilization effects documented here and in our previous study are not M1-specific, there is reason to doubt that microtubule destabilization is the primary gain-of-function effect of mutant spastin, assuming that it is mutant M1 that accumulates and not mutant M87.

Deficits in axonal transport are associated with various neurodegenerative diseases [174-176]. A deficit in axonal transport may be especially problematic for the corticospinal tract because their axons are among the longest in the body [177]. Impairment of axonal transport has been documented not only in HSP associated with SPAST mutations [157, 172], but also in HSP associated with mutations of other genes [178-180]. Our previous work infusing squid axoplasm with truncated mouse spastin demonstrated that axonal transport is impaired by truncated M1 but not by comparable levels of truncated mouse M85[117]. Here, instead of using mouse spastin and instead of using a truncation to approximate pathogenic mutation, we used human spastin with actual mutations (such as E442Q, E112Q, L195I and C448Y) that occur in HSP patients. In all such mutations tested, infusion of mutant M1 impaired axonal transport in squid axoplasm, while the corresponding mutant M87 did not. Consistent with these data, alterations in vesicle transport were observed in neuroblastoma cells expressing truncated M1 but not truncated M87, and the same was true of organelle mis-localizations in fibroblasts expressing mutant M1 but not mutant M87.

How might mutant M1 inhibit axonal transport and cause organelle mis-localization? It was previously speculated that mutant spastin bind along microtubules and prevent them from interacting with molecular motor proteins [181]. We think this is an unlikely explanation because some mutant spastin do not have a microtubule-binding domain, and the squid work demonstrates
that mutant M1 is toxic to axonal transport at levels too low to do so simply by coating the surface of microtubules. The stoichiometry is also problematic for mutant spastin molecules to directly interact with and thereby inactivate molecular motor proteins. It is more feasible that an amplification process would be involved to magnify the biological action of mutated spastin on molecular motor proteins. There is precedent for aberrant activation of protein kinases by disease-related misfolded proteins such as Huntingtin [155], Presenilin [182] and fibrillar Tau [183]. While the theme is the same, the particular kinase varies, depending on the disease. Our present studies demonstrate the reversal of the mutant M1-specific transport deficits and organelle mislocalizations by inhibitors of CK2 but no other kinase tested, and this was true for a range of different SPAST mutations. CK2 is known to phosphorylate both cytoplasmic dynein [184] and kinesin-1 [185], and at least in the case of the latter, in a manner that mediates its release from vesicles. These results are consistent with a scenario whereby mutant M1 elicits its effects on axonal transport and organelle distribution by aberrant phosphorylation of molecular motor proteins resulting from hyperactivation of CK2. On this basis, we propose that CK2 inhibitors may offer a novel strategy for alleviating symptoms of SPG4-HSP, although we acknowledge that the inhibitors would have to be delivered in moderation and targeted to the particular neurons that suffer most, given that CK2 is important for many different signaling events.

In the case of tau fibrils, it has been suggested that the relevant domain of tau that causes kinase hyperactivation normally influences motor protein phosphorylation in a regulated fashion [183]. The same general principle may apply in the case of M1 and CK2, with M1 normally influencing the phosphorylation of CK2 targets. M1 is believed to insert into membranes such as endoplasmic reticulum and endosomes, and contribute to their shape [142, 186, 187]. Perhaps M1 does so in part by influencing the phosphorylation of CK2 targets relevant to membrane modeling. Of course, M1 can also join with M87 to form the hexamers that sever microtubules, and CK2 targets might also be relevant to that process. Reid’s laboratory has posited that spastin might functionally
link microtubule severing to membrane modeling during axonal branch formation and cytokinesis [188], and it is not unreasonable to surmise that such a linkage may involve CK2 targets such as molecular motor proteins.
Material and Methods

*In vitro* translation. For studies on vesicle motility in squid axoplasm, spastin polypeptides were produced by *in vitro* transcription/translation (Promega; TnT T7 coupled Reticulocyte Lysate system), as previously described [189, 190]. Typically, 1-2 µg of plasmids were transcribed in a 50 µl reaction mix, following the manufacturer’s procedures. *In vitro* translation products were briefly centrifuged to eliminate translation machinery, and supernatants were frozen in liquid N\textsubscript{2} until use. Spastin polypeptides were typically perfused at 1-10 nM levels. To ensure that the same levels of spastin peptides were perfused for each experimental condition, parallel reactions were performed using \textsuperscript{35}S-labeled methionine (Amersham), and relative levels were quantified using phospho-imaging scanning methods [190].

