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Dynamic Behaviors of α-Synuclein and Tau in the Cellular Context: New Mechanistic Insights and Therapeutic Opportunities in Neurodegeneration

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Abstract

α-Synuclein (αS) and tau have a lot in common. Dyshomeostasis and aggregation of both proteins is central in the pathogenesis of neurodegenerative diseases: Parkinson's disease, dementia with Lewy bodies, multi-system atrophy and other'synucleinopathies' in the case of αS; Alzheimer's disease, frontotemporal dementia, progressive supranuclear palsy and other'tauopathies' in the case of tau. The aggregated states of αS and tau are found to be (hyper)phosphorylated, but the relevance of the phosphorylation in health or disease is not well understood. Both tau and αS are typically characterized as 'intrinsically disordered' proteins, while both engage in transient interactions with cellular components, there by undergoing structural changes and context-specific folding. αS transiently binds to (synaptic) vesicles forming a membrane-induced amphipathic helix; tau transiently interacts with MTs forming an 'extended structure'. The regulation and exact nature of the interactions are not fully understood. Here we review recent and previous insights into the dynamic, transient nature of αS and tau with regard to the mode of interaction with their targets, the dwell-time while bound, and the cis and trans factors underlying the frequent switching between bound and unbound states. These aspects are intimately linked to hypotheses on how subtle changes in their transient behaviors may trigger the earliest steps in the pathogenesis of the respective brain diseases. Based on a deeper understanding of transient αS and tau conformations in the cellular context, new therapeutic strategies may emerge, and it may become clearer why existing approaches have failed or how they could be optimized.

1. Introduction

In a sense, the study of protein folding is a quandary: conventional NMR, cryo-EM, or X-ray crystallography can solve the structure of a purified protein but does so by taking the protein

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out of its biological context. On the other hand, various cell biological and biochemical techniques can provide insight into a protein's behavior with in a cellular context but the information on its structure at atomic-level resolution is limited. That does not seem to be a problem for many proteins because their *in vitro* structures are a good approximation of those in the cell. However, it becomes a greater challenge when studying 'intrinsically disordered' proteins. Such proteins (or at least significant portions of them) adopt a more or less random-coil ('unfolded') structure in solution *in vitro*, but this does not rule out that context-specific folding/assembly states can arise inside cells. On the contrary, for many 'intrinsically disordered' proteins, transient folding has been proposed, but the dynamic nature of such states can render the proteins elusive to precise characterization. The stakes arguably become higher when the proteins under investigation are implicated in disease. Indeed, two key proteins in neurodegenerative diseases, α-synuclein (αS) in Parkinson's disease (PD), Dementia with Lewy bodies (DLB) and other 'synucleinopathies' and tau in Alzheimer's disease (AD), frontotemporal dementia (FTD) and other 'tauopathies', are still not yet well understood regarding their dynamic conformational behavior in cells. αS and tau, which both form insoluble aggregates in the brain under disease conditions, have been described to be largely soluble in their native states while transiently interacting with cellular targets: αS with (synaptic) vesicles, tau with MTs. This indicates that a contextual understanding of the transient and dynamic behavior of both proteins at the chemical, molecular, and cellular level may be required to understand synucleinopathies and tauopathies and develop therapeutic strategies through new perspectives. Here, we discuss recent advances in understanding cellular αS and tau dynamics in the context of the previous literature. Our special focus will be molecular'cis' and 'trans' determinants undergirding αS and tau transient behavior. We conclude with some remarks on possible therapeutic strategies in light of our analysis.

2. αS

2A. αS in health and disease

Since the first discoveries of αS aggregation in Lewy bodies[1] (see below), hundreds of studies have probed the precise structure and characteristics of physiological αS. There are many fine reviews on structure/function[2–6], genetic interactions[7,8], and neurotoxicity[9,4,10–13] of αS, but what is significant to highlight here is that despite years of research, the precise function, subcellular distribution, and context-specific behavior of αS are still under investigation. It is likely that these properties are all intimately linked.

In mature neurons, including dopaminergic neurons[14,15], αS predominantly resides in presynaptic nerve terminals, which has prompted the hypothesis that αS may be involved in regulating dopamine release.[16–18] Considerable evidence suggests that αS helps regulate (synaptic) vesicle trafficking.[19–25] This role of αS is consistent with its well-documented propensity to bind to small vesicles *in vitro*, which is mediated by its formation of transient amphipathic helices at highly curved membranes (see below).[26–29] PD and DLB cytopathology and neurodegeneration are characterized by αS accumulation into Lewy bodies, large intracellular aggregates whose major componentis αS. However, the exact relationship of these lesions with dopaminergic neuronal death in the substantia nigra pars

compacta and eventual PD symptoms, such as bradykinesia and muscular tremors[30], is not fully understood. Similarly, we lack understanding of the exact pathogenic significance of cortical Lewy bodies in DLB. How and under what conditions αS aggregates have been areas of much inquiry and debate.

