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The impact of α-synuclein on microtubules: from dynamics to ultrastructure

Francesca O. Cantele

PhD Thesis

Scientific tutor: **Prof. Graziella Cappelletti**

Co-tutor: **Prof. Francesco Demartin**

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Part I

Abstract

α-Synuclein is a presynaptic protein supposed to be involved in the control of neuronal synapse functions. It is widely expressed in brain tissue and associated to Parkinson's disease. When free in the cytoplasm, α-synuclein is unstructured, while it adopts a α-helical conformation when bound to vesicles. Its variable structure allows α-synuclein to interact with multiple partners and makes difficult to understand its physiological role, which remains elusive despite decades of intense study. Here, we looked at the interaction between α-synuclein and microtubules, using both wild type and mutated α-synuclein. We investigated the influence of α-synuclein on microtubule nucleation and dynamics and on microtubule structure.

We found that α-synuclein is a novel, foldable, microtubule dynamase, which could participate in the organization of the microtubule cytoskeleton at the pre-synapse, through its binding to tubulin and its regulation of microtubule nucleation and dynamics. We also showed that α-synuclein mutants are much less sensitive than wild type α-synuclein to fold upon tubulin binding and are more prone to cause tubulin aggregation rather than polymerization. Next, we found that α-synuclein deeply affects the structure of microtubules assembled in vitro causing changes in some of the parameters that define it, namely microtubule diameter and tubulin periodicity. Wild type α-synuclein increases the microtubule diameter, but has no effects on tubulin periodicity. A30P α-synuclein, instead, increases both these parameters and A53T and E46K α-synuclein decrease them. We also analysed 3D reconstructions of microtubules and unravelled some very particular structures assembled in the presence of mutated α-synuclein. Next, we carried out an extensive study of the protofilament number distribution among the microtubule population by use of cryo-electron microscopy and we discovered that α-synuclein increases the presence of microtubules with uncommon structures, especially highly twisted microtubules and microtubules with a small number of protofilaments. We also found that the protofilament distribution changes with the time of polymerization. Finally,

we showed that the amount of E46K α-synuclein bound to microtubules is significantly higher than that of the other variants.

These results support the idea that the interaction of α-synuclein with microtubules heavily impacts on microtubules and, consequently, could play an important role in modulating synaptic physiology. In addition, its alteration can reasonably cause neuronal dysfunction via impairment of the proper microtubule organization and structure. The interaction between α-synuclein and microtubules seems to be very complex, changes over time and depends on the proper folding of α-synuclein. Finally, our results suggest that pathological variants of α-synuclein impair the microtubule system promoting tubulin aggregation more than polymerization, but also changing the structure and the stability of the microtubules that are formed. Thus, this work provides new evidences for looking at the regulation of microtubule as a crucial step in the pathogenesis of Parkinson's disease.

State of the Art

α-Synuclein

α-Synuclein is a presynaptic protein widely expressed in brain tissue, where it is about the 1% of total cytosolic protein (Iwai *et al.*, 1995; Stefanis, 2012). It was discovered in 1988 (Maroteaux *et al.*, 1988) and in 1997 it was identified as the major component of Lewy bodies and neurites (Spillantini *et al.*, 1997; Goedert *et al.*, 2017), the defining pathological hallmark of Parkinson's disease. Besides Parkinson's disease, it is also involved in other neurodegenerative pathologies, like dementia with Lewy bodies and multi systemic atrophy (Spillantini *et al.*, 1997; Baba *et al.*, 1998; Gai *et al.*, 1999; Spillantini *et al.*, 1998; Recchia *et al.*, 2004). Mutations in the synuclein gene (SNCA) directly cause Parkinson's disease and dementia with Lewy bodies (Jellinger, 2009).

The amino acid sequence of α-synuclein was found in 1993 (Ueda *et al.*, 1993). It is a soluble naturally unfolded protein (Weinreb *et al.*, 1996; Burré *et al.*, 2013) of 140 amino acids. α-Synuclein can acquire different conformations depending on the environmental conditions and/or the interaction with several partners. The structure of α-synuclein includes three domains (fig. 1).

The N-terminal domain is an amphipathic lysine-rich domain and it includes seven imperfect repeats of 11 amino acids. In solution, this region is disordered, but can acquire a conformation with two α-helices by interaction with phospholipids. This domain binds to membranes and modulates their interactions with α-synuclein (Ulmer *et al.*, 2005). All the missense mutations of the α-synuclein gene that are linked to Parkinson's disease were found in this domain.

The central domain is called "non-amyloid component" (NAC). It is hydrophobic and allows α-synuclein to acquire a β-sheet structure enabling it to produce fibrils. This latter ability is greatly diminished by deletion of large segments of the NAC domain (El-Agnaf *et al.*, 1998; Giasson *et al.*, 2001).

The C-terminal domain is a disordered, acidic and solubilizing domain, which contains several acidic amino acids and phosphorylation sites. It is also rich in prolines, which interfere with the acquisition of a secondary structure. The C-terminal domain is important for the chaperone-like activity of α-synuclein. If this domain is extensively phosphorylated, the protein loses the ability to form oligomers.

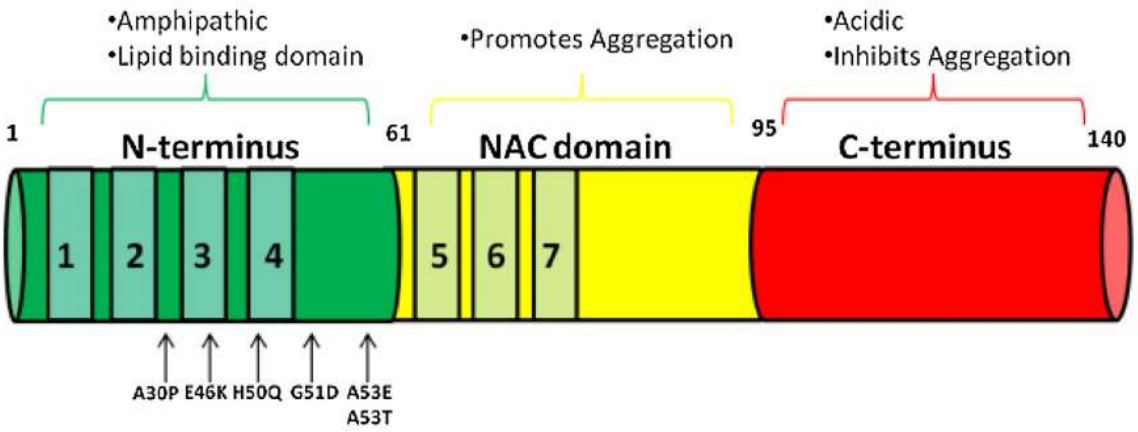


Figure 1. Schematic representation of the wild type α-synuclein structure. The N-terminus domain is a lipid-binding domain, while the NAC domain and C-terminus domain promote and inhibit aggregation, respectively. All the missense point mutations are present in the N-terminus domain, while the seven imperfect repeats of 11 amino-acids are found throughout the N-terminus and NAC domains. (From Butler *et al.*, 2016)

The predominant status of α-synuclein is the monomer (Binolfi *et al.*, 2012) and under physiological conditions it can adopt a tetrameric state with a high helical content (fig. 2).

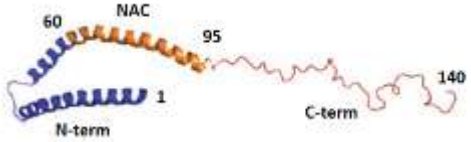


Figure 2. Schematic representation of micelle-bound α-synuclein. The N-terminal region with antiparallel α-helices is shown in blue, the NAC region is also an α-helix and is shown in orange, and the unstructured C-terminal part is shown in red. Numbers refer to amino acid residues. (Adapted from Gallegos *et al.*, 2015)

In the tetrameric form, α-synuclein is resistant to aggregation and fibrillation (Bartels *et al.*, 2011). In these states it is mainly bound to membranes (Lee *et al.*, 2002) and when bound to lipid vesicles it has two α-helices (Eliezer *et al.*, 2001).

In pathological conditions, α-synuclein fibrillates in an antiparallel beta-sheet structure (Conway *et al.*, 2000), as a typical amyloid protein.

The exact function of α-synuclein is still unknown, but several partners were found, suggesting a number of different functions.

In physiological conditions, its principal partners are membranes (Lee *et al.*, 2002) and synaptic vesicles (Larsen *et al.*, 2006). These findings suggest a role in docking and recycling of vesicles in mature presynaptic terminals (Hunn *et al.*, 2015). α-Synuclein also has a role in modulating synaptic level of neurotransmitter via direct interaction with synaptobrevin-2, a major component of SNARE complex assembly (Burré *et al.*, 2010). This function is confirmed by the observation that triple synuclein knockout mice show a significant decrease in SNARE complex assembly (Burré *et al.*, 2010). In addition, α-synuclein directly interacts with dopamine transporter (Sidhu *et al.*, 2004).

In the mitochondria, α-synuclein is involved in the stabilization of electron transport chain proteins. Mitochondria can also be the major target of the α-synuclein antioxidant activity (Zhu *et al.*, 2006). α-Synuclein is also able to bind microtubules (Alim *et al.*, 2004) and actin (Sousa *et al.*, 2009). Interaction between α-synuclein and microtubules will be discussed in more detail later on.

Respect to what is known about α-synuclein in physiological condition, more is known about pathological ones, because of its relationship with Parkinson's disease, the second most diffuse neurodegenerative disease after Alzheimer's disease.

In pathological conditions, α-synuclein is found mainly in an insoluble aggregated form. The aggregation process starts from the formation of less soluble oligomers containing β-sheets, called protofibrils, that eventually became insoluble fibrils (Chen *et al.*, 2015). The accumulation of the fibrils leads to the formation of Lewy bodies and neurites.

The conversion of α-synuclein into a toxic oligomeric form might be promoted or accelerated by several factors: post-translational modifications, interactions with lipids or small molecules, oxidative stress, C-terminal truncation (Fauvet *et al.*,

2012), overexpression (Lee *et al.*, 2006), and missense point mutations (Conway *et al.*, 2000; Greenbaum *et al.*, 2005).

The formation of Lewy bodies was first supposed to be the pathogenic mechanism, but now it seems to be a cellular protective response to aggregated and misfolded proteins (Ross and Poirier, 2005), while the most toxic form of α-synuclein are "insoluble" oligomers/protofibrils. Then, the fibril formation in Lewy bodies seems to be an attempt to isolate the oligomers and convert them into stable and less toxic structures (Roberts and Brown, 2015).

α-Synuclein aggregation induces both intracellular and intercellular effects. The intracellular ones are reduction of presynaptic vesicle size (Cheng *et al.*, 2011), interference with axonal transport of synaptic proteins (Scott *et al.*, 2010), mitochondrial damage (Elkon *et al.*, 2002), increasing of intracellular levels of reactive oxygen species (Junn and Mouradian, 2002), intracellular increase of calcium concentration by formation of membrane pores (Volles and Lansbury, 2002), and inibition of ubiquitin-proteasome system (Emmanouilidou *et al.*, 2010).

The intercellular effects arise when toxic α-synuclein oligomers were secreted and then transferred to neighbouring neurons and glia, where they trigger the formation of new aggregates (Desplats *et al.*, 2009; Lee *et al.*, 2010; Prymaczok *et al.*, 2016). These effects, together with the finding that autopsy of patients transplanted with fetal nigral mesencephalic cells showed that the surviving neurons had the typical Lewy pathology (Kordower *et al.*, 2008), led to the hypothesis that extracellular α- synuclein oligomers might behave like prion proteins. As a consequence, Parkinson's disease etiopathogenesis seems to rely on a retrograde and transneuronal system, from the peripheral nerves, to the susceptible cortical areas of the brain (Rietdijk *et al.*, 2017).

Other pathological effects can be found when α-synuclein is misfolded. Misfolded α-synuclein inhibits the proteasome system and the mitochondrial complex I activity. It also mediates the mitochondrial fission and inhibits autophagy (Bobela *et al.*, 2015).

α-Synuclein and Parkinson's disease

As already mentioned, α-synuclein is the major component of the Lewy bodies, the pathological hallmark of Parkinson's disease. In addition, different α-synuclein mutations are related to some familial cases of Parkinson's disease. In particular, the α-synuclein mutation A53T (Polymeropoulos *et al.*, 1997; Chen *et al.*, 2015) is also the first identified mutation related to Parkinson's disease. The three most common point mutations of α-synuclein, namely A53T, A30P (Krüger *et al.*, 1998) and E46K (Zarranz *et al.*, 2004; Íñigo-Marco *et al.*, 2017), produce a pathology that is more progressive and tends to have an earlier onset than sporadic Parkinson's disease (Cookson, 2005). Moreover, the truncated form (Tofaris, 2006) and the overexpression of the wild type α-synuclein (Singleton *et al.*, 2003) are also pathological. These findings suggest that α-synuclein can play a central and key role in Parkinson's disease pathogenesis.

It was found that in Lewy bodies the majority of α-synuclein is present in the phosphorylated form, while in the healthy brain only a small fraction is phosphorylated (Gallegos *et al.*, 2015; Fujiwara *et al.*, 2002). This suggested that phosphorylation can have an important role in the regulation of α-synuclein aggregation, Lewy bodies formation and neuronal degeneration, but whether it suppresses or enhances α-synuclein aggregation and toxicity *in vivo* is controversial.

In Lewy bodies, several kinases were also found and they were found to be able to phosphorylate aggregated forms of α-synuclein (Waxman and Giasson, 2011; Walker *et al.*, 2013), leading to the hypothesis that kinases may catalyse α-synuclein phosphorylation after Lewy bodies formation. In addition, the accumulation of insoluble α-synuclein phosphorylated forms is mainly observed at the advanced stages of synucleinopathies (Anderson *et al.*, 2006). Taken together, these observations suggested that α-synuclein phosphorylation can be the consequence of α-synuclein aggregation and Lewy bodies formation instead that the cause.

Pathological α-synuclein can also be found in the peripheral nervous system, including the enteric nervous system, cardiac and pelvic plexus, nerve terminals in adrenal glands, salivary glands, and skin (Wakabayashi and Takahashi, 1997; Beach *et al.*, 2010). Thanks to that, several very recent studies have tried to use detection and measurements of α-synuclein in different peripheral tissues as biomarkers for the Parkinson's disease and other synucleinopathies (Schneider *et al.*, 2016; Lee *et al.*, 2017).

Microtubules

Microtubules are emerging as cellular partners of α-synuclein. They are highly dynamic polymers and one of the major components of the cytoskeleton, together with actin filaments and intermediate filaments. Microtubules are particularly important in neurons, where they control many aspects of neuronal function: they provide a scaffold to sustain axonal and dendritic architecture and supply the railway for axonal transport (Conde and Cáceres, 2009).

Microtubules are polarized and dynamic polymers built up by tubulin, a highly conserved protein. Tubulin is a globular protein of about 450 amino acids containing many α-helices and β-sheets. It consist of three domains: i) the N-terminal one contains the nucleotide binding domain, ii) the intermediate one is involved in contact between monomers and binding to taxol, and iii) the C-terminal domain is implicated in interactions with proteins (Nogales *et al.*, 1998). There are many classes of tubulin, and the most common are α- and β-tubulin, that also exist in many different isoforms. Together α- and β-tubulin constitute a heterodimer that is the structural subunit of microtubules. The αβ-tubulin heterodimer associates head to tail to form long protofilaments, which further associate laterally to form a hollow tube: the microtubule (fig. 3).

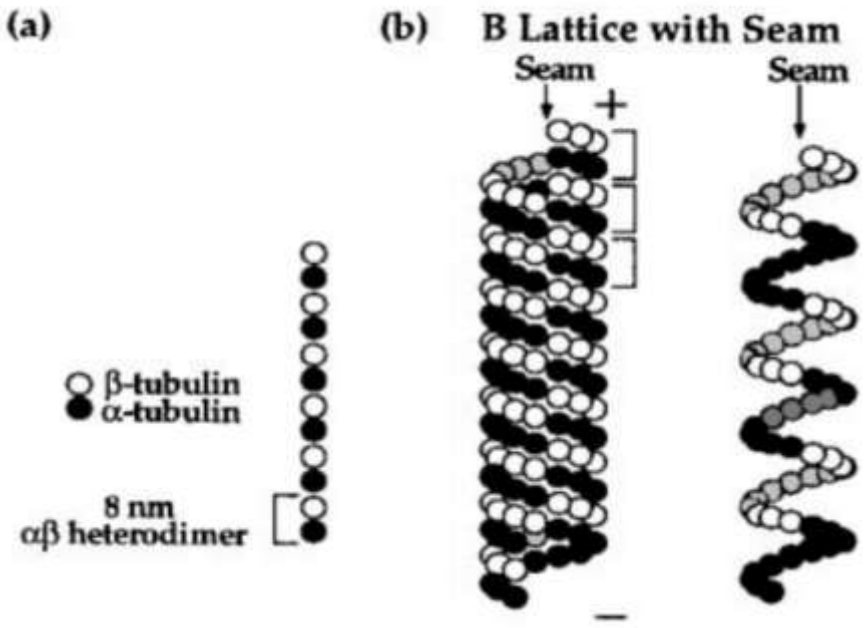


Figure 3. (a) αβ-tubulin dimer (left) and a protofilament formed by head-to-tail interactions between dimers (right). (b) A 13 protofilament microtubule with a B-type lattice with seam (left). Lateral interactions between protofilaments are α to α and β to β, except at the seam. The seam is formed because one turn of a 3-start helix results in a rise of 1.5 αβ-tubulin dimers, or 3 tubulin monomers. Plus and minus signs indicate microtubule polarity and the brackets delineate αβ-dimers within the microtubule lattice. On the right a single 3-start helix is shown. (Adapted from Desai and Mitchison, 1997)

The most common microtubule structure is made by 13 protofilaments (Tilney *et al.*, 1973), but other numbers can be found for example in sperm axonems of insects (Afzelius *et al.*, 1990; Dallai *et al.*, 1993).