Vesicle motility assays in isolated squid axoplasm. Axoplasm was extruded from giant axons of the squid *Loligo pealeii* (Marine Biological Laboratory) as described previously [191-193]. Axons were 400-600 µm in diameter and provided ≈5 µl of axoplasm. Recombinant *in vitro* translated spastin peptides were diluted into X/2 buffer (175 mM potassium aspartate, 65 mM taurine, 35 mM betaine, 25 mM glycine, 10 mM HEPES, 6.5 mM MgCl\textsubscript{2}, 5 mM EGTA, 1.5 mM CaCl\textsubscript{2}, 0.5 mM glucose, pH 7.2) supplemented with 2-5 mM ATP. 20 µl of this mixture was added to perfusion chambers [194]. Preparations were analyzed on a Zeiss Axiomat with a 100X, 1.3 n.a. objective, and DIC optics. Hamamatsu Argus 20 and Model 2400 CCDs were used for image processing and analysis. Organelle velocities were measured with a Photonics Microscopy C2117 video manipulator (Hamamatsu). All experiments were repeated at least 3 times. Unless otherwise stated, the data were analyzed by ANOVA followed by post-hoc Student-Newman-Keul’s test in order to make all possible comparisons.
**Cell culture.** Primary embryonic rat cortical cultures were obtained from rat embryo at age E18-E20 following the same culture procedure described in our previous work [103]. Briefly, rat cortices were separated from the dissected brain, and neurons were dissociated in a single suspension prior to be plated in a 35 mm glass bottomed dish (14 mm diameter In Vitro Scientific™) at a density of 25-35.000 per dish. The glass bottoms of the dishes had been coated with poly-L-lysine (P2636, Sigma) prior to adding the cells. Neurons were kept in DMEM-astroglia conditioned media and transfected at 48 DIV, and morphological analysis was performed 24 h post-transfection. RFL-6 rat fibroblasts were cultured in F12K/FBs 10% media as previously described [152]. Two novel stably transfected cell lines based on mouse neuroblastomas (N2A) cells were generated, one which expresses human truncated M1 and the other which expresses the corresponding human truncated M87.

**Transient transfection of cultured cells.** Adherent primary cortical cells were transfected by using Cellaxess CX1 device (Cellectricon) allowing the analysis of neuronal structures such as axons, neuritic processes and neuronal contacts, formed before the ectopic expression of the interest protein. We follow the transfection protocol previously described from our lab [195] with some minor modifications on culture condition and total amount of DNA used. In particular, 18 µg of DNA was used per electroporation, and 3 individual electroporation per dishes was used to allow the efficient transfection of an average 5-10 neurons per dish. The transfection methods, created areas of distressed cells that were carefully avoided in our analysis. Neurons were transiently transfected with plasmid encoding with mutated M1C448Y or M87C448Y human spastin or control RFP. In this experimental regimen 30 cells per condition in each experiment was used.

Adherent RFL-6 cells were transfected by using Lipofectamine 2000 (Invitrogen), using a procedure previously described from our lab [138]. Cells were plated 48 hrs prior transfection at
density of 10,000 cells per dish, 1 µg of DNA /2.5 µl of Lipofectamine® was used per dish. This method allows an efficient transfection of 10-15% of the overall cell population in each dish. For analysis of ectopic expression of mutated spastin (M1C448Y; M87C448Y; or control RFP) an average of 100 cells per condition per experiment was used.

**Kinase inhibition.** As for the cultured cells, they were treated with vehicle (2 µl of DMSO) or TBCA (Calbiochem®, 218710), a cell permeable competitive inhibitor of Casein Kinase 2 for 24 hrs.

**Western blotting and immunocytochemistry.** For immunocytochemistry, cells were fixed with a microtubule stabilizing buffer (PHEM) Formaldehyde 4%, and glutaraldehyde and extracted post-fixation with 0.1% Triton. All the quantitative immunofluorescence data were obtained by using identical exposure setting allowing comparison across groups. For western blotting was performed by using standard protocol using as GAPDH to balance loading. For immunocytochemistry and western blot we use the following antibodies: Anti-Acetylated tubulin (Sigma T-6793); Anti-Tyrosinated Tubulin (Millipore, Mab1864); Anti-βtubulin (Abcam, Ab6046) Anti-GM130 (Abcam ab52649).

**Mitochondria distribution analysis.** Mitochondria visualization was performed by incubating neurons or fibroblast with a working solution of Green Mitotracker® at the final concentration of 20 nM for 20 minutes in 37C. In this analysis we considered *clustered* a mitochondria distribution in which great part of the organelle localize asymmetrically in one pole of the nucleus. We consider instead a mitochondria distribution as “dispersed” a spatial organization of these organelles in a disseminate pattern, losing the characteristic asymmetrical, perinuclear clustering.

To perform vesicle trafficking analysis in human neuroblastoma cell line, cell were transfected by using a nucleofector device in a cell suspension system as already described previously [103]. After transfection cells were plated at density of 15,000 cells per dish and analysis of RFP
fluorescently labeled vesicles were obtained 48 hrs post-transfection. For the analysis of vesicle trafficking an average of 30 cells per condition per experiment was used.

**Live cell imaging.** Vesicle transport assay experiment was performed by using live cell imaging techniques. To visualize movement of membrane bound elements, neuroblastoma cells were transfected with RFP-Synaptophysin. Movies of mobile vesicles were obtained by using the following parameters modified by another laboratory [196]: 5 frame per second for a total length of 20 seconds. We consider transport events occurring in neuritic processes which have a minimum length of 30 mm. We exclude the first 5 mm of neurite most proximal to the cell body. Any traceable vesicle that moves for more than 3 mm during the overall length of the movie was considered as mobile. Conversely any vesicle moving for less than 3 mm during the entire analysis was considering as stationary.