2B. αS transiently binds to synaptic vesicles

Early biochemical characterizations of purified bacterially-expressed αS identified the protein to be soluble[14–31], which confirmed previous immunogold-EM data that detected αS throughout the cytoplasmic matrices in axon terminals.[3] Subsequent biophysical and biochemical studies, however, reported that αS can bind to small unilamellar and multilamellar vesicles[33,34] and detergent micelles[35]. Chemically crosslinked αS in SH-SY5Y cell homogenates was also identified in vesicle fractions by flotation centrifugation,[36] but this observation did not seem to directly contradict the generally soluble nature of αS, because fractionated brain extracts revealed only a weak association of αS with synaptic vesicles[37,38]. Similarly, photobleaching microscopy indicated that αS interacts only weakly with membranous elements of the nerve terminal, and each molecule appears to switch rapidly between the aqueous cytosol and the membrane.[38,39] While methods to interrogate the function of αS vis-à-vis synaptic vesicles[40–42,25,43,15] became a natural implication of such studies, this function is still not well understood (for other proposed functions, see review by Bendor et al.[2]). Nevertheless, what has emerged is a nuanced picture of αS – a aqueously soluble protein that can transiently interact with vesicular membranes.

Recent studies have supplemented established biophysical and microscopy techniques by using new technologies to quantify the transient nature of αS. Single-molecule microscopy combined with photo-bleaching identified an average of seventy αS molecules bound to each vesicle and situated 10 nm apart from each other.[46] Moreover, the use of phospholipid bilayer nanodiscs (which had been previously used to study αS-Ca^{+2} interactions)[47] and bacterially purified αS allowed for αS-membrane association and dissociation kinetics to be calculated *in vitro* (e.g., an off-rate of $0.015 \pm 0.006\ s^{-1}$).[48] However, data generated by *in vivo* multiphoton fluorescence recovery after bleaching (FRAP) coupled with murine cranial window surgery were consistent with the existence of at least two pools of αS in terminals with lower levels of mobility than measured previously. The observed $t_{1/2}$ for axonal terminal photobleaching recovery (~2 min) in the mice was much slower than that measured in a photobleaching study of GFP-tagged αS at presynaptic terminals in an acute dissociated hippocampal cell culture system (<10sec)[39], or YFP-tagged αS in *C. elegans* body wall muscle (<10sec)[49]. In addition to the presynaptic terminal, the turnover and mobility of αS in the somatic compartment of cortical neurons was measured by Unni et al. [51,52] In contrast to the discrepancy between their presynaptic terminal results and those reported in the literature, the rapid mobility of αS-GFP measured within the soma (<5 sec) was in good agreement with these previous studies of αS mobility. This suggested that the cellular context of αS may influence transient binding affinity and kinetics.[50,51]

This state of equilibrium between soluble and membrane-associated forms of αS is expected to be finely balanced and tightly regulated. Familial PD mutations as well as engineered variants have been observed to shift the balance towards misfolding, insolubility, inclusion

formation and cell toxicity[52–55] (discussed in further detail below). Consequently, such adverse changes in neuronal cells pose functional problems in vesicle trafficking, recycling, and neurotransmitter release[56–59] (even overexpressed wildtype αS has been shown to undergo accelerated aggregation[60,61] and display many of the aforementioned phenotypes[62,18,63–65]). In short, these recent observations highlight the transient nature of wildtype αS and the importance for the molecule to remain dynamic to keep cell toxicity at bay.