Tubulin can be purified from brain of various animals and this allows to obtain tubulin able to polymerize *in vitro*. When polymerized *in vitro*, tubulin forms protofilaments that can associate in two different ways leading to parallel and anti-parallel conformations (Amos and Baker, 1979). The parallel conformation directs to microtubules, while the anti-parallel, obtained in the presence of zinc ions, leads to sheets.

The *in vitro* assembled microtubules are formed by different number of protofilaments, generally from 12 to 15 (Chrétien *et al.*, 1992; Arnal and Wade, 1995; Hyman *et al.*, 1995; Chrétien and Fuller, 2000; Sui and Downing, 2010) and were

extensively studied by cryo-electron microscopy. They are characterized by several structural parameters: i) protofilament number, ii) microtubule diameter, iii) tubulin periodicity, iv) supertwist helix pitch (fig. 4), and v) number of helix start (fig. 3,4).

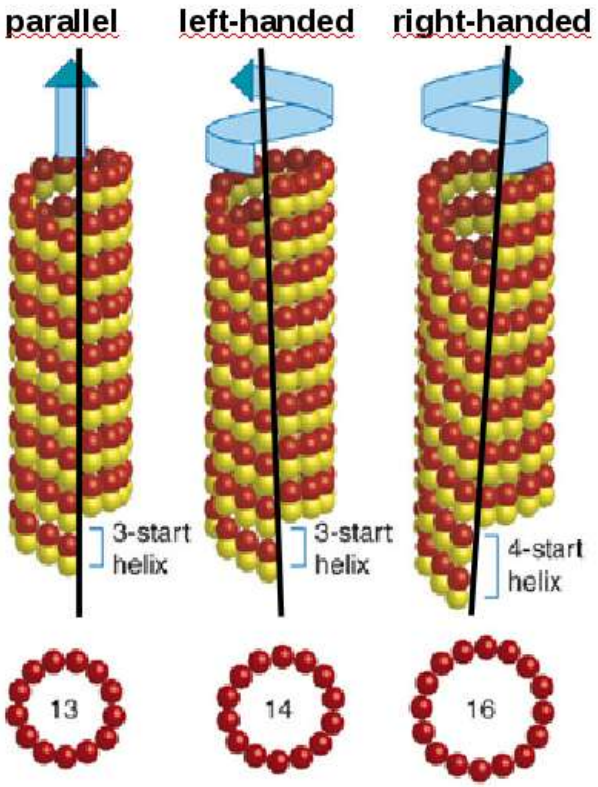


Figure 4. Schematic representation of microtubule's surface for 13, 14 and 16 protofilaments. The protofilaments in a 13-protofilament microtubule are perfectly straight, whereas in all the others microtubules they are skewed and become helical. The straight black lines highlight the "skewness" of the protofilaments and for 13- and 14-protofilament microtubules corresponds to the seam. The protofilament helix is the so-called supertwist helix of the microtubule. On the top, blue arrows indicate the handedness of the supertwist helix. (Adapted from Pampaloni and Florin, 2008)

Microtubule diameter can be easily determined from electron microscope images and is directly linked to protofilament number (Chrétien and Wade, 1991; Wade and Chrétien, 1993; Chrétien *et al.*, 1998). Tubulin periodicity, that is the spacing of tubulin monomers in the protofilament, can be calculated from the power spectrum of the microtubule image (Hyman *et al.*, 1995). Supertwist helix pitch can be determined from the protofilament number and the moiré period measured on the images (fig. 5). The moiré period is often measured as half moiré period, that is the distance between two fuzzy regions along the microtubule length (Chrétien and

Wade, 1991; Chrétien and Fuller, 2000). Between three fuzzy regions there is the so-called fringe pattern, a combination of longitudinal strands that is the result of an optical effect due to the superimposition of the protofilaments on the projected image of the microtubule, that is the electron microscope image, when it is taken at high defocus (fig. 5).

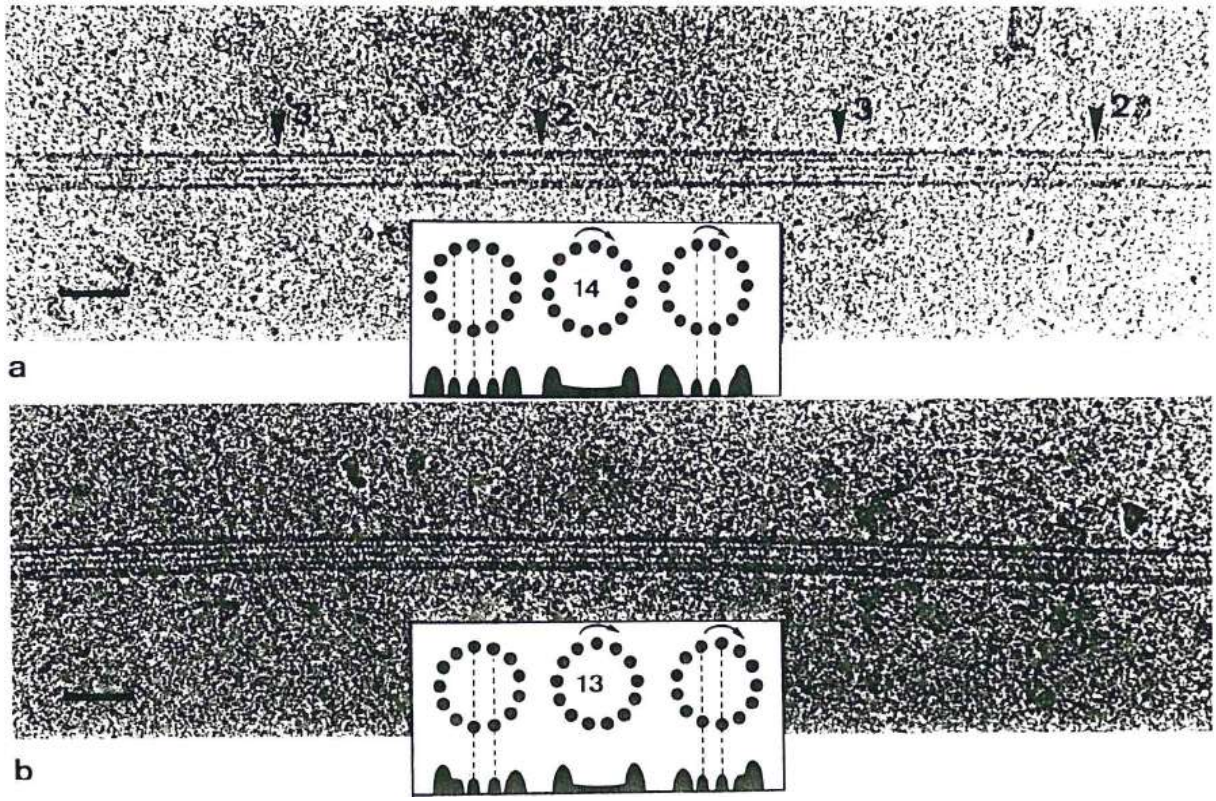
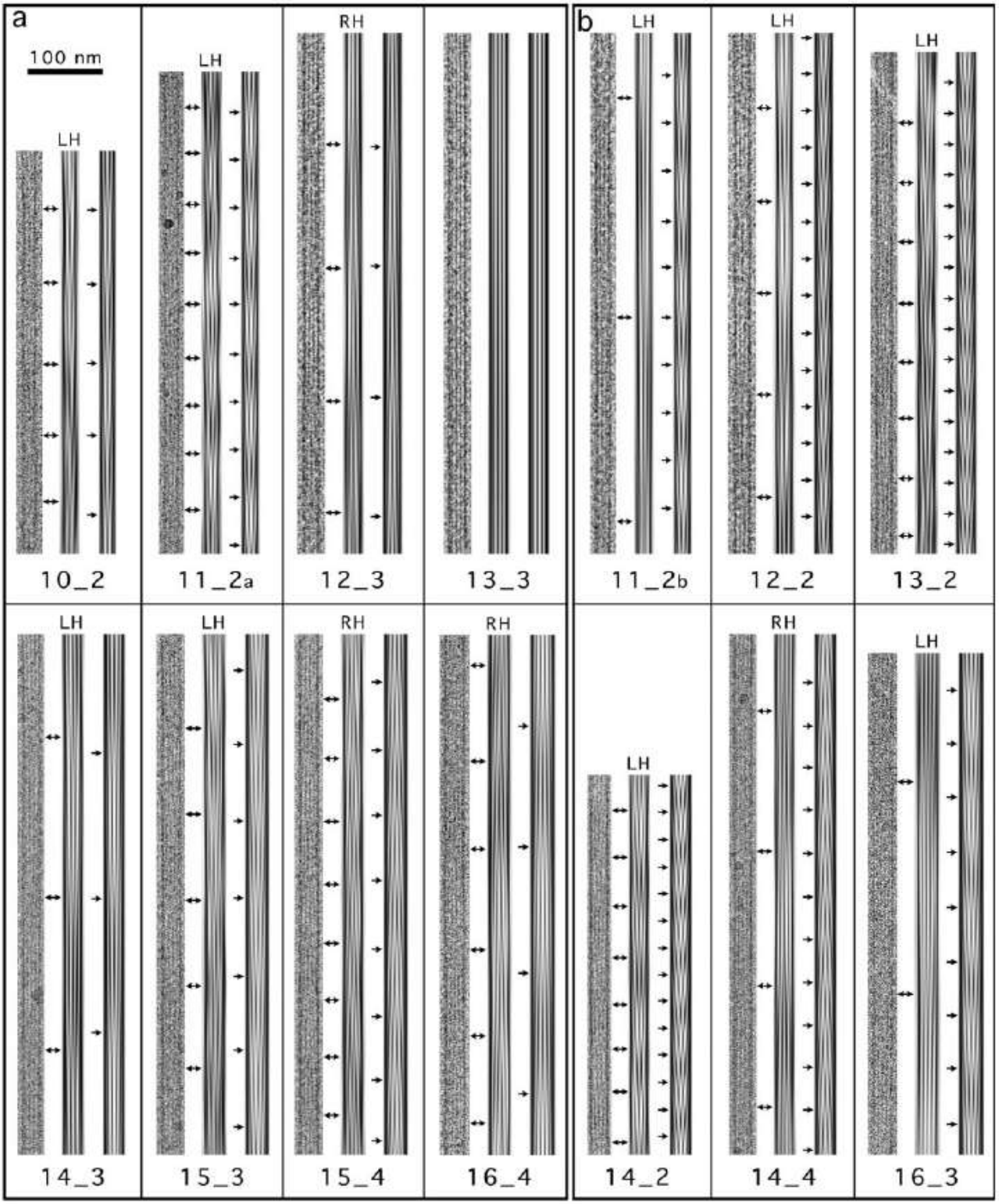


Figure 5. Images of (a) 14- and (b) 13-protofilament microtubules in vitreous ice. The insets show the formation of the major features of the microtubule image by projecting the protofilament structure along the electron beam direction (from top to bottom). The strong edge contrast is due to the superimposition of several protofilaments, while the finer inner fringes by the superimposition of one top and one bottom protofilament. When the top and bottom protofilaments are not in register, the fringe pattern disappears (fuzzy regions). The twist of the protofilaments produces a sequence of fringed and fuzzy regions, which are repeated every time the rotation brings a protofilament in the same position of the previous one, with respect to the electron beam. The complete twist of the protofilament is then reached when the fringe motif repeats itself the same number of time of the number of protofilaments composing the microtubule. The 14-protofilament microtubule shows repetitions of the typical motif: 3 fringe/2 fringe intercalated by fuzzy regions (arrowheads). The 13-protofilament microtubule shows its characteristic asymmetric, constant and long contrast. Scale bars: 50 nm. (From Wade and Chrétien, 1993)

Each type of microtubule has a typical repeat of two fringe regions, that together constitute the fringe pattern and their total length is the moiré period (fig. 6). From the fringe pattern it is then possible to determine the protofilament number (Chrétien and Wade, 1991; Chrétien and Fuller, 2000). Number of helix start can be deducted from the protofilament number and the moiré period (Chrétien and Fuller, 2000) and the inspection of the 4 nm lines of the power spectrum (Chrétien *et al.*, 1996). From the fringe pattern it is also possible to determine the microtubule polarity (Chrétien *et al.*, 1996; Sosa and Chrétien, 1998).

Figure 6. Microtubules assembled in vitro observed by cryo-electron microscopy. (a) Favourable microtubule types. (b) Unfavourable microtubule types. Each microtubule is characterized by its protofilament and monomer helix-start number (N_S). For each microtubule type, a raw image after digital unbending (left) and its filtered version with the fringe pattern enhanced (middle) are presented, next to a simulation of this pattern based on the lattice accommodation model (right). The arrows indicate fuzzy regions corresponding to sections of the microtubule wall where the protofilaments are exactly intercalated in projection (the moiré period is twice the spacing between arrows). When looking, better at a shallow angle, along the microtubule images, it can be seen that the fringes show a directionality that reflects microtubule polarity. The fringe pattern points towards the minus end of microtubules with left-handed protofilaments (LH), and towards the plus end with right-handed protofilaments (RH). All the microtubules have been oriented with their plus ends toward the top of the page. (From Chrétien and Fuller, 2000)


a
100 nm
LH
10_2
LH
11_2a
RH
12_3
13_3
b
LH
11_2b
LH
12_2
LH
13_2
LH
14_3
LH
15_3
RH
15_4
RH
16_4
LH
14_2
RH
14_4
LH
16_3

All these microtubule parameters can be determined from the power spectrum or diffraction pattern of the imaged microtubule by use of the helical reconstruction method (DeRosier and Moore, 1970; Lanzavecchia *et al.*, 1994; Arnal *et al.*, 1996; Sosa *et al.*, 1997; Hirose *et al.*, 1997), which allows to obtain the 3D reconstruction of a microtubule from one of its projections, that is an image produced with a transmission electron microscope (fig. 7).

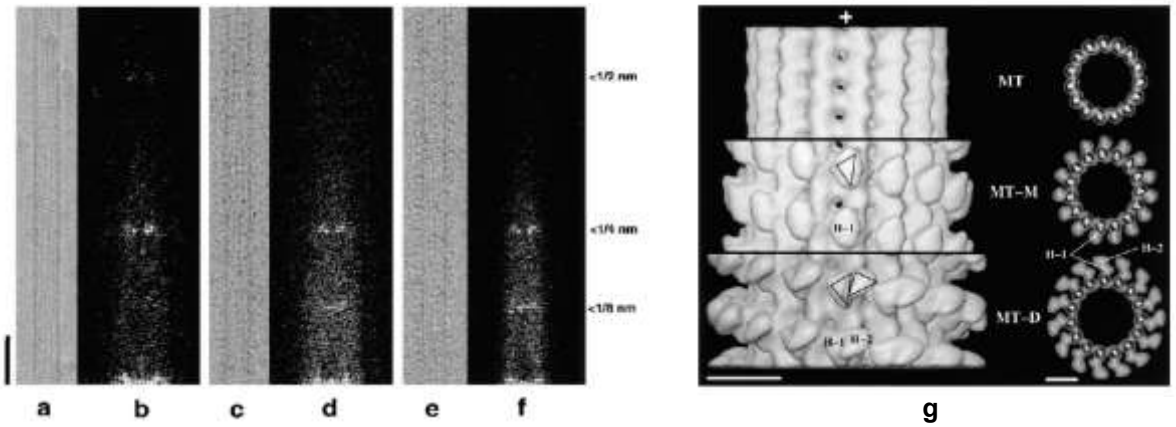


Figure 7. Cryo-electron micrographs of an undecorated 15 protofilament microtubule (a) and microtubules decorated with recombinant monomeric (c) and dimeric (e) Ncd motors (nonclaret disjunctional kinesin-14). The top halves of the corresponding diffraction patterns are shown in (b), (d), and (f), respectively. It is interestingly to note that the 8 nm line is difficult to see and disappear in the case of the undecorated microtubule. Scale bar: 50 nm. (g) Surface representations (left) and end-on projections (right) of the 3D maps of the undecorated microtubule (MT, top) and the microtubule decorated with monomeric (MT-M, center) and dimeric Ncd (MT-D, bottom). The surface representations are shown in side view and are oriented with the microtubule plus end at the top. The end-on projections represent the view from the plus end. In the MT-M and MT-D maps, triangles represent the motor domains associated with a single-tubulin heterodimer. H-1 (head 1) is the microtubule-attached head. H-2 is the detached head. Scale bars: 10 nm. (From Sosa *et al.*, 1997)

The main limitation of the helical reconstruction method is that it can be applied only to real helical structures, such as microtubules with 2- or 4-start helix. Despite this, the reconstructions of almost all 3-start helix microtubules, that are the majority, can be obtained at low resolution. In such a case, α- and β-tubulin are not distinguishable and so also 3-start microtubules result to be real helix, as 2- and 4-start helix are,

allowing the use of the reconstruction algorithm. The only microtubule at which the algorithm still remains not applicable is the 13_3 one, because it does not have a supertwist, a characteristic necessary to apply the helical reconstruction method. The microtubule supertwist, in fact, causes a small shift around the microtubule wall of each tubulin with respect to the one that precedes or follows it along the protofilament. This allows to consider the image of two adjacent tubulins as two different views of the same protein (for real helical microtubules the term tubulin stays for the αβ-dimer, while for not real ones it stays for the monomer), giving the possibility to apply the helical reconstruction method to microtubules.