**Statistical Analyses.** All of the data analyses, statistical comparisons, and graphs were obtained by using SPSS 20 (IBM) and Excel (Microsoft). Data represent, if not otherwise specified, mean ± SD. Mean difference was considered to be significant at the 0.05 level (*p ≤ 0.05). Multiple group comparison was performed by one-way ANOVA followed by Bonferroni post hoc, and for pair comparison, Student’s t test.
Figure 7: Effects of M1 and M87 mutant spastin on axonal transport. (A) Schematic representation of mutant spastin proteins used in this study. Specific domains are indicated in the top graph. The ATPase associated with various cellular activities (AAA, in red), microtubule-binding (MTB, in yellow), and microtubule-interacting and trafficking (MIT, in X) domains are shown, as well as the nuclear localization [125] and export (NLS) signals are indicated. M1 spastin mutant proteins were generated by mutating M87 to in plasmid cDNA templates (red asterisk)*, whereas M87 spastin proteins were generated by mutating the M1 residue (red asterisk)*. The locations of non-sense and missense mutant residues are indicated by a red asterisk. Note that truncated M1-STOP and M87-STOP lack the MTBD. (B) In vitro translation of cDNA constructs in A was performed in the presence of 35S-methionine. Autoradiogram analysis of reactions after SDS-PAGE confirms expression of M1 and M87 mutant spastin proteins. Control reactions (no cDNA) did not result in detectable protein synthesis. (C-H). Plots depicting results from vesicle motility
assays in isolated squid axoplasm. In vitro translated reactions as in B were perfused and fast axonal transport (FAT) rates monitored by video microscopy*. Individual velocity measurements (arrowheads) are plotted in function of time. Anterograde (blue arrows and line) and retrograde (reverse green arrows and line) FAT rates are shown. Perfusion of spastin mutants M1-C448Y (C), M1-E442Q (D), inhibited both anterograde (kinesin-1-dependent*) and retrograde (cytoplasmic dynein-dependent) FAT rates. In contrast, their corresponding mutant M87 isoform counterparts showed no effect on FAT (E and F). Similar results were observed using M1 and M87 versions of HSP-related E112K and L195V mutants (see Suppl. Fig. 1). Inhibition of FAT by M1-STOP (G), but not M87-STOP (H) truncated spastin further suggested a toxic mechanism independent of spastin binding to microtubules. Quantitation of data in (I) indicates that the inhibitory effect of mutant spastin on FAT is M1 isoform-specific. n: number of independent experiments.
Figure 8: Cytotoxic effect of mutated M1 spastin in vertebrate cells. Ectopic expression of truncated form of spastin (M1/M87 STOP) or missense mutation (M1C448Y) causes a series of detrimental effect in different cellular compartments. A Human neuroblastoma cell line (SHS5Y), stably transfected for truncated M1 isoform
**Figure 6 (continue):** isoform of spastin (M1-STOP) or M87 (M87-STOP), were transiently transfected with a RFP-Synaptophysin plasmid to measure vesicles trafficking along processes. Ectopic expression of M87 isoform of spastin does not significantly change the vesicles trafficking compared to our control cell line ectopically expressing GFP (fraction of mobile vesicles in M87-STOP: 78.5 ± 4.1%, GFP control: 69.5 ±5.5%). Conversely the expression of M1-STOP mutation decreases the vesicles trafficking reducing the number of moving vesicles of about the half of the value of GFP control (42.5 ±5.9%). The impairment of vesicles transport is evident by comparing two representative kymograph of vesicles transport from a cell expressing M1-STOP (top left panel) and M87-STOP (lower panel). **B** Effect on Golgi distribution on the same cell line described in A. The expression of mutated M1 causes an expansion of its surface (M1 STOP: 5.6 ± 0.47 % Golgi surface over the total cell area area) compared to control and cell expressing the less toxic M87-STOP isoform (3.8 ± 0.15 % and 3.9 ± 0.11 % respectively), suggesting a possible dynein inhibition mechanism of action. **C** Stably-transfected SH-SY5Y cells expressing M1-STOP and M87 STOP were metabolically labeled using radiolabeled P 32, and lysates derived from these cells processed for immunoprecipitation using antibodies that selectively immuno-precipitate conventional kinesin (kinesin-1) and cytoplasmic dynein (CDyn). Autoradiogram analysis of whole cell lysates confirmed similar levels of P32 incorporation for M87- STOP and M1-STOP- expressing cells. However, autoradiogram analysis of immune-precipitates revealed increased phosphorylation of kinesin heavy [197] and light chain (KLCs) subunits, as well as dynein intermediate chain (DIC) subunits in cells expressing M1-STOP, compared to M87-STOP cells. **B** Quantitation of data in A indicates a 43%, 27% and 40% increase in net phosphorylation of KHC, KLC, and DIC, respectively. **C** Western blot analysis confirmed similar levels of KHC and DIC expression for M87-STOP and M1-STOP- expressing cells.
Figure 9: Inhibition of axonal transport by M1 spastin mutants is mediated by CK2. Results from vesicle motility assays in co-perfusion experiments. When co-perfused with M1-STOP, the pharmacological CK2 inhibitor TBCA (200 nM) completely prevented the effects of M1-STOP (A) on FAT (compare to Fig. 5C). Extending these findings, the inhibitory effect of M1-STOP on FAT was also prevented by the
unrelated CK2-specific peptide substrate (CK2pept, 500µM) that acts as a competitive inhibitor (B). Further, TBCA also prevented the inhibitory effect of M1-C448Y (C) and M1-E442Q (D) on FAT. Together, these results indicate that CK2 mediates the toxic effect of mutant M1 spastin on FAT. n: number of independent experiments.
Figure 10: Constitutive expression of truncated M1 human spastin causes organelle distribution and transport alteration through a CK2-dependent mechanism. Human neuroblastoma cell line SHS5Y constitutively expressing truncated M1 spastin (M1 STOP) and treated with vehicle DMSO (C), (H) have a significant increase of Golgi dispersion compared to cells expressing shorter truncated isoform M87 STOP (A), (H) or GFP treated with DMSO (M1 STOP+ DMSO: 5.6 ± 0.47 %; control GFP+DMSO : 3.8 ± 0.15 % and M87 STOP +DMSO3.9 ± 0.11 % Golgi surface over the total cell area area). Selective blocking of CK2 activity via chemical inhibition by TBCA revert the dispersion phenotype bringing the Golgi morphology back to normal (M1-STOP  +TBCA: 3.8±0.26 ; GFP +DMSO: 3.9±0.38). Neither TBCA nor M87 expression seems to affect significatively the Golgi morphology in this cell line (GFP +TBCA: 3.6± 0.329; M87 STOP + DMSO 3.8±0.49 ; M87 STOP +TBCA: 3.8±0.268, (H)). (E-G, I) Expression of the M1-STOP causes also a vesicle transport impairment in the same cell line, here
measured as percentage of vesicles moving along processes (M1-STOP +DMSO (F): 42.53 ± 5.56; (E)M87 STOP +DMSO : 69.49 ± 5.7; GFP +DMSO : 78.25 ± 4.1). This effect is also reverted by TBCA, which increase the number of moving vesicles in M1-STOP expressing cells (G) 59.68 ± 10.71). Treatment with TBCA has negligible effect on the other experimental groups (I) M87 STOP +TBCA: 51.91 ± 5.5; GFP +DMSO: 65 ± 5.5).
Figure 11: Mutated M1 spastin alters mitochondria distribution in neuronal and non-neuronal cells.