2C. αS transiently exists in a variety of monomeric and multimeric forms

Soluble αS had long been believed to exist solely as an unfolded monomer, both *in vitro* and *in vivo.* Less than a decade ago, emerging studies on the conformation of αS began to posit that the protein can exist in part physiologically as a soluble, higher-ordered multimer, apparently primarily a tetramer.[66–71] Several biochemical as well as *in-cell* NMR studies responded by defending the classical view of αS as a natively unfolded monomer in cells[72–75]. A review on αS homeostasis[76] highlighted the debate and suggested specific reasons for why certain studies reported the successful purification of tetrameric/multimeric αS whereas others did not: 1) multimer stability might be tissue-specific,[67,74,77,71] 2) αS purification *in vitro* under non-denaturing conditions could stabilize multimers, but the exact parameters for successful multimer purification are not fully understood [67,78,3]; and 3) under certain conditions an undiscovered multimer-stabilizing molecule (perhaps one or more lipid molecules) may be lost during purification.[71] Because of the difficulty of αS multimer purification and stabilization, intact-cell methods such as the application of cell-penetrant cross-1 inkers to cells and brain tissue[68,79–81,54,82] as well as fluorescent-protein complementation[79,83–86] became increasingly important for documenting multimeric αS arrangements. Intact-cell NMR, an elegant method that was used to propose the *in vivo* relevance of unfolded soluble αS[75], is 'blind' to multimeric or membrane-associated αS molecules[87]. A crosslinking study that compared the outcome of crosslinking intact cells vs. lysates demonstrated that αS and βS putative tetramers (60 kDa) were prominent in intact cells but largely disassembled in crude cell lysates; only in highly concentrated ('crowded') cell lysates could some multimeric αS be trapped by crosslinking.[88] These findings were consistent with a model in which αS multimerization is highly dependent on the intact cellular environment. Relevant factors that promote αS multimerization inside cells could be general 'molecular crowding' or the transient but constant interaction with specific cellular factors such as membrane lipids, organelles or other biomolecules. Obviously, dilution by cell lysis reduces crowding and disrupts any weak transient interactions that may exist. Recently, an elegant *in vitro* study demonstrated that transient αS-membrane interactions may indeed be the first step towards native αS-αS assembly.[89] The authors investigated the interplay of αS with 13:0 phosphatidyl choline small unilamellar vesicles. By modulating αS binding through phase transitions of the vesicle lipids, soluble helical αS species could be reconstituted that behaved as multimers. While folding of certain 'intrinsically disordered proteins' upon binding to ligands is well established, no previous report had described folding assisted by intermittent contact with a cofactor (in this case: membrane lipids). This remarkable study is in agreement with several reports on soluble αS multimers[67,66,90,88,91,77,78,71,70,79,80,92,54,82,93]. The relevance of multimeric αS at membranes has also been highlighted in other studies (e.g., in the context of mediating

SNARE complex assembly[94,95] and vesicle clustering[23]). Fig. 1A illustrates a model of dynamic cellular αS behavior in health, centered around the idea of (vesicle) membrane-assisted, transient αS folding and assembly[89]. In this model, αS monomer and multimers levels are highly transient, dynamic, and sensitive to environmental factors. Fig. 1B highlights potential mechanisms of synucleinopathy initiation based on this model; the illustration takes into account reports on excess soluble αS monomers as the starting point of cytosolic fibrillar αS aggregation[95] (top left), fibrillar αS aggregation that is mediated by excess membrane binding via 'nucleatin'[96] (bottom right), as well as membranous non-fibrillar αS aggregation as an emerging concept[82,97,98] (bottom left).

2D. Molecular determinants of the transient nature of αS

Across the 140 amino acids that span αS, the protein is often subdivided into three regions: the N-terminal region (amino acids 1-60), the 'NAC' (non-amyloid component) domain (amino acids 61-95), and the C-terminal region (amino acids 96-140).[3] The N-terminal region has been of immense interest to better understand the molecular determinants for αS transient membrane-binding behavior, because early studies identified this region to contain amino acid repeat motifs that resemble lipid-binding domains often observed in apolipoproteins[14]. Biochemical, cell biological and biophysical studies have shed much light on how αS 'cis' determinants[99–101,54] and 'trans' factors such as vesicle membrane composition[102–104,89,105] may govern αS transient binding to artificial and biological membranes.

Different regions of the protein have been shown to have different binding affinities for charged lipid membranes. The first 25 residues in the N-terminus have been suggested to 'anchor' into the bilayer[106], with certain key residues (M1, V3, F4, and L8) stabilizing αS at membranes via Van der Waals interactions, while other residues (K6, K10, and K12) interact electrostatically with negatively-charged lipid head groups.[107,108] Due to the repeated structure of αS (6-7 imperfect repeats of 11 aa with the consensus sequence of the repeat motifs being KTKEGV), this mechanism can be extended to the first ~95 aa of the protein. The positively-charged Lys residues have been suggested to interact with negatively-charged lipid head groups when the N-terminus of αS forms an α-helix (3.67 amino acids per turn)[109], which allows the Lys residues to lie perpendicular to the helical axis on a membrane.[110] In contrast, nonpolar amino acid residues can partially dip into the membrane bilayer (~1-5 Å below lipid head groups)[111–114], especially where lipid packing defects exist, and interact with the lipid carbon chains.[115,116] However, in the αS membrane-induced 3-11 helix, some non-polar residues are exposed to the aqueous phase, and some polar residues are embedded into the nonpolar lipid bilayer, most prominently threonine residues (see Fig. 2), leading to imperfect amphipathic helix formation. Consequently, the specific arrangement of charged residues, polar lipid head groups, and nonpolar carbon chains seem to manifest the transient nature of αS-membrane binding[5,54] (see further discussion in Dettmer et al. 2017[97]).