In any case, the microtubule reconstruction obtained with the helical reconstruction method had not atomic resolution and so pseudo-atomic resolutions are achieved by fitting the atomic structure of tubulin into the low resolution 3D-EM map. The hybrid method was first used in Nogales *et al.* (1999) (fig. 9A, top), coupling a EM reconstruction at 2 nm resolution with a tubulin structure at 3.7Å resolution, obtained in 1998 by the same group (Nogales *et al.*, 1998) from zinc sheets by electron crystallography (fig. 8, left). In 2000, the first X-ray crystallography structure of tubulin was obtained using a microtubule depolymerizer, such as the stathmin-like domain of RB3, to block tubulin αβ-dimers in a "curved", inhibited state, that allows the formation of crystals (Gigant *et al.*, 2000). This tubulin structure can be used as surrogate for the peels of curved protofilaments observed at depolymerizing microtubule ends (fig. 8, right), or for the tubulin dimers in an unassembled state, not for fully formed microtubules.

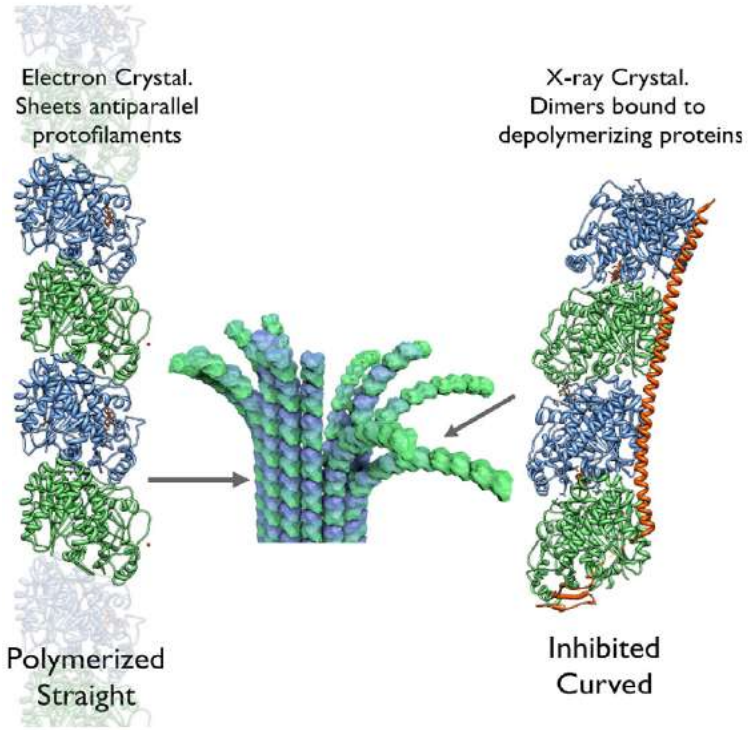


Figure 8. A cartoon of a depolymerizing microtubule end (centre) together with the crystallographic surrogate of a tubulin protofilament of a full-formed microtubule (left) and that of protofilaments peels at the end of depolymerizing microtubules. The surrogate structures are the electron crystallographic structure of tubulin in zinc-induced sheets and the X-ray crystallography structure of tubulin dimers bound to a microtubule depolymerizing protein, respectively. (From Nogales, 2015)

In 2002, Li and co-workers introduced a novel strategy that allows to improve the resolution of 3D reconstruction obtained from cryo-electron microscope images: the use of a single-particle strategy. They obtained an 8 Å resolution map (Li *et al.*, 2002) that allows to identify elements of secondary structure and to see the differences between the tubulin in the microtubule and that in the zinc sheet (fig. 9A, centre). With this approach, the microtubules were not yet considered helical structures, but were divided into small pieces and each piece is considered an image of a small microtubule portion. In this way, the averaging of different microtubules and the corrections of small imperfections of the helical structure can be more easily accounted, together with the possibility of reconstructing also the non-helical 13_3 microtubule, but more images are needed. In the last years, this approach had brought

to a resolution of 3.3Å (Nogales and Zhang, 2016) and rearrangements of the atomic structure of tubulin became possible (fig. 9A, bottom). At this resolution, became also possible the direct view of lateral contacts between adjacent protofilaments (fig. 9B) and the differences in tubulin conformation and interactions when it is bound to different nucleotides or associated proteins.

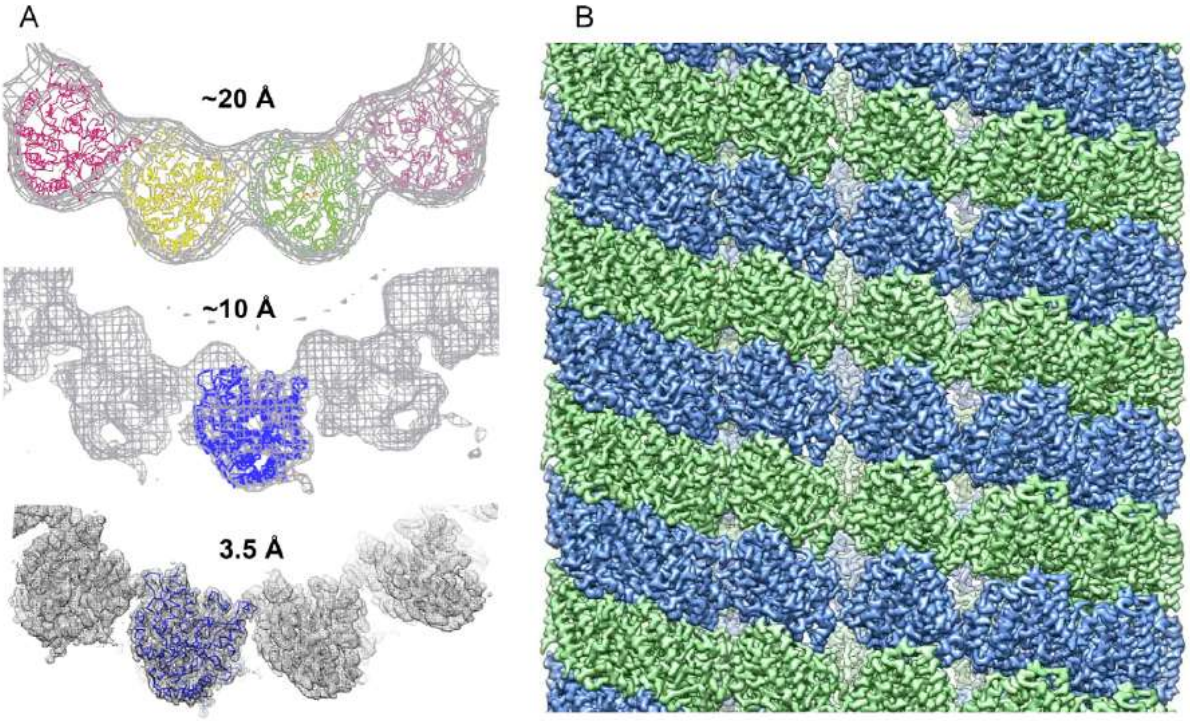


Figure 9. (A) Cryo-EM reconstructions of microtubules at increasing resolutions, from Nogales *et al.* (1999, top), Li *et al.* (2002, centre), and Zhang *et al.* (2015, bottom). The electron crystallographic atomic model of tubulin is fitted into the density maps as it is (top and centre panels) or refined directly into the density map (bottom). (B) Outside view of the 3.5 Å map of microtubules from Zhang *et al.* (From Nogales, 2015)

These techniques allowed the study of tubulin/microtubule interactions with several partners (Kellogg *et al.*, 2017; Morikawa *et al.*, 2015) and were able to show a mechanistic origin of microtubule dynamic instability (Zhang *et al.*, 2015), a very important feature of microtubules.

Microtubule dynamics and regulation

Microtubules are highly dynamic polymers continuously subjected to polymerization and depolymerization and this behaviour is called dynamic instability (Mitchison and Kirschner, 1984; Conde and Cáceres, 2009). Dynamic instability is described by 5 parameters: polymerization rate, shrinkage rate, catastrophe frequency, rescue frequency, and pause time. Catastrophe frequency is the frequency of transitions from polymerizing to depolymerizing microtubules, while rescue frequency is the frequency of transitions from depolymerizing to polymerizing microtubules. Pause time is the time spent by microtubules neither polymerizing nor depolymerizing (fig. 10).

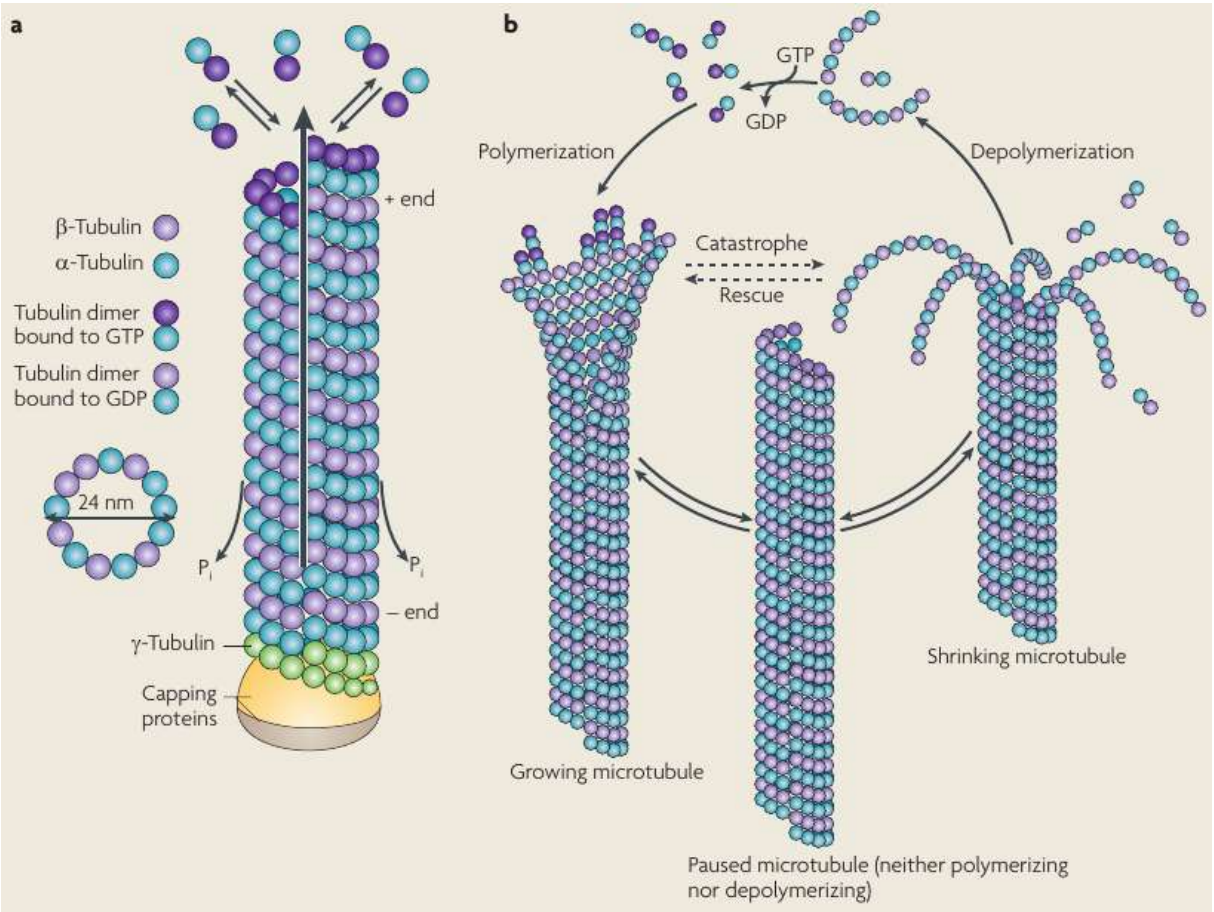


Figure 10. (a) Components and structure of a microtubule. (b) Scheme of dynamic instability of microtubules. (From Conde and Cáceres, 2009)

The polarized nature of the microtubules gives them different polymerization rates at the two ends: higher at the "plus end" and lower at the "minus end". This difference

comes from the different ability of α- and β-tubulin to hydrolyze GTP (guanosine-5'-triphosphate). At the plus end, the GTP located on the exposed β-tubulin monomer is easily hydrolysed, while at the minus end, where the exposed monomer is α-tubulin, the GTP hydrolysis is slower (Carlier *et al.*, 1987). During or shortly after polymerization, the GTP is hydrolysed and becomes non-exchangeable. As a result, the microtubule is mainly composed of GDP-tubulin, with the GTP presents only at the plus end, where it forms a "GTP cap". The GTP cap is generally made of minimum one tubulin layer and stabilizes the microtubule structure. The GDP-tubulin, instead, destabilises the microtubule, because it is more stable in a curved state. The lost of the GTP cap causes the opening of the microtubule that rapidly shrinks (Burbank and Mitchison, 2006). Shrinking microtubules then undergo to a rapid loss of GDP-tubulin, dimers or oligomers, from their plus ends. In conclusion, microtubules grow when the addition of free tubulin is faster than the GTP hydrolysis on incorporated tubulin, leading to the formation of GTP cap that stabilizes MT.

The polymerization rate of tubulin dimers into microtubules is proportional to the concentration of free tubulin and is regulated by the presence of GTP and Ca^{2+} at intracellular level (Keith *et al.*, 1986) and by temperature. As long as Mg^{2+} and GTP are present, it is possible to follow the *in vitro* kinetics of tubulin assembly into microtubules by light scattering measurements. The resulting polymerization curve (fig. 11) shows the characteristic shape, with an initial lag phase, corresponding to nucleation, followed by a fast growing phase, microtubule elongation, and concluding with an equilibrium phase where a plateau level is reached (steady state).

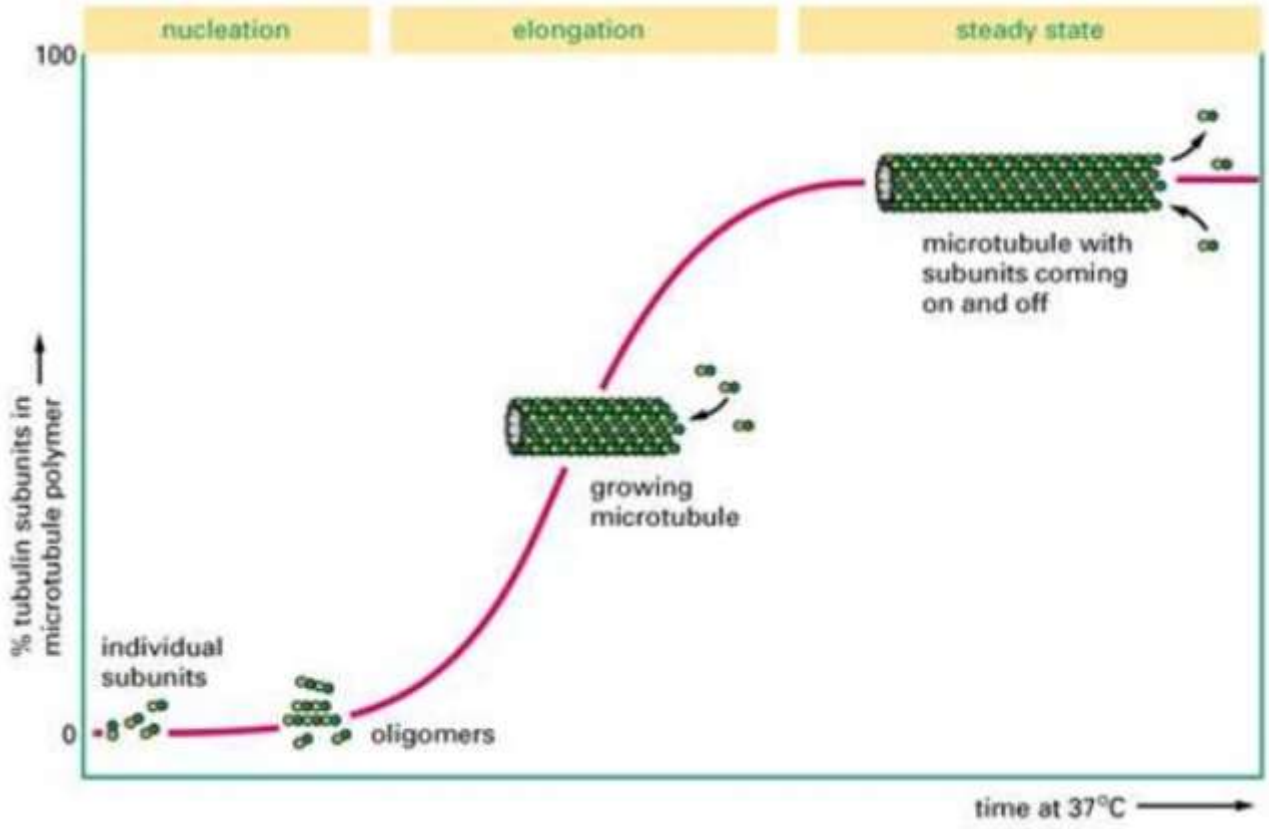


Figure 11. The polymerization curve of tubulin *in vitro*. The amount of microtubules, measured by light scattering, follows a sigmoidal curve, with the three typical phases of polymerization: nucleation, elongation and steady state. (From Alberts *et al.*, 2008)

During the lag phase tubulin dimers associate to form metastable aggregates, that can become polymerization nuclei. The lag phase reflects a kinetic barrier to the nucleation process, that is slow, and can be reduced or abolished by adding pre-made nuclei, such as fragments of polymerized microtubules. During the elongation phase, tubulin dimers add to the free ends of growing microtubules. In this phase, the rate of polymerization drops with time because it is proportional to the concentration of free tubulin. Finally, during the plateau phase, polymerization and depolymerization are balanced, because the amount of free tubulin has dropped to the point where a critical concentration has been reached and subunits are dissociating from the ends of microtubules as well as adding to them (Alberts *et al.*, 2008).