Analysis of mitochondria distribution in Rat fibroblast transiently over-expressing M1 C448Y (Ac), M87 C448Y (Aa) or RFP. Expression of M1C448Y with DMSO vehicle (Ac), results in an increase of mitochondria dispersion (number of cell with dispersed mitochondria: (62% ± 3.61), meanwhile expression of the shorter mutated isoform M87C448Y treated with DMSO (Aa) causes minimal mitochondria re-distribution (41±4.16%) comparable to the effect obtainable by expressing RFP (and 31.6 ± 2.08 %, (C)). Treatment with specific CK2 inhibitor (TBCA) reverses the redistribution effect observed in M1C448Y (Ad) over-expressing cells lowering the number of cells with dispersed mitochondria to level similar to GFP untreated cells (38.6 ± 6.65 (C)). Casein kinase inhibition has a negligible effect on distribution pattern ((C)RFP+TBCA: 30.6 ± 11.62; (Ab) M87C448Y +TBCA: 40.56 ± 10.6) . (Ba-Bd)The reversion of the mitochondria distribution phenotype induced by M1C448Y via a CK2 mechanism has been observed also in primary cortical neurons ((Bc) M1C448Y + DMSO: 63.3 ± 3.21; (Ba)M87C448Y + DMSO: 37.0 ± 4.0;
RFP + DMSO: 12.28; (Bd) M1C448Y + TBCA: 38 ± 2.64; (Bb) M87C448Y + TBCA: 32.67 ± 3.52; RFP + TBCA: 14.03 ± 11.27 (D)).
Figure 12: Mutated spastin has a minor or negligible effect on microtubule PTMs: A-D A minor increase (roughly 30%) of tubulin tyrosination has been observed by immunocytochemistry in cells expressing M1-STOP isoform of spastin (2.6 ± 0.11 tyrosine-tubulin /total tubulin ratio) compared to control cell line and cell expressing the shorted M87 isoform (1.94 ± 0.11 and 1.92 ±0.15 ratio value respectively). Treatment with TBCA does not alter significantly acetylated tubulin ratio in any groups (M1-STOP + TBCA: 2.1 ± 0.10; M87-STOP + TBCA: 2.0 ± 0.10; CTRL GFP + DMSO: 1.95 ±0.08). (E-F)
Quantitative Western blot (top right panel) shown no significant changes on tubulin acetylation. The basal level of tubulin acetylation, here expressed as ratio for total tubulin, does not significantly change between control cell line and M87 STOP + DMSO (1.18 ± 0.47) and M1-STOP + DMSO (0.9 ± 0.31). No significant changes were also observed after treatment with TBCA (Control + TBCA: 0.9 ± 0.25; M1-STOP + TBCA: 1.0 ± 0.35; M87-STOP + TBCA: 1.2 ± 0.29).
Recommendation and final remarks

Microtubule organization and regulation play a pivotal role in the physiology of neuronal development in the adult and developing nervous system. Morphology, appropriate connectivity, intracellular and cellular movements are all essential phenomena for the normal neuronal functionality which function would be just not possible without the presence and re-arrangement of microtubules [6].