This principle can be confirmed by observing how rationally engineered mutants within the canonical repeat motif, KTKEGV, that'correct'the imperfect hydrophobicity within the hydrophobic half of the αS membrane-induced amphipathic helix can there by abolish the

transient nature of αS.[80,54,117,118] Six or 7-repeats of a KLKEGV or KTKEΛV or KTKKGV mutant can destabilize native tetramers/multimers in neurons and presumably lead to the accumulation of helical monomeric αS at membranes and perhaps some unfolded cytosolic αS, as these mutants remained monomeric when tested by DSG crosslinking and were highly enriched in PBS-insoluble fractions that required Triton-X 100 to solubilize membranes.[80] Beyond such artificially engineered αS, an amplification of the E46K fPD-linked mutant (i.e., KTKKGV in repeat 4) in the two adjacent repeat motifs strongly reduces tetramers/multimers, as suggested by intact cell-crosslinking and YFP-fluorescent-protein complementation assays, and it also accumulates in PBS-insobluble fractions.[79,97] In addition, the mutant H50Qwas reported to decrease αS solubility 10-fold.[119] However, also the membrane-binding deficient, cytosol-enriched fPD variants A30P and G51D cause PD, and it was suggested that they expose their hydrophobic core in solution, thus enabling other αS molecules to bind and aggregate.[120] A study based on intact-cell crosslinking and YFP complementation concluded that all known fPD-linked αS variants lead to a relative decrease of αS multimers and a gain in aggregation-prone monomers (either in the cytosol or at membranes, depending on the specific mutants[121]). In short, certain regions of αS as well as specific residues have been shown to play a role in either increasing or decreasing binding affinities to lipid bilayers through electrostatic or Van der Waals interactions; increasing αS dwell-time either in the cytosol or at membranes both seem to decrease multimerization, with negative consequences on cell health, as evidenced by the αS fPD-causing variants.

Lastly, post-translational modifications add nuance to αS proteostasis. αS phosphorylation is of particular interest because *90%* of aggregated αS present in Lewy bodies were reported to be phosphorylated at serine 129 (pS129),[122] implying that this specific phosphorylation event is connected to pathology (Fig. 1B). In addition to the largely insoluble Lewy bodies, however, soluble pS129 αS has been found in CSF samples from PD patients and even controls (e.g., [123]). Growing evidence also indicates that Lewy bodies can contain large amounts of pS129-positive αS without being fibrillary (e.g., a very recent study[98]). The interplay between αS tetramerization/multimerization has not yet been studied. Moreover, it has been proposed that pS129 may actually be a neuroprotective mechanism to accelerate the clearance of aggregated αS.[124,125] This may be achieved at least with the aid of PLK-2[126]. Still other groups have concluded that rather than neurotoxic or neuroprotective roles for pS129, it serves a normal regulatory function in αS turnover[127] and even in controlling gene expression.[128] In short, whether pS129 unequivocally indicates pathology under all circumstances is unclear. The trigger for pS129, whether certain kinases phosphorylate only when αS interacts with membranes, or whether a “master kinase” exists may all be important questions, the answers to which might further reveal whether pS129 plays multiple roles in contributing to synucleinopathy.

3. Tau

3A. Tau in health and disease

One of the hallmarks of AD, frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), Pick’s disease and other neurodegenerative diseases collectively called