The dynamic instability of microtubules allows the cell to rapidly adapt to environmental changes and to respond to cellular needs. For example, cells promptly

reorganize the cytoskeleton throughout mitosis or during the extension of growth cones from neurons (Burbank and Mitchison, 2006). This complex process is probably controlled by the combination of a number of proteins and post-translational modifications of α- and β-tubulin (Dubey *et al.*, 2015).

The proteins that interact with microtubules are divided in two categories: motor proteins and (non-motor) microtubule associated proteins (MAPs). Motor proteins include dyneins and kinesins and are involved in different intracellular functions, first of all intracellular transport (Janke and Chloë Bulinski, 2011). Dyneins are involved in retrograde transport along the microtubules, while kinesins in anterograde. Due to the viscosity of the cytosol, active transport is necessary and so the motor proteins use hydrolisis of ATP (adenosine 5'-triphosphate) to generate the necessary force (Luby-Phelps, 1999). Microtubule transport is particularly important for neurons, where it needs to work over long distances.

The non-motor MAPs include proteins that can both stabilize or destabilize microtubules, by changing the frequencies of transition between growing and shrinking state. Structural MAPs bind and stabilize microtubules and, among them, tau, MAP1 and MAP2, were specifically found in neuronal cells, while MAP4 is expressed in others cell type (Conde and Cáceres, 2009). One of the mechanisms implicated in MAP regulation is phosphorylation, which promotes the detachment from microtubules and impairs their stabilizing function (Trinczek *et al.*, 1995).

A second group of MAPs consists of the plus end tracking proteins that specifically accumulate at the plus end of growing microtubules. These proteins help to control microtubule dynamics, interaction with cellular organelles and subcellular domains and signalling molecules (Akhmanova and Steinmetz, 2008). Some kinesins, which belong to that family, regulate microtubule dynamics and depolymerize microtubules, promoting spindle assembly and chromosome segregation (Kline-Smith and Walczak, 2004). On the other side, end-binding proteins usually promote microtubule polymerization and inhibit catastrophes, increasing the rescue frequency and decreasing the depolymerization rate (Lansbergen and Akhmanova, 2006). They are

often used conjugated to fluorescent protein as a tool in live cell imaging approach to follow MT growth (Akhmanova and Steinmetz, 2008).

Another class of MAPs includes spastin and katanin, severing enzymes able to destabilize the microtubule lattice generating internal breaks. They are involved in the regulation of neurite outgrowth and branching formation (Roll-Mecak and McNally, 2010).

Besides interacting proteins, microtubule functions are also regulated trough the presence of different tubulin isoforms and a number of their post-translational modifications. Post-translational modifications of tubulin mark subpopulations of microtubules and selectively affect their functions. They are preferentially made on microtubules other than on free dimers (Conde and Cáceres, 2009) and the majority of them are on the C-terminal domains of α-tubulin. The first discovered post-translational modification is detyrosination/tyrosination. Detyrosination was mostly found on stable and long-lived microtubules, especially in neurons, and it was assumed that promotes or correlates with microtubule stability. The opposite modification, tyrosination, happens exclusively on free tubulin dimers and is associated to dynamic microtubules, with the newly assembled microtubules almost formed of tyrosinated tubulin (Janke and Chloë Bulinski, 2011). By the removal of its two C-terminal glutamate, detyrosinated tubulin can be further converted to the so-called Δ2 tubulin. This is considered an irreversible post-translational modification, because it cannot undergo retyrosination (Janke and Chloë Bulinski, 2011). Δ2 tubulin is typical only for very stable microtubules and is very frequent in neurons, where it occurs in about 35% of brain tubulin. It seems that its function is simply to lock microtubules in the detyrosinated state (Janke, 2014). The second tubulin post-translational modification to be discovered was the acetylation of lysine 40 (K40) on α-tubulin (L'Hernault and Rosenbaum, 1985). It is applied on microtubules and is enriched on stable microtubules.ε

Microtubules in the neuron

Microtubules are particularly important for neurons. Neurons are highly polarized cells generally consisting of a cell body with several protrusions, a long axon and many short dendrites (fig. 12). Besides morphology, these neurites also differ in function, with the dendrites specialized to receive signal and the axon to transmit it.

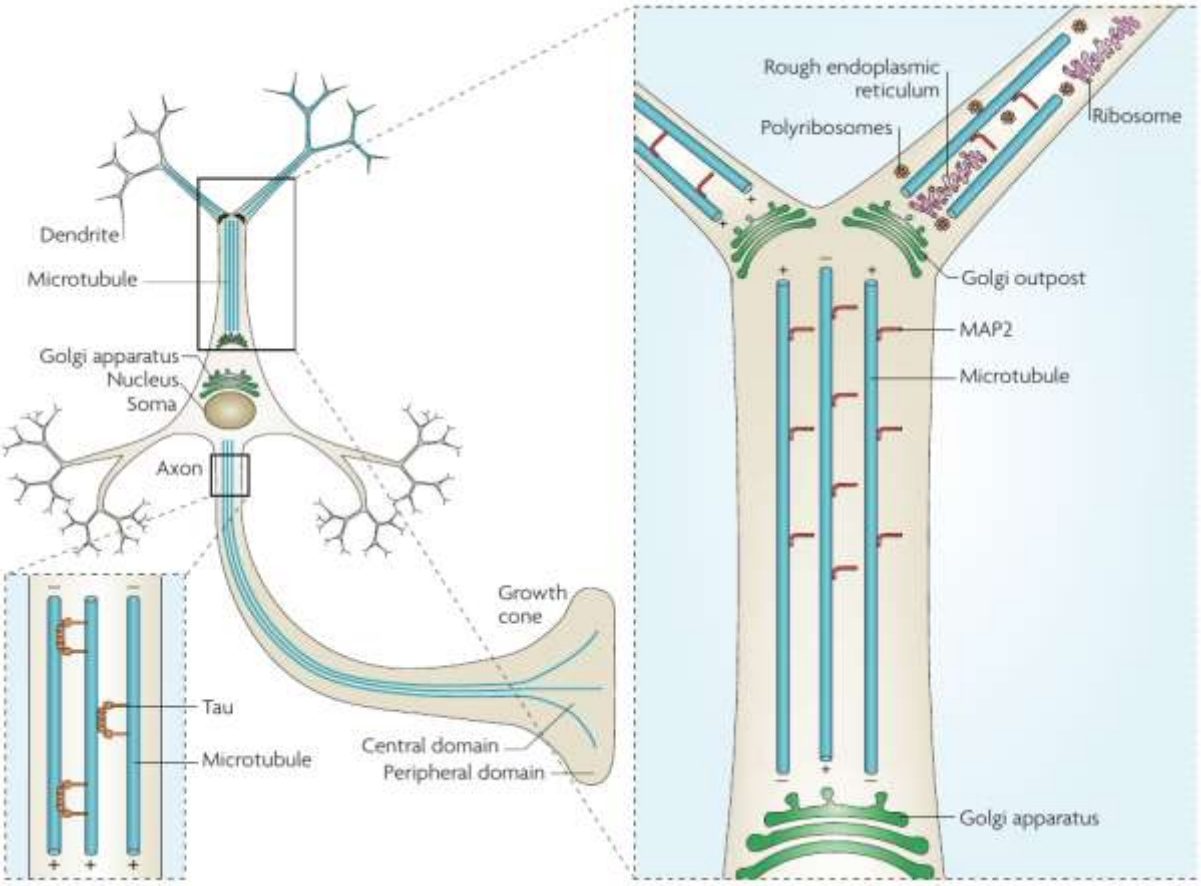


Figure 12. Microtubule organization and organelle distribution in axons and dendrites. Axons have tau-bound microtubules of uniform orientation, whereas dendrites have microtubule associated protein 2 (MAP2)-bound microtubules of mixed orientation. Dendrites also contain organelles that are not found in axons, such as rough endoplasmic reticulum, polyribosomes and Golgi outposts. (From Conde and Cáceres, 2009)

In axons and dendrites, microtubules form dense parallel arrays that are required for their growth and maintenance. In the early stages of neuronal differentiation, microtubules pilot the growth cone and axon elongation and during the life of the neurons the shape changes during neuroplasticity.

Microtubule organization differs between axons and dendrites in at least two major aspects (fig. 12). The first one is the orientation: axonal microtubules have uniform orientation, with their plus ends facing the axon tip, whereas dendritic microtubules have mixed orientation, with their plus ends facing either the cell body or the dendritic tip (Baas *et al.*, 1988). The plus end of microtubules is a crucial site for tubulin polymerization, while the minus end is often anchored to a microtubule-organizing center (MTOC) that constitutes the major centre of microtubule nucleation. In animals, the most important microtubule-organizing center is the centrosome (Vinet and Zhedanov, 2010). The nucleating structure is called γ-tubulin ring complex (γ-TuRC) and is made by γ-tubulin, a tubulin isoform that functions as a template for the correct assembly of microtubules (Erickson, 2000) and establishes their polarity orientation. Microtubule polarity allows the selective trafficking of cargo inside the neurons and its disorganization can result in the incorrect localization of cargo (Dubey *et al.*, 2015) and can impair the transport system. Some microtubules remains attached to the nucleating structure while others are rapidly released by the microtubule severing protein katanin (Ahmad *et al.*, 1999; Yu *et al.*, 2005). The released microtubules are then transported away from the centrosome through the molecular motors as short polymers (Vale, 2003; Baas *et al.*, 2005). The second aspect of the differences between axons and dendrites is in their complement of MAPs: for example, MAP2 is found mostly in dendrites and tau in axons. MAPs and post-translational modifications modulate the microtubule stability, thus influencing their functions. As a consequence, the expression of post-translational modifications varies throughout neuron compartments and changes during neuronal differentiation. Older microtubules are the most stable because post-translational modifications of tubulin accumulate over time, whereas the newly synthesized ones are more dynamic. For instance, in developing neurons, with an extending axon and not yet differentiated dendrites and synapses, detyrosination and Δ2 tubulin are relatively low. In the growing axon, acetylation and detyrosination are high, while they are reduced in the growth cone that is enriched in tyrosinated tubulin. The minor neurites

have a low level of acetylated tubulin, but a relatively high level of detyrosinated tubulin. In mature neurons there is an enrichment of tubulin post-translational modifications associated to stable microtubules. Detyrosinated and Δ2 tubulin remain relatively high in axonal microtubules and acetylation increase in axon, dendrites and also in the synaptic region (Song and Brady, 2015). As for post-translational modifications, during the differentiation and maturation of neurons, there are also changes in MAPs (Song and Brady, 2015).

Tubulin post-translational modifications are involved in regulating most of the processes in which microtubules could directly or indirectly lead to disease, especially those involving neurons where microtubules are normally subjected to high levels of post-translational modifications, such as neurodegeneration (Janke and Chloë Bulinski, 2011).

Many human neurodegenerative diseases, including Parkinson's disease, have been shown to display axonal transport impairment, particularly important due to the extreme polarity and size of these cells. In these disorders, some cargoes that are usually conveyed along the axons can accumulate in the proximal segment of the axon, the perikaryon, or in the distal part of it. Long-range microtubule-based transport is the main mechanism to deliver cellular components to their actions site. Anterograde axonal transport is used to supply proteins, lipids and organelles to the distal synapse, while retrograde transport is involved in removing misfolded and aggregated proteins from the axon and in the intracellular transport of distal signals to the soma. The major components of this system are molecular motors and microtubules, the railway on which molecular motors run. Thus, the disruption of the system could occur via damage of molecular motors, microtubules, transported cargoes or ATP supply, the fuel for the activity of molecular motors. They all contribute to neurodegeneration (De Vos *et al.*, 2008) and it is also possible that they lead to a convergent pathway. The idea that defective intracellular transport can directly trigger neurodegeneration is strongly supported by the identification of mutations in genes encoding proteins involved in axonal transport and by the

observation that chronic exposure to some chemicals can provoke axonal transport perturbation and subsequent neurodegeneration (Millecamps and Julien, 2013). For example, tauopathies are neurodegenerative diseases with alterations in the microtubule-associated protein tau and as a consequence a reduction in microtubule stability that leads to impairment in axonal transport (Forman *et al.*, 2004). Other neurodegenerative diseases that affect microtubule stability and microtubule-based transport are α-synucleinopathies, that result in protein aggregates inclusion bodies, the Lewy bodies. All these observations support the idea that in neurodegenerative disorders associated with axonal transport, alteration of microtubule system is an early event that can cause cell death by accumulation and mislocalization of different organelles within neuron.

Microtubules and Parkinson's disease

The molecular mechanism underlying neuronal death in Parkinson's disease results still unknown and current therapies offer just the management of symptoms. Several pathogenic pathways, correlated each others, have been implicated in dopaminergic neurons degeneration such as, oxidative stress, mitochondrial dysfunctions, accumulation of misfolded proteins and local inflammation (Kumaran and Cookson, 2015). Understanding which mechanism might be the primary insult leading to Parkinson's disease is a major challenge to deal. Notably, the early microtubule dysfunction is becoming established as a key alteration in Parkinson's disease pathogenesis (Pellegrini *et al.*, 2017). Within neurotoxins associated to Parkinson' disease, MPTP (1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine) and rotenone are implicated in microtubule dysfunctions. MPTP has many effects: i) its toxic metabolite, MPP+, affects MT dynamics in vitro, acting as catastrophe promoter, increasing its frequency (Cappelletti *et al.*, 2005); ii) in MPP+-exposed PC12 cells, alterations of microtubule stability have been shown to precede mitochondria transport defect and neurite degeneration (Cartelli *et al.*, 2010); iii) the systemic

injection of MPTP to mice induces microtubule alteration very early, before tyrosine hydroxylase depletion, neuron degeneration and axonal transport impairment, suggesting an important role of microtubule dysfunction in triggering neurodegeneration (Cartelli *et al.*, 2013); iv) MPP+ has also been proved to decrease anterograde and increase retrograde axonal transport of membranous vesicles in squid axoplasm (Morfini *et al.*, 2007). The effect of rotenone, instead, is to depolymerize microtubules, both *in vitro* and in the cell (Marshall and Himes, 1978; Ren *et al.*, 2005), thus disrupting vesicular transport in dopaminergic neurons, with their subsequent accumulation in the cytoplasm. As a consequence, the leakage of dopamine from the vesicles leads to the generation of oxidative stress, induced by its oxidation, and to neuronal death (Ren *et al.*, 2005; Choi *et al.*, 2011). Other proteins linked to Parkinson's disease that affect the microtubule system are LRRK2 (leucine-rich repeat kinase 2), parkin and DJ1. LRRK2 modulates microtubule stability by interacting with and phosphorylating β-tubulin isoforms in the brain (Law *et al.*, 2014). In addition, fibroblasts obtained from Parkinson's disease patients carrying LRKK2 mutations show an altered microtubule stability (Cartelli *et al.*, 2012). DJ-1 deficiency reduces microtubule dynamics by downregulation of β-tubulin III and causes a decline in dendritic complexity and the loss of dendritic spines in striatal medium spiny neurons (Sheng *et al.*, 2013). Finally, parkin affects microtubules in many ways: i) it is a tubulin-binding protein, as well as a MT-associated protein, which increases the ubiquitination and degradation of both α-and β- tubulin (Ren *et al.*, 2003); ii) it stabilizes microtubules trough a strong binding with both tubulin and microtubules (Yang *et al.*, 2005); iii) it protects midbrain dopaminergic neurons against PD-causing substances by stabilizing microtubules (Ren *et al.*, 2009); iv) its mutations decrease microtubule stability, an effect restored by pharmacological microtubule stabilization (Cartelli *et al.*, 2012) or overexpression of native parkin, but not its mutants (Ren *et al.*, 2015); v) its absence accelerates microtubule ageing, affects mitochondria mobility via an alteration of the MT system that is rescued by paclitaxel, and causes the fragmentation of stable microtubules (Cartelli *et al.*, 2018).