During the life of any organism, neurodevelopment is the time where incredible complex morphological and physiological changes occur in a very short time frame. However the importance of the cytoskeleton, particularly microtubule cytoskeleton, is not limited to the developmental phase of nervous system, but it is absolutely necessary during the entire life of the organism. In this case the morphological changes and related physiological alterations are still intensively occurring, but on the light of fine scale tuning, where subtle changes are capable to induce great alteration on neuronal functionality [198-200].

As already mentioned, microtubules, thanks to their plastic configuration, are key elements for directing a large series of processes in the nervous system [6]. This conditional plasticity, is the result of a balanced interaction between several regulatory proteins which will shift the microtubule organization toward a more dynamic polymer or more stable structure during a particular spatial-temporal window [7].

Microtubule-severing proteins are key regulators of microtubule cytoskeleton, and by reflection, of numerousness neuronal functions [106, 109]. In this dissertation we have focused on two members of this family in two different scenarios: the role of fidgetin in developing of the CNS and the role of spastin in neurodegeneration.

In neurodevelopment our present study indicates that fidgetin depletion from vertebrate neurons is accompanied by an increase in the axon dynamic of microtubule mass. We pose that fidgetin is present in the axon of vertebrate neurons to manage the length of the labile domains of the
microtubules, and thereby also control the relative proportions of labile and stable microtubule mass. In this fashion, we speculated that fidgetin contributes to regulating axon growth in the fetus, as well as the cessation of growth when an axon has reached its target or the pruning back of supernumerary axons or axonal branches that occur during development. The way by which this occurs is potentially complex, because the labile domains of microtubules are not only more dynamic, but are also very different compositionally than the stable domains. Stable domains are rich in post-translationally modified tubulin subunits [44] that establish the affinity of the microtubule for various molecular motors as well as other proteins that influence the various duties and properties of the microtubule array [201]. Thus changes in the levels or activity of fidgetin in the axon can have a profound impact on the axon at many levels. We have not studied dendrites in the present studies, but minor processes become dendrites at a later stage [12], and hence the role of fidgetin in determining the number of minor processes could become a major factor in the complexity of the dendritic arbor as well.

Spastin and katanin preferentially sever microtubules in their stable domains [129]. A break in an axonal microtubule’s stable domain will create two new microtubules, each with a stable domain that will ensure that it does not completely depolymerize [130]. Katanin and spastin are important for increasing the number of microtubules in the axon and also for supplying the axon with microtubules that are sufficiently short so that they can undergo rapid transport events [145]. For these reasons, depletion of katanin or spastin is deleterious to axonal growth, while their mild overexpression can increase axonal growth and branching [104, 128, 129]. Compared to katanin and spastin, vertebrate fidgetin appears to be an unusual microtubule-severing protein. When overexpressed in cells, vertebrate fidgetin does not create obviously greater numbers of short microtubules in neurons or other cell types we have studied. We suspect that when vertebrate fidgetin is overexpressed, an elevation in the severing of labile domains of microtubules occurs, but is not obvious unless the cell is in a phase of growth, such as building an axon, wherein labile domains expand rather than maintain a certain length. In the latter instance, over-expressed fidgetin
would tamp back the growth of labile domains such that fewer processes would form and the axon would grow more slowly, and this is exactly what we observed.

While katanin and spastin activity is important for generating new microtubules to underlie axonal growth [118], their activity could also prune back the levels of stable microtubule mass [104]. This is presumably why inhibition of any of the severing proteins in Drosophila results in a greater number of axonal branches and synapses [122, 136]. Inhibition of vertebrate fidgetin might well also result in overgrown axons with more synapses, not because of abnormal microtubule stabilization, but rather because of uncontrolled expansion of the labile domains of microtubules without their normal paring back process. However, while these phenotypes may have morphological similarities, the underlying microtubule arrays would be very different in terms of their composition and stability properties. Recently it was discovered that the third most commonly mutated gene in autism is katanin-like2, a protein closely related to katanin [202, 203]. If the mutations in this protein are causative of autism, the mechanism may be a result of too many synapses resulting from insufficient severing of microtubules either in their stable or labile domains. Future anatomical studies on the brain of the fidgetin knockout mice as well as future studies on patients with autism or animal models with katanin-like-2 mutations will be of enormous interest. We suspect that a more thorough understanding of the microtubule-severing proteins within humans will be instrumental to a better understanding of certain developmental disorders such as autism.