α-Synuclein and microtubules

Among the proteins that interact with microtubules, it has been included α-synuclein, whose intraneuronal accumulation is the pathological hallmark of Parkinson's disease. α-Synuclein interacts with tubulin (Alim *et al.*, 2002; Zhou *et al.*, 2004) and with the microtubule interacting protein tau (Jensen *et al.*, 1999; Zhou *et al.*, 2004).
Regarding the influence of microtubules on α-synuclein, it is known that tubulin promotes α-synuclein fibrillation *in vitro* (Alim *et al.*, 2002; Kim *et al.*, 2008). The *in vivo* effects, instead, are not clear. Esteves and colleagues (Esteves *et al.*, 2010) had found that the destabilization of the microtubule cytoskeleton potentiates α-synuclein aggregation and that a treatment with taxol, a microtubule stabilizer, decreases this aggregation. On the contrary, Nakayama and co-workers (Nakayama *et al.*, 2009) reported that the destabilization of the microtubule cytoskeleton prevents α-synuclein aggregation. Conflicting results had been also obtained overturning the roles of α-synuclein and microtubules. It was found that α-synuclein induces polymerization of purified tubulin into microtubules (Alim *et al.*, 2004), as well that monomeric and oligomeric α-synuclein have not significant effects on tubulin polymerization *in vitro* (Chen *et al.*, 2007). In addition, it was found that the α-synuclein mutants A30P and A53T do not promote microtubule polymerization, but that they induce the formation of amorphous tubulin aggregates (Alim *et al.*, 2004). However, a very recent study reported that the direct assembly of microtubules by α-synuclein in vitro is not reproducible (Oikawa *et al.*, 2016). Looking at cell cultures, it was found that the treatment with both extracellular α-synuclein (Zhou *et al.*, 2010) or α-synuclein oligomers (Prots *et al.*, 2013) destabilizes microtubules. Besides, it was shown that experimental overexpression of α-synuclein disrupts microtubule-dependent vesicle trafficking in cultured cells (Lee *et al.*, 2006).
These conflicting findings, especially about how α-synuclein affects microtubules, suggest that the correct target and function of α-synuclein are still unknown and need to be further investigated. In addition, evidence of the destabilizing effect of α-

synuclein on the microtubule system were found, but very little is known about how this effect arise and particularly about the involvement of possible changes in the microtubule structure and dynamics.

Aim of the Project

α-Synuclein is a presynaptic protein supposed to be involved in the control of neuronal synapse functions. Intraneuronal accumulation of α-synuclein is the pathological hallmark of the so-called synucleinopathies, including Parkinson's disease, and has been widely studied. However, due to the variable structure and multiple interactions of α-synuclein with a number of proteins, its physiological role remains elusive.

The overall aim of this project is to investigate the interaction of α-synuclein with the microtubule network, which is very important for any cell but especially for neurons. Nowadays, conflicting results have been obtained about their interaction whereas the potential impact of α-synuclein on microtubule structure and dynamics has been mainly neglected.

Our first aim was to assess if α-synuclein interacts with tubulin/microtubules and affects microtubule dynamics, both *in vitro* and *in cell*. The interaction between α-synuclein and tubulin has been investigated *in vitro*, using multiple approaches as mass spectroscopy and ^{1}H-NMR diffusion measurements, which allow the analysis of their binding, and circular dichroism, which reveals the descending changes in the α-synuclein secondary structure. The interaction between α-synuclein and microtubules, instead, has been investigated by light microscopy, both *in vitro* and *in cell*, using live cell imaging and VE-DIC light microscopy.

Next, we aimed at deeply analysing the influence of α-synuclein on microtubule structure by use of transmission electron microscopy. The structural parameters of microtubules assembled in the presence of wild type α-synuclein and of their variants linked to Parkinson's disease were investigated starting from microtubule diameter and tubulin periodicity. To address this task, we carried out negative staining, a relatively simple and fast technique. As a second step, we computed a number of 3D reconstructions from the best quality images obtained and calculated almost all the

structural parameters of the microtubules, including protofilament number, number of helix start and supertwist helix pitch.

Furthermore, since negative staining method produces variable results, due to the presence of staining itself, our succeeding aim was to employ cryo-electron microscopy, a challenging technique that allows the observation of biological samples in quasi-natural conditions. In this way, we were also able to extend the large-scale examination to the protofilament number, which is a crucial parameter defining microtubule structure.

Finally, we aimed at assessing the hypothesis that the impact of α-synuclein on microtubule ultrastructure depends on its direct binding to the microtubules themselves throughout immuno-gold strategy.

Main Results

The interaction between α-synuclein and microtubules is one of the current topics in our laboratory. α-Synuclein co-localizes with the most dynamic microtubules, at the pre-synapse in PC12 cells and human neurons, and it has conflicting effects on microtubules, such as enhancing both microtubule growth rate and catastrophe frequency, *in vitro* and *in cell*. Interestingly, α-synuclein changes its conformation when binds to $\alpha_2\beta_2$-tetramers, acquiring some α-helix content. Furthermore, α-synuclein mutants are much less sensitive than wild type to fold upon tubulin binding (Cartelli *et al.*, 2016, in PART II).

This project investigated the effects of α-synuclein on microtubule structure. We analysed microtubule assembly *in vitro* by use of transmission electron microscopy (TEM), both with negative staining methods and cryo-electron microscopy.

In the first part we added our contribution to the ongoing project of the laboratory and inspected the presence of aggregates following microtubule polymerization, by use of negative staining methods. We found that in the absence of α-synuclein only few and small aggregates were present. We obtained the same situation when wild type α-synuclein was present, while, when α-synuclein mutant A30P, A53T and E46K were used, we found some aggregates. The amount of the aggregates was inversely proportional to the ability of the mutant to fold upon tubulin and they were formed by crowds of microtubules, but also by other protein aggregates. These protein aggregates should contain α-synuclein, because they were recognized by the anti-α-synuclein antibody, as shown by other laboratory members (Cartelli *et al.*, 2016, in PART II).

Next, we investigated how α-synuclein influences microtubule ultrastructure. We first investigated the effect of folded α-synuclein on microtubule diameter and tubulin periodicity in negative stained microtubules by TEM and 2D-analysis. We found that microtubules assembled in the presence of wild type α-synuclein displayed the significant increase in diameter while no changes were observed in tubulin

periodicity. About mutants, A30P α-synuclein increased both microtubule diameter and tubulin periodicity, while A53T and E46K mutants had the opposite influence, reducing both parameters. The E46K mutant is the one that showed the most interesting results, with some very low values of microtubule diameter and a shift toward low values of tubulin periodicity with respect to all the other samples (Cantele *et al.*, manuscript in preparation, in PART III).

From the best quality images of microtubules we obtained by TEM, we computed 3D reconstructions using the 3D helical reconstruction method (DeRosier and Moore, 1970; Lanzavecchia *et al.*, 1994; Arnal *et al.*, 1996; Sosa *et al.*, 1997; Hirose *et al.*, 1997). The obtained reconstructions had very variable parameters and did not give statistical significance, but some peculiar results were obtained. In particular, microtubules assembled in the presence of E46K mutant had high number of protofilaments, even if they had small diameters (Cantele *et al.*, manuscript in preparation, in PART III).

We then inspected the variation of protofilament number among the microtubule population by use of cryo-electron microscopy and we found different distributions of the microtubule protofilament number for each sample. The microtubules of the control, assembled from purified tubulin alone, had a very high percentage of 13-protofilament microtubules, the most stable ones, while for α-synuclein variants, we found a relatively low amount of 13-protofilament microtubules and a high percentage of 12-protofilament ones, with a particularly high presence of highly twisted microtubules for mutant A53T. In these experiments, we found a high number of flattened microtubules that are not analysable and thus reduced the size of the microtubule population to be inspected. Thus we decided to analyse the microtubule population also at a higher time of polymerization, 4 hours instead of 1 hour, to be sure to reach the assembly plateau, where more and more stable microtubules should be present. After 4 hours of polymerization, we actually found a higher amount of microtubules respect to that found after 1 hour of polymerization, with only few flattened microtubules. Interestingly, in this condition, we found

significant differences between almost all the samples. In particular, for microtubules assembled in the presence of α-synuclein mutant E46K, we found significant differences with respect to all the other α-synuclein variants, but not with those assembled from tubulin alone. Nevertheless, it is interesting to note that, as after 1 hour of polymerization, the microtubules assembled in the presence of E46K α-synuclein were the only ones that include protofilament numbers lower than 12. The lack of significant differences between the number of protofilaments in microtubules assembled in the presence and in the absence of E46K α-synuclein seems in contrast with the differences observed in the microtubule diameter. However, this could be explained by the reduction in the space between adjacent protofilaments, which might lead to a change in microtubule diameter without affecting the protofilament number, as suggested by the comparison of 3D reconstructions. The microtubules observed after 4 hours of polymerization showed a further characteristic, namely the relatively high presence of highly twisted microtubules. The overall high amount of highly twisted microtubules, which have been shown to be energetically unfavourable configurations, could be explained by the presence of glycerol in the assembly buffer that stabilizes the microtubule structure. The particularly high values obtained for the α-synuclein variants, on the contrary, can be explained by a "stabilizing" effect of α-synuclein, that is the change in the microtubule structure making more stable the energetically unfavourable ones (Cantele *et al.*, manuscript in preparation, in PART III).

In conclusion, we found that α-synuclein variants induce significant changes in the structural parameters of microtubules, increasing their variability and the presence of generally energetically unfavourable structures. In addition, α-synuclein, particularly the mutant variants, makes the overall microtubule population less stable.

In the last part of the thesis, we faced up the hypothesis that the effect on microtubule structure strictly depends on the amount of α-synuclein bound to the microtubules. With this aim, we performed immuno-gold labelling experiments with anti-α-synuclein antibody. We then count the number of gold particles marking

microtubules and the number of that "free" or marking aggregates. We found the significant differences between microtubules assembled in the presence of E46K α-synuclein and all the other groups. This should confirm the high propensity of E46K α-synuclein mutant to bind to tubulin. In addition, we found a labelling of amorphous aggregates and a remarkable presence of "free" gold particles that are always significantly different from the number of particles linked to microtubules. The "free" gold particles should be linked to free α-synuclein or tubulin/α-synuclein small oligomers and their presence suggested that the sucrose cushion we used for separating microtubules from small aggregates and free α-synuclein, did not work well enough. Another possible explanation was suggested by the fact that, at the end of the immunogold-labelling process, the microtubule ultrastructure was not well conserved. This could mean that the procedure damages the microtubules, leading to amorphous aggregates and "free" α-synuclein (Cantele *et al.*, manuscript in preparation, in PART III).

Conclusions and Future Prospects

This thesis is centred in one of the current topics in our laboratory: the study of the interaction between microtubules and α-synuclein. Therefore, in the first part of the thesis we contributed to this project by investigating the gross ultrastructure of microtubules assembled in the presence or in the absence of wild type and mutated α-synuclein. The main result of this project is the evidence that α-synuclein, whose accumulation is the pathological hallmark of Parkinson's disease, is as a novel, foldable, microtubule dynamase, which could participate in the organization of the microtubule cytoskeleton at the pre-synapse, through its binding to tubulin and its regulation of microtubule nucleation and dynamics (Cartelli *et al.*, 2016, in PART II). The term dynamase raised from data showing that α-synuclein co-localizes with the most dynamic microtubules, in PC12 cells and human neurons, and that it has conflicting effects on microtubules, such as enhancing both microtubule growth rate and catastrophe frequency, *in vitro* and *in cell*. Interestingly, α-synuclein mutants are much less sensitive than wild type α-synuclein to fold upon tubulin binding. Looking at the structures formed at the end of tubulin assembly we found that mutated α-synucleins are more prone to cause tubulin aggregation rather than polymerization into conventional microtubules (Cartelli *et al.*, 2016, in PART II). Altogether these results support the idea that interactions between α-synuclein and microtubules play an important role in modulating synaptic physiology and that its alteration can cause neuronal dysfunction via impairment of the proper microtubule organization.

In the second part of the thesis, we deeply investigated the effect of α-synuclein on microtubule ultrastructure (Cantele *et al.*, manuscript in preparation, in PART III). We summarized the multifaceted impact that wild type and mutated α-synucleins exert on the most common parameters defining the structure of microtubules in Figure 13.

Wild type α-synuclein induces changes in microtubule diameter and protofilament number, increasing the presence of microtubules with uncommon structures. On the

other end, it has no effect on tubulin periodicity. The increasing presence of microtubules with uncommon structures, that have energetically unfavourable configurations, could suggest a mild "stabilising" effect of wild type α-synuclein on microtubules. First, this supports the results of Alim and colleagues (2004) showing that α-synuclein induces microtubule polymerization. Second, our data also support the findings of Toba and colleagues (2017) that wild type α-synuclein promotes the assembly of twisted microtubules, like 14-protofilament ones, and stabilizes them. In addition, the lack of effects on tubulin periodicity suggests that wild type α-synuclein could not directly impair the functionality of microtubules.

Focusing on α-synuclein mutants, we found that they all affect microtubule structure, although with some peculiarities. A30P α-synuclein increases both microtubule diameter and tubulin periodicity, but does not affect protofilament number. In addition, the high number of flattened microtubules we found in samples analysed by cryo-electron microscopy suggests that A30P α-synuclein leads to the assembly of less robust microtubules. These results are in agreement with the findings of Eguchi *et al.* (2017), who showed that overloading wild type α-synuclein into presynaptic terminals has an inhibitory effect on vesicle endocytosis, while the overloading of A30P α-synuclein has no effect (Eguchi *et al.*, 2017). They suggested a mechanism based on microtubule overassembly, which is an excess of microtubule assembly, since vesicle endocytosis was rescued by the pharmacological block of tubulin polymerization by nocodazole and the photo-switchable microtubule inhibitor photostatin-1. Our results suggest that A30P mutant reduces the overall stability of microtubules. Thus, the A30P mutant might prevent the achievement of the microtubule "overassembly", advising for a different mechanism for its pathological effect. Looking at A53T and E46K α-synuclein, we observed that they exert similar effects by decreasing microtubule diameter and tubulin periodicity, but differently impact on microtubule protofilament number. In addition, E46K mutant induces the strongest effect, being the one that binds more to microtubules and leads to the formation of peculiar microtubules, with high protofilament number and small

diameters. The fact that the two mutants have similar effects on microtubule diameter and tubulin periodicity supports the idea that the functional site of α-synuclein is in the same region of the two mutations and suggests this might be the region devoted to the interaction with tubulin (as previously hypothesized in Cartelli *et al.*, 2016, in PART II). The differences arise from the fact that E46K mutation is "stronger" that A53T one, because it changes an acidic amino acid into a basic one and just in the middle of a α-helix domain (Zarranz *et al*., 2004). Our results suggest that this change in α-synuclein can affect the microtubule structure in the inter-protofilament contact region, changing the inter-protofilament distance (Cantele *et al.*, manuscript in preparation, in PART III). This can lead to microtubules that retain some but not all of their functions.

Taken together, the effects of mutant α-synucleins we found are in contrast with those of Toba and colleagues (2017), which found the loss of interaction between mutated α-synucleins and microtubules and the consequent accumulation of α-synuclein in the cell body. On the other hand, our findings suggest an active role of both wild type α-synuclein and its mutated forms, due to the fact that they all have different effects on microtubule ultrastructure and that the mutated forms do not merely reduce the effect of wild type α-synuclein. To our opinion, this is in agreement with the idea that the toxic species of α-synuclein are monomers or small oligomers (Plotegher *et al.*, 2017), like those we used in our experiments, while the accumulation of α-synuclein in the cell body has a neuroprotective role achieved by consumption of toxic free α-synuclein (Pinotsi *et al.*, 2016). Such a hypothesis favours an active role of α-synuclein, both wild type and mutated, compared to a loss of interaction, this last being a result that can also be obtained by consumption of free α-synuclein.

From a global point of view, our results confirm the findings that α-synuclein has a complex function, more probably a regulatory function, with a generally mild initial effect on the microtubule network, which can accumulate over the time. We also suggest that the impairment of the transport function of microtubules could be the most important effect of α-synuclein. In addition, they provide hints for the

pathogenesis in Parkinson's disease, suggesting that microtubule-stabilizing strategies may offer an opportunity for treating it, but also that different strategies should be required for different mutants.

Future work will be focused on the further characterization of microtubule assembly and dynamics in the presence of α-synuclein mutants by spectroscopy and VE-DIC light microscopy. Next, we will complete the ultrastructural study for assessing the effect of α-synuclein on microtubule using cryo-electron microscopy and 3D reconstructions of microtubules decorated with α-synuclein, both wild type and mutated. Finally, we will inquire the effects of α-synuclein presence on the microtubule transport power by use of in vitro microtubule translocation.

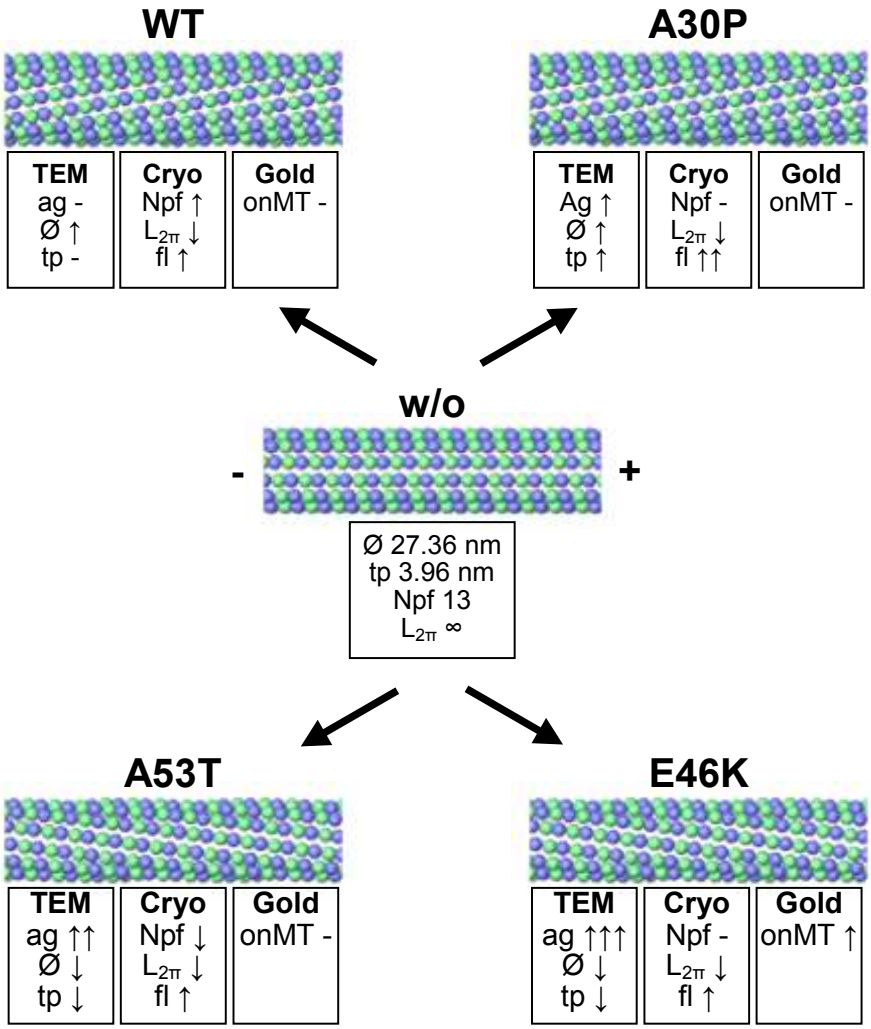


Figure 13. Schematic representation of the impact of α-synuclein variants on microtubule ultrastructure. The characteristics inspected are: the presence of aggregates (Ag), the microtubule diameter (Ø), the tubulin periodicity (tp), the protofilament number (Npf), the supertwist helix pitch ($L_{2\pi}$), the presence of flattened microtubules (fl) and the amount of gold particles associated to microtubules (onMT). They are grouped by the methodology used to study them: negative staining electron microscopy (TEM), cryo-electron microscopy (Cryo) and immuno-gold labelling (Gold). The variation of each parameter found respect to the microtubules obtained in the absence of any α-synuclein variant (w/o) is indicated with the symbols: − (no variation), ↑ (increase) and ↓ (decrease).

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Part II

Contents

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Part III

Contents

The impact of α-synuclein on microtubule structure *in vitro*.

Francesca Cantele[1], Valentina Budroni[1], Jacopo Marangon[1], Emmanuelle Neuman[2], Elisabetta Onelli[1], Alessandra Moscatelli[1], Isabelle Arnal[3] and Graziella Cappelletti[1,4].
[1]Department of Biosciences, Università degli Studi di Milano, Milano, Italy.
[2]Institut de Biologie Structurale, Université Grenoble Alpes, Grenoble, France.
[3]Grenoble Institut des Neurosciences, Grenoble, France.
[4]Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Milano, Italy

ABSTRACT

α-Synuclein is a presynaptic protein supposed to be involved in the control of neuronal synapse functions. It is widely expressed in brain tissue and associated to Parkinson's disease. When free in the cytoplasm, α-synuclein is unstructured, while it adopts a α-helical conformation when bound to vesicles. Its variable structure allows α-synuclein to interact with multiple partners and makes difficult to understand its physiological role, which remains elusive despite decades of intense study.

Here, we looked at the interaction between α-synuclein and microtubules. We investigated the influence of α-synuclein, both wild type and mutated, on the structure of microtubules assembled *in vitro* using transmission electron microscopy. We found that α-synuclein deeply affects microtubules causing changes in some of the parameters that define their ultrastructure, namely microtubule diameter and tubulin periodicity. It also impacts on the number of microtubule protofilaments, increasing the presence of some microtubules with uncommon structures.

INTRODUCTION

α-Synuclein, a presynaptic protein widely expressed in brain tissue, is supposed to be involved in the control of neuronal synapse functions. It is a soluble

naturally unfolded protein (Burré *et al.* 2013), being able to adopt multiple conformations. Its physiological localization is mainly at neuronal synapses, where it can be present as a monomer or as a tetramer (Burré *et al.* 2013), whereas it forms fibrils and intracellular aggregates in neurodegenerative diseases (Spillantini *et al.* 1998). In particular, α-synuclein was the first mutated protein identified in familial Parkinson's disease cases (Krüger *et al.* 1998; Polymeropoulos *et al.* 1997). The absence of a unique and rigid structure allows α-synuclein to interact with multiple partners, including synaptic vesicles (Larsen *et al.* 2006) and actin cytoskeleton (Sousa *et al.* 2009). Nevertheless, despite decades of intense study, conflicting results have been reported and the physiological role of α-synuclein remains elusive.

Microtubules have been proposed to be cellular partners of α-synuclein. They are highly dynamic polymers that control many aspects of neuronal function: they provide a scaffold to sustain axonal and dendritic architecture and supply the railway for axonal transport (Conde and Cáceres 2009). In spite of the fact that the regulation of microtubule organization and dynamics has been extensively studied during axon and dendrite formation and maintenance, much less is known about the regulation of microtubule dynamics at synaptic terminals. Regarding the interaction of microtubules with α-synuclein, it is known that tubulin promotes α-synuclein fibrillization *in vitro* (Alim *et al.* 2002), although it is not clear whether destabilization of the microtubule cytoskeleton potentiates (Esteves *et al.* 2010) or prevents (Nakayama, Suzuki, and Yazawa 2009) α-synuclein aggregation *in vivo*. Recently, it was also found that tubulin binds α-synuclein *in vitro*, causing its partial folding due to α-helix formation (Cartelli *et al.* 2016). On the other side, it was shown that α-synuclein induces polymerization of purified tubulin into microtubules (Alim *et al.* 2004) and that, even if unfolded monomeric α-synuclein has not significant effects on tubulin polymerization (Chen *et al.* 2007) folded α-synuclein promotes

microtubule nucleation and increases microtubule dynamics (Cartelli *et al.* 2016).

Here we investigate the influences of α-synuclein, wild type and some mutated forms, on the microtubule structure using transmission electron microscopy (TEM) of both negative stained and frozen samples. We found that α-synuclein causes significant changes in the parameters that define microtubule structure, in particular with the apparition of abnormally unstable microtubule conformations. In addition, one of the mutants shows a high aggregation propensity that leads to the formation of microtubule piles. Collectively, these alterations could have a direct impact on microtubule functions.

MATERIALS AND METHODS

Protein purification.

Tubulin was purified from young cow brain according to Castoldi and Popov (2003) by two cycles of polymerization/depolymerization in high molar Pipes buffer. Then it was suspended in BRB80 buffer (80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) and stored at -80°C.

Recombinant wild type α-synuclein, A30P α-synuclein and A53T α-synuclein were produced and purified according to Martinez *et al.* (2003), and recombinant E46K α-synuclein (kindly gifted by prof. Luigi Bubacco, University of Padova, Italy) was produced and purified according to Plotegher *et al.* (2017). Synuclein variants were suspended in Hepes buffer (20 mM Hepes, pH 7.4, 100 mM KCl) and stored at -80°C.

Proteins aliquots were clarified by ultracentrifugation (tubulin: 124000 × *g* at 4°C for 30 minutes; α-synuclein: 230000 × *g* at 4°C for 45 minutes) immediately before use.

Microtubule assembly.

Tubulin was pre-incubated for 10 minutes at 20°C, alone or in the presence of one of the α-synuclein variants, at concentrations of 80 μM tubulin and, when present, 20 μM α-synuclein, in BRB80 buffer. Then the polymerization was started by adding an equal volume of the polymerization buffer (BRB80, GTP 2 mM and glycerol 20%) and rising the temperature to 37°C.

Negative stain electron microscopy.

After 1 hour of polymerization, each sample was quickly but gently diluted with warm polymerization buffer (BRB80, GTP 1 mM and glycerol 10%, at 37°C) to the final concentration of 2 μM tubulin, and, when present, 0.5 μM α-synuclein. Then, 5 μl drops of sample were placed on glow discharged 200 mesh Formvar/carbon coated copper grids (Electron Microscopy Sciences), negative stained with 1% aqueous uranyl acetate and observed with a Philips CM10 transmission electron microscope at 80 kV equipped with a Morada Olympus digital camera. Images were taken at 23500×.

2D-image analysis and 3D reconstruction.

Microtubules were first straightened using STIRA, an interactive unpublished software developed in the Laboratory of image elaboration of the Department of Chemistry. Then the microtubule diameter was computed from the average image of each microtubule, calculated along its length, using the values obtained with the intercept at 0.25 of the maximun intensity (Chrétien and Wade 1991). The tubulin periodicity was calculated from the position of the "4 nm" line of the power spectrum of the straightened microtubules. 3D reconstructions were computed from the straightened image of microtubules using FT3D and its updates, a software allowing to compute 3D reconstruction of helical structures (Lanzavecchia *et al.* 1993, 1995).

Cryo electron microscopy.

After 1 hour or 4 hours polymerization, 4 µl of sample were placed on glow discharged 400 mesh holey carbon copper grids (Quantifoil) and automatically blotted and plunged into liquid ethane with a Vitrobot (FEI Vitrobot MARK IV) set at 100% humidity, 37°C, 2 seconds and force 1 or 0. The grids were observed with a FEI Tecnai F20 cryo-electron microscope at 200 kV equipped with a 4kx4k Ceta FEI camera. Images were taken at 29000×.

Immuno-gold labelling.

1 hour assembled microtubules were fixed with 0.5% glutaraldehyde by adding equal volumes of fixation buffer (BRB80, GTP 1mM, glycerol 10% and glutaraldehyde 1%). Then, they were centrifuged through a sucrose cushion (30% w/v sucrose in BRB80) for 1 hour at 150000 × *g* and 20°C. The pellet microtubules were retrieved and resuspended in BRB80 and 5 µl drops were placed on glow discharged 200 mesh Formvar/carbon coated nickel grids (Electron Microscopy Sciences). The grids were first incubated with anti-α-synuclein antibody (polyclonal rabbit antibody, S3062, Sigma Aldrich) for 45 minutes at 37°C and then with 5 nm gold-conjugated secondary antibody (anti-rabbit antibody, 5 nm gold conjugate, EM.GAR5, BBI Solutions) for 30 minutes at room temperature. They were then post-fixed with glutaraldehyde 1% in BRB80 for 10 minutes at room temperature, washed with 5 mM EDTA and negative stained with 1% aqueous uranyl acetate. Negative controls were obtained incubating the grids with only the secondary antibody.

Statistical analysis.

The statistical significance was assessed by one-way or factorial ANOVA with Tukey HSD or Fischer LSD *post hoc* testing (STATISTICA software, StatSoft Inc., Tulsa, OK) or by 2x2 contingency table analysis with two-tailed Fisher's

exact test (GraphPad Software, QuickCalcs tool, "www.graphpad.com/quickcalcs/contingency1.cfm").

RESULTS

In order to investigate how α-synuclein influences microtubule structure, we analysed microtubules assembled *in vitro* using transmission electron microscopy. We assembled the microtubules in the presence of wild type α-synuclein or A30P, A53T and E46K mutants, with a tubulin/α-synuclein ratio of 4:1. The assembly was carried out for at least 1 hour at 37°C. At the end of the assembly, samples were either fixed by negative staining or quickly frozen to obtain vitrified samples. As a control, we assembled microtubules from tubulin alone, in the same conditions.

Investigating the influence of α-synuclein on microtubule diameter and tubulin periodicity.

We first investigated the effect of folded α-synuclein on microtubule diameter and tubulin periodicity in negative stained microtubules by TEM (fig. 1) and 2D-analysis (as described in Materials and methods). We found that microtubules assembled in the presence of wild type α-synuclein display a significant increase in diameter (fig. 2a) while no changes were observed in tubulin periodicity (fig. 2b). We investigated also the effects of mutant α-synuclein A30P, A53T and E46K, and we found that they significantly impact both the microtubule diameter and tubulin periodicity (fig. 2a,b). In particular, A30P α-synuclein increases both microtubule diameter and tubulin periodicity (fig. 2a,b,c,d). Mutants A53T and E46K, instead, have the opposite influence, reducing both parameters. Other differences among the samples arise from the inspection of the distribution plots of the two parameters (fig. 2e,f). The profile of the microtubule diameter shows, for the E46K mutant, two almost equal

peaks (at about 25 and 27 nm) around the average value (about 26 nm) that is almost unrepresented and, in addition, some very low values (less than 20 nm). The other α-synuclein variants, instead, show a principal peak that includes the average value. About the tubulin periodicity, the E46K α-synuclein mutant shows a peak of values shifted to the lowest ones that is also asymmetric and a second small peak at high values, whereas all the other samples show only one almost symmetric peak of values.

In conclusion, these first results unravel different effects of synuclein variants on microtubule structures as wild type α-synuclein and A30P mutant significantly induce the increase in microtubule diameter, while A53T and E46K mutants significantly reduce both microtubule diameter and tubulin periodicity.

Evaluating the α-synuclein effects on microtubule 3D structure and its parameters.

From the best quality images of microtubules we obtained by TEM, we computed 3D reconstructions using the 3D helical reconstruction method (Arnal *et al.* 1996; Lanzavecchia *et al.* 1994). Using this approach, we were able to compute three principal parameters of the microtubule structure: protofilament number (Amos and Klug 1974), number of helix start and supertwist helix pitch (Mandelkow and Mandelkow 1985). The obtained reconstructions have very variable parameters and we did not find any statistically significant difference in these parameters (data not shown) but, interestingly, some peculiar results were obtained. In particular, we found a relevant difference between two microtubules obtained in the presence of A30P and E46K α-synuclein: they have the same protofilament number but very different microtubule diameter and tubulin periodicity, which are lower for the microtubule assembled in the presence of the E46K mutant (fig.3). This suggests a specific effect of E46K α-synuclein on

the lateral interaction of protofilaments during the building up of the microtubule.

Assessing the α-synuclein impact on the microtubule population through an analysis of their protofilament number.

To inspect in detail the protofilament number of the microtubule population we moved to cryo-electron microscopy (cryo-EM), a challenging technique that allows the observation of biological samples in quasi-natural conditions. The microtubules were, in fact, fixed in vitrified ice directly in the assembly buffer, without the addition of any external substance to enhance the contrast, and so what is observed is only the sample itself (fig. 4). We performed microtubule assembly in the same conditions than those used for negative staining experiments, i.e. 1 hour assembly at 37°C with tubulin/α-synuclein ratio of 4:1, and at a longer time, 4 hours. After one hour of assembly of tubulin in the absence of α-synuclein, we found a good number of intact microtubules, while for microtubules assembled in the presence of α-synuclein variants we found a lot of flattened microtubules (fig. 4, asterisks), which do not show the characteristic fringe pattern that allows the recognition of the protofilament number. This greatly reduced the population of the overall analysable microtubules. In the case of E46K α-synuclein mutant, we also found some parallel microtubule co-alignements (fig. 4) where the microtubules are not flattened. These piles were also evident in all the samples after 4 hours of assembly. We analysed the protofilament number distribution in the microtubule population by inspecting the microtubule fringe pattern (Chrétien and Wade 1991). The total microtubule length of the microtubule populations we analysed for each sample is shown in figure 5a,b. To evaluate the statistical differences of the microtubule populations assembled in absence and in presence of α-synuclein variants, we divided the different types of microtubules into two

groups: i) microtubules with less than 13 protofilaments and ii) microtubules with 13 or more protofilaments. We then applied 2x2 contingency table analysis with two-tailed Fisher's exact test. After 1 hour of assembly we found significant differences between microtubules assembled in the presence of wild type, A53T and E46K α-synuclein versus microtubules assembled in the absence of α-synuclein (fig. 5c).