Depression of fidgetin levels and boosting the overall dynamic microtubule mass could be beneficial also for another class of clinical applications such as degeneration of CNS neurons. A growing number of work indicates the tight correlation between microtubule alterations and neuronal degeneration caused either by external insults (traumatic such as spinal cord injury or brain injury [204-207]) or internal (such as hereditary neurodegenerative diseases [208, 209]). Neurodegenerative diseases are often associated with a decrease in microtubule levels, which is believed to cause a series of pathological effects such as impairment of axonal transport, extreme
reduction of dendritic spines and aberrant mis-localization of organelles especially at the mitochondria level [209, 210]. All these alterations will ultimately cause the premature death or loss of functionality of neurons. A good example is amyotrophic lateral sclerosis (ALS), in which the expression of mutated SOD is related with an increase of Reactive Oxygen species (ROS) of a NO radical [211]. Liberation of such high reactive species hits particularly sensitive targets such as tubulin, which in the nitrosylated form, has a dramatic change on its polymerization rate causing an overall impoverishment of microtubule level in the cell. The pathological nitrosylation has also a drastic consequence in the capability of Dynein to bind tubulin and consequently to correctly dock on to microtubules [212]. It is believed that the convergence of those two events, the reduced microtubule mass, indispensable for axonal transport, and reduced binding on molecular motor proteins could greatly contribute to the detrimental effect of mutated SOD in ALS [211].

Even more interestingly, in Alzheimer disease and other related cognitive dysfunction such as Tauopathy, the altered tau protein functions through a not-yet-defined mechanism (gain/loss of function), resulting, again, in a net loss of microtubules [213, 214]. This microtubules loss leads to consequential alteration of intracellular transport in the axonal compartment, and a drastic diminution of synaptic spines in the dendritic component [214, 215].

Therefore, it appears that a common denominator for many (if not all) neurodegenerative diseases is the loss of microtubule architecture, and regulation in neurons [209-211]. This aspect, especially in the last decade, has become an object of intense attention to the scientific community due to the possible translational aspect. In several clinical treatments, especially for anti-cancer therapy, the use of microtubule-targeting drugs, such as taxol that could change the dynamic profile of microtubule, becomes a very common and very well characterized clinical treatment [216].

However, the clinical potential of this class of drugs has recently been extended to the neuroscience field. In particular, CNS permeable derivatives of taxol seem to be particularly promising for the treatment of several neuropathological conditions [217]. The reasons are various: first for the potential wide range of applications of these drugs in different neurological diseases. Indeed, very
different neurological pathologies, from CNS injury to neurodegeneration, are commonly characterized by a selective loss of microtubules. The use of a taxol derivative could collectively correct all these diverse conditions independently from their nature [217-219]. Another advantage is the safety profile of these drugs: their extensive use in oncological applications allows a thorough characterization under the pharmacokinetic and toxicological profile, making this drug safer for clinical purposes [220].

Because of this, during a relatively short amount of time, a large number of effort has been focused on testing the putative beneficial effect of microtubule-stabilizing agents (taxol and derivatives) to contrast the pathological loss of microtubules in degenerating neurons. The use of this approach has brought several promising results, Zhang in 2005 demonstrated the beneficial effect of epothilone D, a CNS permeable form of taxol, under the histological (protein deposits), behavioral (motor improvement) and cellular (axonal transport) aspect [221]. Few years later Sengottuvel observed some degree of functional recovery and nerve regeneration in spinal cord injured rats [222, 223]. However, the discontinuation of clinical trials for treatment of mild Alzheimer patients (ClinicalTrials.gov Identifier: NCT01492374) could suggest more than just a problem of the pharmacokinetics of this drug. A note of concern has already been mentioned in the past by our lab regarding the balance of risk over benefit of this therapeutic approach [16]. Taxol and related drugs decrease the depolymerization rate of microtubules, a process that could counteract the pathological loss of microtubules. However, the functionality of microtubule related processes depend upon the contemporary presence of dynamic and stable microtubules at the same time. Although the increase of microtubule mass would compensate for the pathological reduction, the taxol treatment would cause a dangerous conversion of a large part of the dynamic domains in stable domains, that is not compatible with many important neurological processes. An example is EB3: this protein is a plus end tip binding protein that is associated with the most extreme tip of the dynamic domain of microtubules [224]. The dramatic reduction of dynamic microtubules causes a dose-dependent ablation of EB3 protein bound to the plus tip of microtubules. Considering that the presence of
EB3-microtubule complex is absolutely required for normal functioning of dendritic spine, the dramatic reduction of these elements via taxol would preserve the neuronal cytoskeleton, but would not allow the expression of important functions such as spine re-arrangement [225].