On the other side, after 4 hours of assembly we found significant differences between microtubule populations assembled with wild type α-synuclein, A30P and A53T compared to the control, whereas the microtubule population assembled with E46K was similar to the control (fig. 5d). We also found significant differences between the α-synuclein variants. In detail, wild type and A30P mutant had similar effects, while A53T mutant led to a microtubule population that differs from that of all the other α-synuclein variants.

A detailed report of the different distributions we found for each sample is shown in table 1. At 1 hour of assembly the characteristics of the microtubule population for the control condition are i) the presence of a very high percentage of 13-protofilament microtubules, the most stable ones, and ii) the absence of microtubules with less than 12 or more than 14 protofilaments. The population of microtubules assembled in the presence of α-synuclein variants, instead, has less microtubules with 13 protofilaments and more of the others. In details, wild type and A53T α-synuclein have similar distributions, with a relatively low amount of 13-protofilament microtubules and a very high percentage of 12-protofilament ones. The population of microtubules assembled in the presence of A30P α-synuclein has the peculiarity of a large amount of 14-protofilament microtubules, with a still high presence of 12-protofilament ones. Finally, the microtubule population assembled in the presence of the last α-synuclein mutant, E46K, has an intermediate amount of 12- and 13-protofilament microtubules, a very low one of 14-protofilament microtubules and the unique presence of 11-protofilament microtubules. Interestingly, some of the α-

synuclein variants (A53T α-synuclein and to lesser extent, A30P and E46K) also induce the apparition of microtubules with highly twisted protofilaments representing 13 or 14 microtubules arranged on 4-start helices instead of 3-start.
It is interesting to note that microtubules assembled with E46K are the only ones that include protofilament numbers lower than 12, both for 1 hour and for 4 hours of assembly (table 1).

After 4 hours of polymerization time the microtubule population generally revealed more highly twisted microtubules than after 1 hour for all conditions including the control (table 1), which represent energetically unfavourable configurations.

Summarizing, α-synuclein variants significantly impact on the protofilament number distribution in the microtubule population, both with respect to the control population and to the other α-synuclein variants, generally increasing the amount of unfavourable microtubule configurations, like highly twisted ones.

Inquiring the presence of α-synuclein in microtubules using immuno-gold labelling.

In order to investigate the hypothesis that the effect on microtubule structure depends on the amount of α-synuclein bound to the microtubules, we performed immuno-gold labelling experiments, using the anti-α-synuclein antibody (fig. S1). We obtained an evident labelling of microtubules and we carried out a quantitative analysis by dividing each image into squares 100 nm wide. Then we counted the gold particles in each square, considering two different categories: i) squares with microtubules, even with only small portions, and ii) squares without microtubules. The differences between the microtubule samples are generally not significant (fig. S2a) except microtubules assembled in the presence of E46K α-synuclein, which differ from all the other groups. This should confirm the high propensity of E46K α-synuclein mutant to bind to

tubulin. In addition to gold particles linked to microtubules, we found a labelling of amorphous aggregates and a remarkable presence of “free” gold particles that are always significantly different from the number of particles linked to microtubules (fig. S2a). The “free” gold particles should be linked to free α-synuclein or tubulin/α-synuclein small oligomers, because in the negative controls, without using the anti-α-synuclein antibody, only few particles are present, indicating a good specificity for α-synuclein of the labelling process (fig. S2b).

In conclusion, only E46K α-synuclein significantly impact on the amount of α-synuclein bound to microtubule by increasing it.

DISCUSSION

α-Synuclein is a presynaptic protein widely expressed in brain tissue that is supposed to be involved in the control of neuronal synapse functions and is associated to Parkinson's disease. It interacts with multiple partners and despite decades of intense study, conflicting results have been reported, thus its physiological role remains elusive. Here we show that α-synuclein causes significant changes in the microtubule ultrastructure. These changes are more relevant for mutated than for wild type α-synuclein and suggest a reduction in microtubule functionality that could have a direct impact on microtubule functions. Thus, our data gives new hints in understanding the physiological and pathological roles of α-synuclein.

Wild type α-synuclein induces changes in microtubule diameter and protofilament number, resulting in an increased presence of microtubules with uncommon structures. These are energetically unfavourable configurations of microtubules (Chrétien and Fuller 2000) and could suggest a mild “stabilising” effect of wild type α-synuclein, which could result in promoting microtubule assembly. For this reason, our results are in agreement with those from Alim and

colleagues (2004) showing that α-synuclein induces microtubule polymerization, and with the findings of Eguchi and co-workers (2017), who demonstrated that α-synuclein impairs vesicle endocytosis through a microtubule overassembly-dependent mechanism. Our results also support the findings of Toba and colleagues (2017) that wild type α-synuclein promotes the assembly of twisted microtubules, like 14-protofilament ones, and stabilizes them.

Mutations in the α-synuclein gene are linked to Parkinson's disease. Interestingly, the analyses of their impact on the microtubule ultrastructure unravelled some similarities and also differences with respect to wild type α-synuclein.

A30P α-synuclein increases microtubule diameter, like wild type form does, but, unlike wild type, it impacts on tubulin periodicity without affecting protofilament number. Next, using cryo-electron microscopy, we revealed that A30P α-synuclein leads to a large amount of flattened microtubules, thus suggesting a reduction in the overall stability of microtubules. This is exactly the contrary to wild type α-synuclein. Again, this is in agreement with the findings of Eguchi and co-workers (2017), which showed that, unlike wild type, over-expression of A30P α-synuclein has no effect on vesicle endocytosis and does not lead to microtubule overassembly. Thus, the A30P mutant might prevent the achievement of the microtubule overassembly by reducing the microtubule stability.

Looking at A53T and E46K α-synuclein, the impact on microtubules is multifaceted. We observed that they both exert similar effects by decreasing microtubule diameter and tubulin periodicity, the opposite of what wild type and A30P α-synuclein do. The impact on microtubule protofilament number, instead, is similar to that of wild type, suggesting a mild “stabilising” effect. However, for A53T mutant, the presence of flattened microtubules, which could be the result of their weakness, might suggest a mild “destabilising” effect. The

same result is achieved in the case of E46K α-synuclein, but it seems to be caused by its high propensity to aggregate as we found here and previously described (Cartelli *et al.*, 2016). In addition, E46K mutant induces the formation of peculiar microtubules, which have smaller diameter than the ones with the same protofilament number previously described by other authors (Metoz, Arnal, and Wade 1997). This suggests that E46K mutant could affect microtubule inter-protofilament distance thus explaining the lack of significant differences in the number of protofilaments whereas the microtubule diameters significantly differ. The fact that E46K mutation seems to be the more impacting among the mutated forms, both in terms of the number of microtubule parameters that change and of the high significance of the evoked effects, could be dependent on its specific point mutation, i.e. the switch from an acidic amino acid to a basic one just in the middle of a α-helix domain (Zarranz *et al.*, 2004). Otherwise, its similarities with A53T could be due to the fact that they both affect the same α-helix domain.

Taken together, the effects of mutant α-synucleins we found are in contrast with those of Toba and colleagues (2017), which found the loss of interaction between mutated α-synucleins and microtubules and the consequent accumulation of α-synuclein in the cell body. On the other hand, our findings suggest an active role of both wild type α-synuclein and its mutated forms, due to the fact that they all have different effects on microtubule ultrastructure and that the mutated forms do not merely reduce the effect of wild type α-synuclein. To our opinion, this is in agreement with the idea that the toxic species of α-synuclein are monomers or small oligomers (Plotegher *et al.*, 2017), like those we used in our experiments, while the accumulation of α-synuclein in the cell body has a neuroprotective role achieved by consumption of toxic free α-synuclein (Pinotsi *et al.*, 2016). Such a hypothesis favours an active role of α-synuclein, both wild type and mutated, compared to a loss of interaction, this last being a result that can also be obtained by consumption of free α-synuclein.

From a global point of view, the fact that different variants of α-synuclein have opposite effects on the same microtubule parameters, and that they have both "stabilizing" and "destabilizing" effects, confirms that α-synuclein could have a complex and regulatory function on the microtubule network. Since microtubules are the railway along which molecular motors run, their distribution and stability, but also their structure, influence the cellular transport system (Black, 2016). The molecular motors dyneins and kinesins, in fact, run on microtubules by steps, the length of which is linked to tubulin periodicity and the direction to protofilament directionality. Changes in microtubule parameters could then affect the efficiency of the microtubule transport system, suggesting that it could be the most important pathological effect of α-synuclein (Hunn *et al.*, 2015). Besides this, the fact that A53T and E46K α-synuclein affect all the investigated parameters that defines microtubule structure, while A30P does not, supports the idea that the region of these two mutations might be the one devoted to the interaction with tubulin, as recently proposed (Cartelli *et al.*, 2016). The three α-synuclein mutants are linked to different clinical features of Parkinson's disease, with a relatively early disease onset and a severe form of the disease for A53T and E46K mutations and a more typical late-onset and late and relatively mild dementia for A30P α-synuclein (Gasser *et al.*, 2011). The correspondence between the extent of the effects of α-synuclein mutants on microtubules and the severity of the pathology supports the idea that the pathogenesis of Parkinson's disease is strictly linked to the dysfunction of the microtubule network. Microtubule regulating strategies may then offer an opportunity for treating synucleinopathies, including Parkinson's disease (Dubey *et al.*, 2015; Cartelli and Cappelletti, 2017; Cappelletti *et al.*, 2017), but different strategies could be probably advantageous for different mutants, because they sometimes have opposite effects.

Future analysis are required to verify the effects of various α-synuclein variants on microtubule transport capacity and, in addition, further structural analysis,

particularly on the interaction between α-synuclein and microtubules, could give an explanation of the reason why α-synuclein variants differently impact on microtubules.

LEGENDS

Figure 1. Negative staining electron microscope images of microtubules assembled *in vitro* at 40 μM tubulin concentration, alone (tub) or in the presence of 10 μM of wild type (WT) or mutated (A30P, A53T and E46K) α-synuclein.

Figure 2. (**a**, **b**) Box plots of the microtubule diameter and tubulin periodicity of microtubules observed by negative staining. *$p<0.05$ vs tub, $^{\#}p<0.05$ vs WT and A30P and $^{\S}p<0.05$ vs A53T, according to ANOVA, Fischer LSD *post hoc* test. (**c**, **d**) Numeric parameters of the data showed in (**a**) and (**b**). (**e**, **f**) Distribution of microtubule diameter and tubulin periodicity in the microtubule population.

Figure 3. 3D reconstructions and calculated parameters of two selected microtubules following assembly in the presence of A30P and A53T α-synuclein mutants. Both microtubules have the same protofilament number (16), but different diameter (33.1 vs. 30.4 nm), due to a different distance between adjacent protofilaments. Lattice structure corresponds to: protofilament number (16), number of helix start (3 or 5) and handedness (L or R).

Figure 4. Cryo-EM images of microtubules assembled *in vitro* at 40 μM tubulin concentration, alone (tub) or in the presence of 10 μM of wild type (WT) or mutated (A30P, A53T and E46K) α-synuclein. Asterisks mark flattened microtubules.

Figure 5. (**a**, **b**) Total length of the microtubules analysed on cryo-electron microscope images following 1 hour (**a**) and 4 hours (**b**) assembly *in vitro* at 40 μM tubulin concentration, alone (tub) or in the presence of 10 μM of wild type (WT) or mutated (A30P, A53T and E46K) α-synuclein. (**c**, **d**) Percentage of microtubules displaying less than 13 protofilaments (Npf<13) or 13 and more protofilaments (Npf≥13) for each type of sample, at 1 hour and 4 hours polymerization time. *$p<0.05$ vs. tub, $^{\#}p<0.05$ vs. WT, $^{\S}p<0.05$ vs. A30P, $^{+}p<0.05$ vs. A53T and °$p<0.05$ vs. E46K, according to 2x2 contingency table analysis with two-tailed Fisher's exact test.

Table 1. Analysis of the microtubule protofilament number distribution (ranging from 10 to 16) and the percentage of twisted microtubules (including 12, 13 and 14 twists) following 1 hour and 4 hours of polymerization in the absence (tub) or in the presence of wild type (WT) or mutated (A30P, A53T and E46K) α-synuclein. This analysis was performed on cryo-EM images.

Figure S1. (**a**) Negative staining image of microtubules assembled in the presence of wild type α-synuclein and immunolabelled with anti-α-synuclein antibody and 5 nm gold-conjugated secondary antibody. (**b**) 300x300 nm box enlargement of the indicated portion of the image in (**a**).

Figure S2. (**a**) Average number of gold particles found for 100x100 nm squares in negative staining electron microscope images of microtubules assembled in the absence or presence of α-synuclein variants and immunolabelled with anti-α-synuclein antibody and 5 nm gold-conjugated secondary antibody. (**b**) Average number of gold particles found for 100x100 nm squares in negative staining electron microscope images of microtubules assembled in the absence or presence of α-synuclein variants and incubated with 5 nm gold-conjugated

secondary antibody following omission of the primary antibody. *$p<0.05$, according to ANOVA, Tukey HSD *post hoc* test.

FIGURE 1

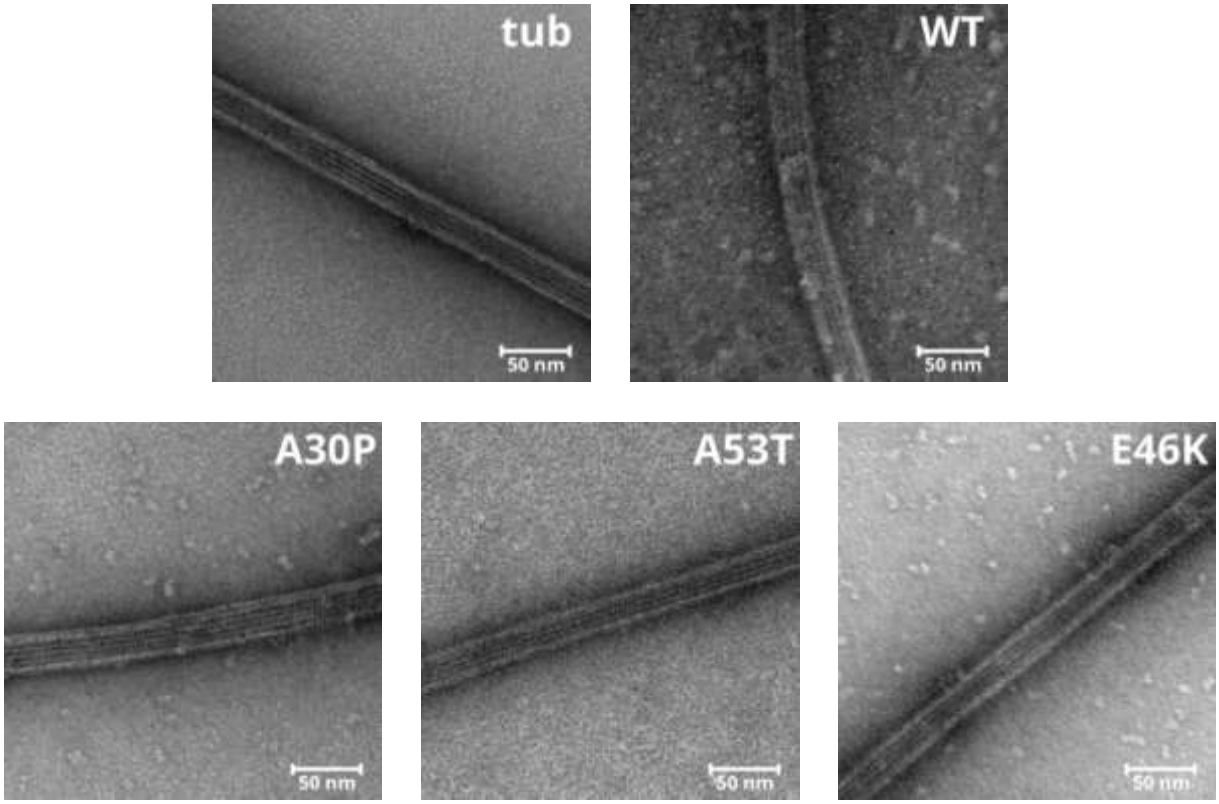
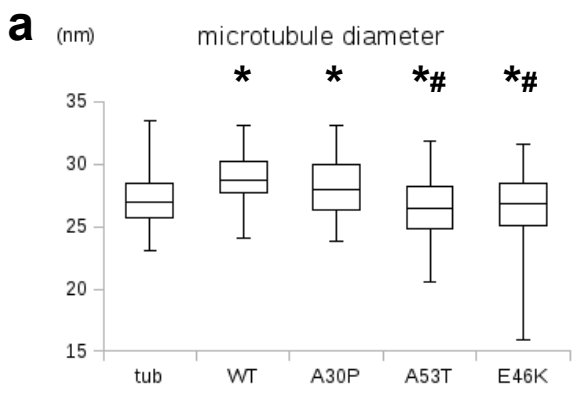
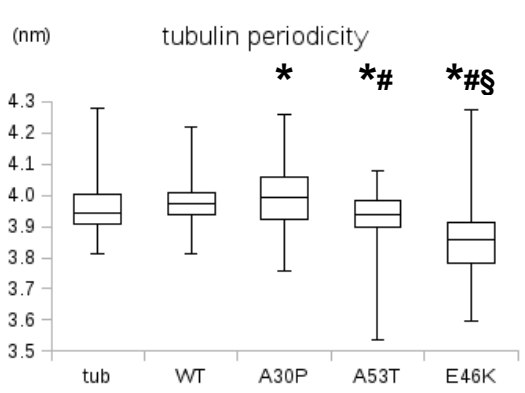


FIGURE 2



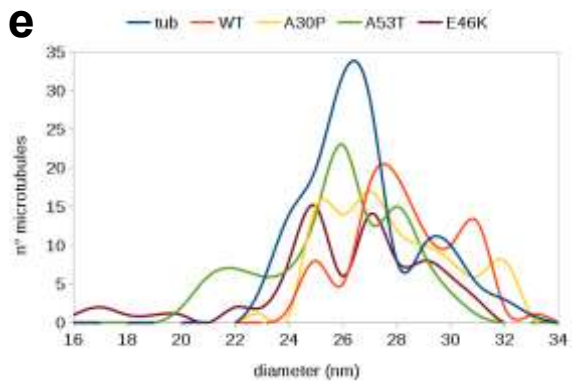


c

Sample	Mean ± Std.Dev.	Valid N
tub	27.36 ± 2.16	123
WT	28.79 ± 1.97	90
A30P	28.32 ± 2.29	93
A53T	26.23 ± 2.5	107
E46K	26.08 ± 4.21	77

d

Sample	Mean ± Std.Dev.	Valid N
tub	3.96 ± 0.08	123
WT	3.98 ± 0.07	90
A30P	3.99 ± 0.10	93
A53T	3.93 ± 0.07	107
E46K	3.87 ± 0.13	77



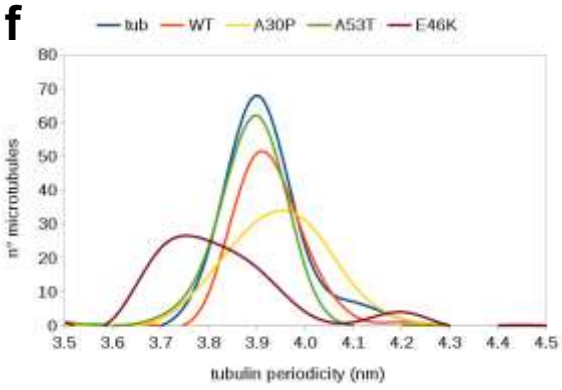


FIGURE 3

A30P	Sample	E46K
33.1	Diameter (nm)	30.4
4.08	Tubulin periodicity (nm)	3.77
16_3 L	Lattice structure	16_5 R
1.6	Supertwist half-helix pitch (μm)	2.4

FIGURE 4

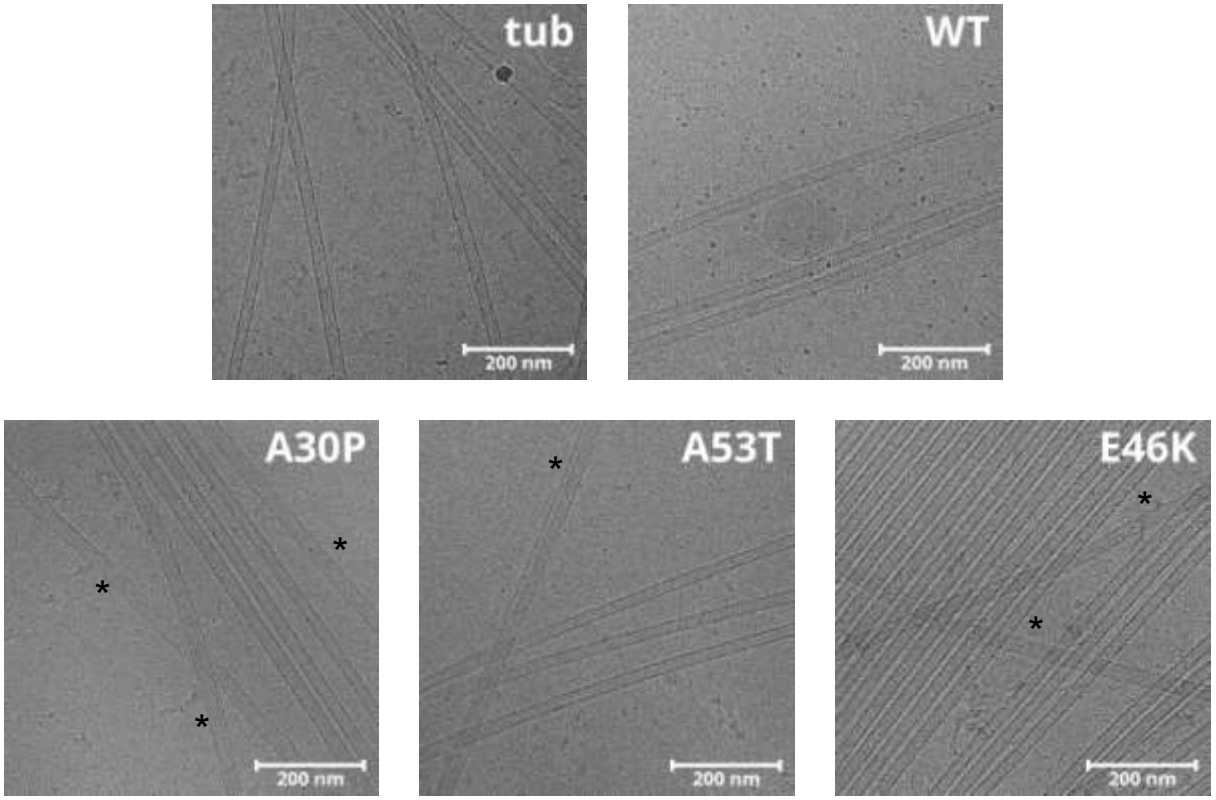


FIGURE 5

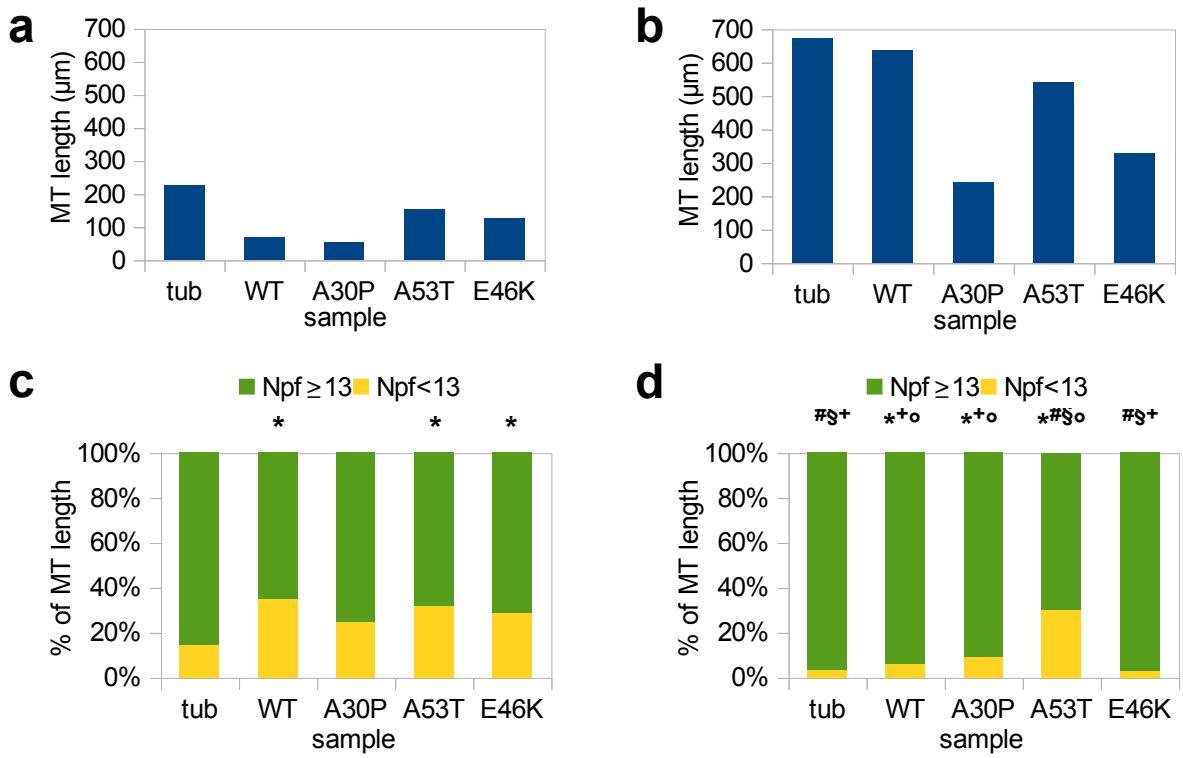

a
MT length (µm)
700
600
500
400
300
200
100
0
tub
WT
A30P
A53T
E46K
sample
b
MT length (µm)
700
600
500
400
300
200
100
0
tub
WT
A30P
A53T
E46K
sample
c
Npf ≥ 13
Npf<13
*
*
*
% of MT length
100%
80%
60%
40%
20%
0%
tub
WT
A30P
A53T
E46K
sample
d
Npf ≥ 13
Npf<13
#§+
*+o
*+o
*#§o
#§+
% of MT length
100%
80%
60%
40%
20%
0%
tub
WT
A30P
A53T
E46K
sample

Table 1

		10	11	12	13	14	15	16	12tw	13tw	14tw
	tub			14,3%	73,1%	12,5%				0,3%	
	WT			34,8%	53,2%	11,6%		0,3%			
1h	**A30P**			24,7%	54,1%	21,2%				3,9%	
	A53T			31,9%	58,3%	9,4%		0,5%	24,9%		
	E46K		1,1%	27,4%	65,3%	6,3%				2,9%	
	tub			3,5%	77,3%	19,0%	0,1%	0,2%		2,4%	3,2%
	WT			5,8%	68,8%	24,9%	0,3%	0,1%	1,6%	7,1%	2,5%
4h	**A30P**			8,9%	77,5%	13,2%	0,4%		6,2%	10,5%	6,3%
	A53T			30,2%	63,0%	6,8%			0,9%	0,4%	3,2%
	E46K	0,2%	0,5%	2,1%	77,9%	18,0%	1,4%			9,5%	4,7%

FIGURE S1

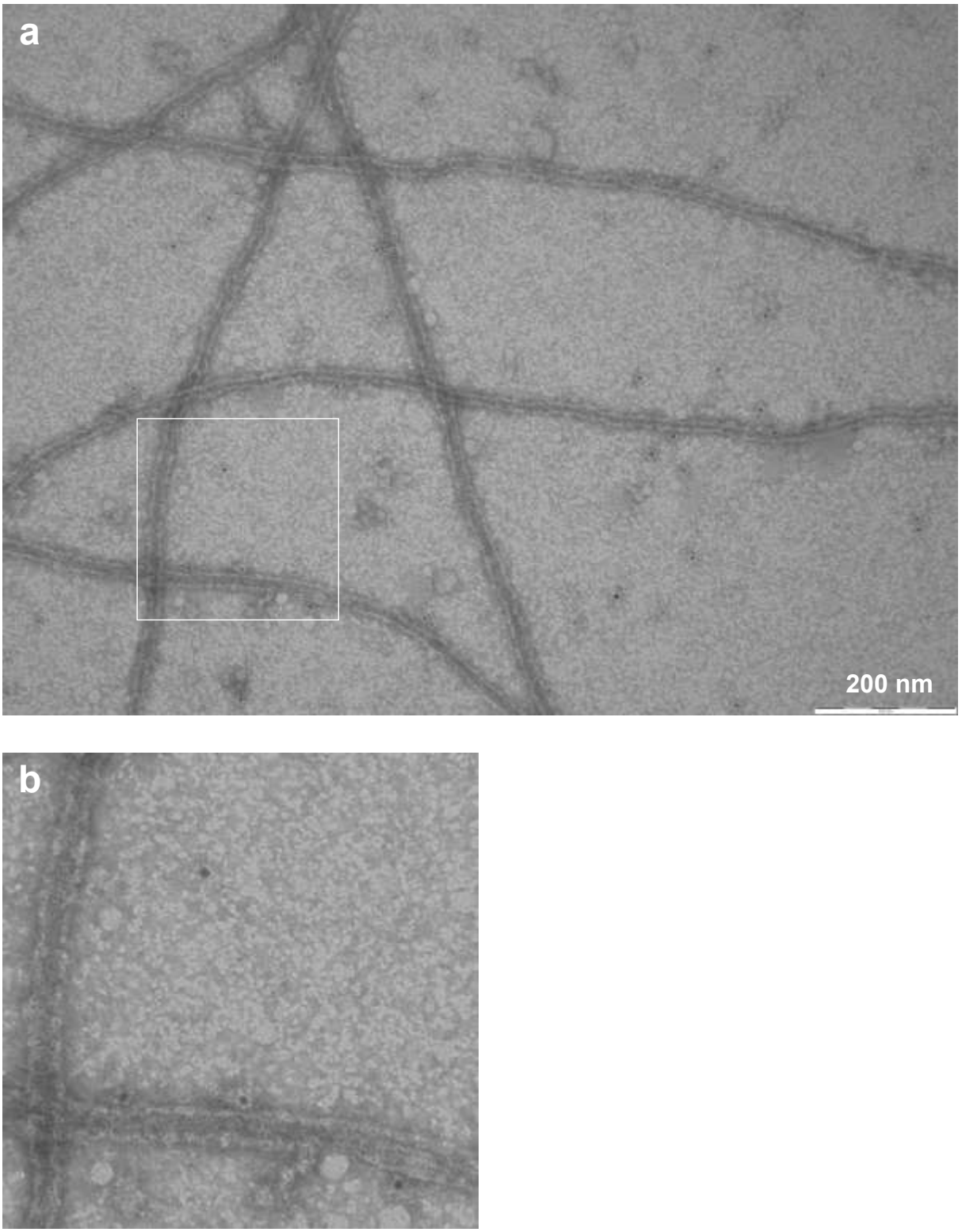
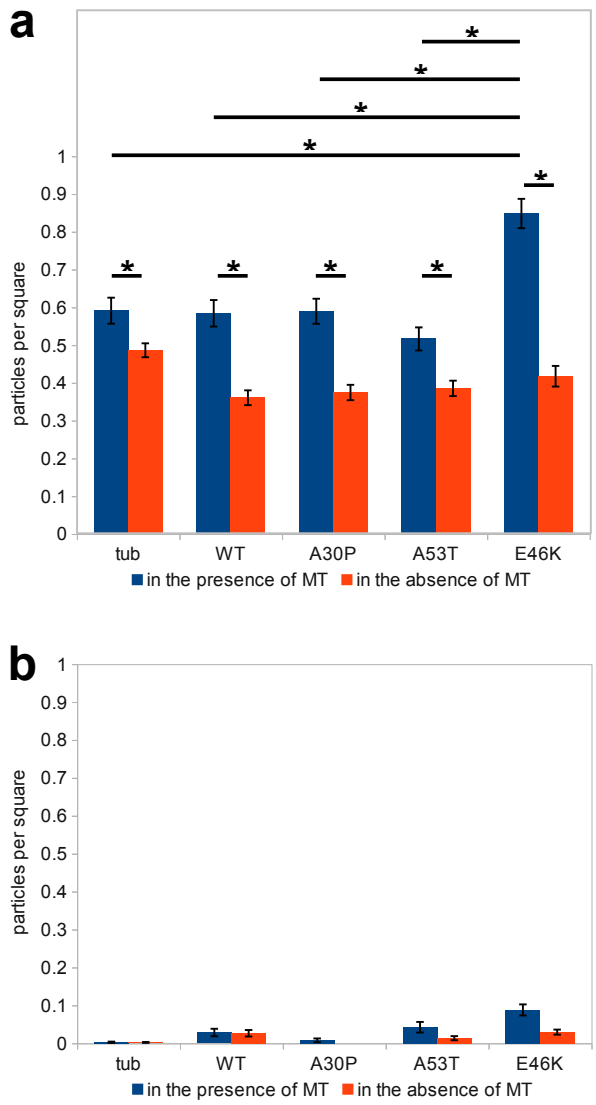


FIGURE S2


a
particles per square
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
tub
WT
A30P
A53T
E46K
in the presence of MT
in the absence of MT
b
particles per square
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
tub
WT
A30P
A53T
E46K
in the presence of MT
in the absence of MT

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