Another important process vital for the proper neuronal functioning and that can be severely changed by taxol treatment is the axonal transport [226]. As already mentioned, molecular motor proteins responsible for intracellular transport recognize the microtubule substrate in virtue of PTMs, such as acetylation. Several reports indicate that taxol treatment causes a substantial increase of acetylation [227]. This indirect effect could have several consequences on microtubule related transport for two reasons: one because the change on PTMs could affect the propensity of the molecular motor protein to transport cargo along microtubules; and the second because PTMs can change the affinity for MAPs to bind and eventually protect / depolymerize the microtubule. An example is the relation between acetylated tubulin, tau and katanin. Increase of acetylation would cause a reduction of affinity for tau to bind microtubules, making it more accessible to katanin, which has a preference for acetylated microtubule causing a potential aberrant severing and impairing the delicate intracellular transport [119].

In our work with Fidgetin, we speculate that we could use another translational strategy to compensate the loss of microtubule mass occurring in these diseases, without passing through the concerns that carries the taxol treatment. The hypothetical ablation of fidgetin in a pathological scenario, through for example the use of locally delivered stabilized siRNA (e.g. intrathecal / intraventricular injection [226]) or by the use of CRISPR technology would increase the overall dynamic microtubule mass [228]. This would eventually restore all the fundamental neuroplastic events that are affected in the CNS pathology abovementioned.

The importance of microtubules in neurophysiology, on the other hand, can automatically bias the interpretation of the patho-physiological mechanism in CNS. In this dissertation we used hereditary spastic paraplegia as a valid example. As previously mentioned, the most common idea about the molecular mechanism underpinning the HSP pathology is the lack of sufficient severing caused by
spastin. This conjecture known as “Loss of function” hypothesis arises from three fundamental reasons: A) The transmission of the disease is autosomal dominant, meaning that all the HSP patients are heterozygote, and consequentially producing at most 50% of WT spastin [121]. This reduction is considered the chief pathological cause by the loss of function hypothesis, which could potentially induce the selective degeneration of particularly sensitive neuronal districts such as motor cortico-spinal neurons.

B) The great majority of the missense mutation (hot-spot) is on the catalytic site, causing enzyme inactivation through a dominant negative mechanism in most of the cases. [121, 229]. According to this mechanism, mutated spastin, once incorporated in the spastin hetero-hexamer (WT + mutated spastin), will cause the complete inactivation of the entire complex [144, 230].

C) HSP can be caused by deletion of the SPAST gene, indicating that the reduced severing activity either by functional inactivation (as described in point 2) or the physical absence (by deletion described in point 3) resulting in the pathological degeneration of the CST [172]. However this hypothetical scenario cannot explain some important inconsistencies of the disease.

The first is the age of onset: considering that SPG4-HSP is a typical disease with adult age onset [231], it is hard to explain how the lack of microtubule severing, can affect only the adult CNS meanwhile no effect can be observed during neurodevelopment, where paradoxically the requirement of plasticity is much higher.

The second incongruity is the explanation of the specificity of the HSP to the CST. Under the loss of function idea, the cortical neurons of the CST degenerate because the lack of severing is particularly detrimental to neurons with extremely long axons, such as the first order neurons from this circuit. However the relation between axon length and microtubule severing should be even more valid in other long nerves in our body such sciatic nerve, where length easily exceeds those from the motor CST neurons.

Third, the loss of function hypothesis cannot give a good explanation of the lack of correlation between the phenotype of patients having a deletion on SPAST gene from the one having a single
nucleotide mutation (missense). Indeed under the dominant negative mechanism, the severing activity of mutated spastin would cause a higher degree of endogenous spastin inhibition compared to the activity inhibited by deletions. Patients bearing deletion mutations have a roughly 50% decrease of the WT spastin protein, with an obvious decrease of the overall severing activity by half. On the other hand, in missense mutated spastin less than 50% of spastin is expected to actually work, considering that the introduction of even a single subunit in the entire hexamer would result in a total loss of the severing activity. Because of this, if the insufficient severing is the ultimate cause of the HSP pathology, patients affected by missense SPAST mutation would have a much more severe phenotype than people affected by a deletion. However no correlation can be made between the type of mutation and the severity of the pathology [231].

In our work we propose that the mutated spastin could act in the context of HSP through a different mechanism of action: through a gain of function mechanism. The idea behind the gain of function hypothesis is that the disease is not caused merely by the lack of microtubule severing, but also by an additional de novo biological activity, or by a misregulated endogenous (different from severing) activity.

This idea is not absolutely new, conversely, several pieces of work suggest that the gain of function mechanism could act in the context of several neurodegenerative diseases, including proteins such as synuclein [232], tau [233] and huntingtin [232]. The central concept of this hypothesis is that the altered structure caused by mutation could form (or deregulate) interactive surfaces on the mutated protein, inducing the aberrant activations of a pathological pathway that should not be activated. The newly acquired biological function results in a disturbed equilibrium of the homeostatic balance within the neuronal cell, causing the progressive degeneration. The importance of understanding which of the two separate mechanisms (loss vs. gain of function) better illustrate the disease progression, do not have just a speculative nature, but have more importantly, a great translational implication. Indeed the possible existence of a gain of function mechanism would suggest completely different therapeutic strategies from the one indicated for a loss of function.
For the latter, the best therapeutic approaches would be essentially two. A direct approach, focused on increasing spastin microtubule-severing activity, activity apparently lost in the disease, through CRISPR strategy or unknown activators. A second approach is to lower microtubule stability with the use of microtubule destabilizing agents such as Nocodazole, in order to contrast the increased microtubule stability caused by the lack of severing of spastin [173].

In the gain of function scenario proposed in this work, the translational approach would be completely different: it would target the gain of function effectors of mutated spastin, such as CK2. Over the past few years, the advancement of research on CK2 has produced excellent inhibitors already used for clinical application [234]. One of the best drugs is CX-4945 used for hematological malignancies, which toxicological and pharmacokinetic profiles have already been extensively described [235, 236]. Interestingly, in a very recent paper, this inhibitor has demonstrated to efficiently cross the blood brain barrier and be capable to reduce several aberrantly phosphorylation substrates of CK2 and closely related kinases, such as Tau in mouse AD model after oral administration [197].

The possibility to use a CK2-blocking strategy, with the administration of very low doses of systematically administered / locally delivered drugs, opens up a completely new avenue for the treatment of HSP. Even more importantly, it can possibly give the pharmacology field a completely new category of anti-neurodegenerative drugs, targeting no longer the aggregation of deposits, but rather the possible biochemical alterations that the misfolded proteins would cause at the signaling level.
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• GSA travel award recipient, spring 2013
• 3rd prize Outstanding junior graduate student poster, Drexel Discovery Day, (fall 2012)
• Recipient of 3 month fellowship from the foundation “Cenci Bolognetti,” Pasteur Institute (2007)
• Recipient of 3 month fellowship from the Italian Work Health and Social Policy (2006)
• Government University Scholarship, Lazio Region, for 5 years (2001-2006)
• Financial award from the University for the score of Bachelor degree (2005)

Foreign Languages
• Spoken and written Italian (native speaker)
• Spoken and written English
• Spoken and written Spanish
Selected Peer-Reviewed Publications
1. Stability properties of neuronal microtubules
Baas PW, Rao AN, Matamoros AJ, Leo L
Cytoskeleton 2016 Feb 16.

2. Vertebrate fidgetin restrains axonal growth by severing labile domains of microtubules.
Lanfranco Leo, Wenqian Yu, Mitchell D’Rozario, Edward A. Waddell, Daniel R.
Marenda, Michelle A. Baird, Michael W. Davidson, Bin Zhou, Bingro Wu, Lisa Baker,
David J. Sharp, and Peter W. Baas;
Cell report, Sept 2015

3. Using siRNA to study microtubule-related proteins in cultured neurons.
Lanfranco Leo, Wenqian Yu and Peter W. Baas
Methods in cell biology, Feb. 2015

4. A. Matteucci, L. Gaddini, M. Villa, M. Varano, M. Parravano, V. Monteleone, F.
Cavallo, L. Leo, C. Mallozzi, F. Malchiodi-Albedi, F. Pricci.
Neuroprotection by Muller glia in in vivo and in vitro models of early diabetic retinopathy through mechanism involving ERK1/2 activation.
Exp Eye Res. Aug 2014

Pigment Epithelium-Derived Factor (PEDF) Peptide Eye Drops Reduce Inflammation, Cell Death and Vascular Leakage in Diabetic Retinopathy in Ins2 (Akita) Mice.
Mol Med Dec 2012;

“Curcumin protects against NMDA-induced toxicity: a possible role for NR2A subunit.”

7. Gaddini L, Villa M, Matteucci A, Mallozzi C, Petrucci TC, Di Stasi AM, Leo L,
Malchiodi-Albedi F, Pricci F.
“Early effects of high glucose in retinal tissue cultures Renin-Angiotensin system dependent and independent signaling.”

Abstracts presented
- Vertebrate fidgetin restrains axonal growth by severing labile domains of microtubules
  *Abstract presented on ECF meeting in August 2015.*

- *Fidgetin restrains axonal growth during neuronal maturation by a microtubule-based mechanism and provides a means for therapeutically enhancing regeneration of injured adult axons.*
  **Lanfranco Leo**, Timothy O. Austin, Wenqian Yu, Daniel R. Marenda, David J. Sharp, and Peter W. Baas
  *Abstract presented at ASCB meeting 2013*
  *Abstract presented at Discovery Day, Drexel university, October 2013*

- Mutations of spastin associated with Hereditary Spastic Paraplegia cause deficits in axonal transport via hyper-activation of CK2.
  **Lanfranco Leo**, Matthew Burns, Peter W. Baas, Gerardo Morfini
  *Abstract presented at Discovery Day, Drexel university, October 2012*
  *Abstract presented at Emerging Concepts on Neuronal Cytoskeleton 2013*

- Efficacy of topical application of PEDF78-121 in the retinal ischemic mouse model.
  Abstract presented at ARVO on June 2010
  **Leo Lanfranco**, Yanling Liu, Colin Barnstable, Joyce Tombran-Tink

Talks
- Vertebrate fidgetin restrains axonal growth by severing labile domains of microtubules
  Presented at ECF meeting in September 2015
• Fidgetin, a novel microtubule-severing protein, regulates neuronal development
Presented at Drexel Discovery Day 2014.
Reference list