tauopathies are the so-called neurofibrillary tangles (NFTs), which consist mainly of aggregated tau protein[129–132]. There are numerous efforts to target tau with the hope of slowing disease progression and subsequent cognitive decline. In the case of AD this is done in addition to targeting amyloid beta, the other, more upstream neuropathological hallmark of AD[133]. Interestingly, the level of dementia in AD patients and AD animal models may correlate well with tau lesions[134–136] –one of several reasons to thoroughly examine the therapeutic potential of tau. Since the discovery of tau from porcine brain MT fractions in 1975[137] and its identification as the main NFT component[129–132], major advances have been made in understanding the biology of tau especially focusing on its aggregation and hyper-phosphorylation in tauopathies[137,138]. Tau is a MT-associated protein (MAP) that may be involved in regulating MT stability (tau function and structure-function relationships are discussed below in sections 3B-D). The tau protein exists in six isoforms ranging in size from 352-441 amino acids as a result of alternative splicing of exons[139]. The isoforms are marked by the presence or absence of exons 2, 3 and 10 and are regulated based on the stage of development of the brain, though their specific function is not known [140]. The isoforms are named based on how many MT binding repeats (R) are expressed, with the 3R isoform having three MT binding repeats and the 4R isoform four binding repeats[138]. The 3R isoform is expressed mainly during the early development stage of the brain and the 4R isoform is expressed later in adulthood [139 140]. Most of what we know about tau originates from its function in neuronal cells. Besides its neuronal expression, tau was found to be expressed in non-neuronal tissues such as liver, muscle, kidney and even cancer cell lines[141,142]. Tau's characterization as an 'intrinsically disordered' protein in solution, while having numerous (proposed) functions within the cell, challenged the thought that proteins have to be pre-folded to exhibit specific functions in a cell.[143,144]

3B. Tau transiently binds to MTs

As one of the three major component of the cytoskeleton, microtubules (MTs) control the formation of mitotic spindles, cilia and flagella and play an important role in cell motility and polarity[145,146]. MTs are built from α- and β-tubulin monomers into a long cylindrical hollow shaped protofilaments. MTs undergo dynamic changes between growth and shortening known as dynamic instability[147]. Individual MT polymers elongate by the addition of GTP-tubulin dimers to the end of the MT and shrink due to the competitive activity of end-binding proteins preventing new tubulin dimers to bind [148,149]. A group of proteins known as MT associated proteins (MAPs) binds to and promotes the assembly and stabilization of MTs. Tau is a MAP that transiently interacts with MTs to regulate their dynamics and spatial organization. In addition, tau is believed to stabilize MTs in axons which serve as 'roads' for transport within the cell.[150] There are many proposed mechanisms for the MT-associated function of tau.[151–153] Several lines of investigation substantiate that tau bridges linear rows of tubulin dimers, by binding to several protofilaments via its MT binding repeats.[154,155] Others have shown that tau stabilizes MTs by bridging the interfaces of tubulin heterodimers.[156] Recent findings, however, suggest that tau may not stabilize axonal MTs at all, but rather modifies the biology of MTs by enabling them to have long labile domains.[157,158] Various techniques such as protein biochemistry and NMR have been used to delineate the physical and structural basis of the transient tau-

MT interaction leading to different hypotheses/models about the interplay between these two binding partners.

It has been suggested that a dynamic equilibrium between free tau and tau bound to MTs is dictated by tau phosphorylation and de-phosphorylation cycles[159–161]. Phosphorylation sites such as Ser262, Ser365, Ser205 and Thr231 are typically considered to reduce the strength of the interaction between tau and MTs when phosphorylated.[159]Tau phosphorylation was proposed to alter its conformation and affinity to MTs, leading to a detachment from MTs[162,163] In line with this idea, dysregulation of certain kinases has been proposed to perturb or even eliminate tau's MT binding in models of tauopathies[164–166]. While tau hyper-phosphorylation is certainly a hallmark of tauopathies, recent studies challenge the view that phosphorylation cycles are the key mechanism underlying transient (instead of stable) tau-MT interaction. In various binding and decay assays, it was shown that tau may be bound tau on each MT filament for only ~40 ms.[167] This dwell-time is ~100 times shorter than previously reported[168] and too short for regulation by kinases: the average catalytic ability of a kinase is approximately 10 interactions per second.[169] Hence, Janning et al. proposed a 'kiss-and-hop mechanism' of regulating tau-MT interaction.[167] Rather than a phosphorylation/de-phosphorylation mechanism of initiating the transient MT-tau interaction, the authors suggest that tau may 'hop' between MTs in an undirected manner. Despite its high mobility, it was postulated that tau can shifts the tubulin equilibrium toward polymerization: the short interaction time between tau and MTs was proposed to suppress the natural dissociation of tubulin dimers, allowing the addition of extra GTP cap size to initiate an interaction. Janning et al. further speculate that the rapid kiss-and-hop interaction that they observe may explain why tau, although binding to MTs, does not interfere with axonal transport. [167] It is worth noting that the 'kiss and hop' model was developed in PC12 cells which do not express axonal components. Therefore, the distance between two MTs is ~70 nm instead of the average ~20 nm for MT-MT interactions[170]. It is conceivable that details of the protein conformation and the effect of phosphorylation amongst others are model-dependent.[171] While probably not regulating the binding/unbinding cycles per se, its exact phosphorylation state is still expected to contribute to tau's overall probability MT binding affinity (see 3B). Reduced MT binding may in turn cause accumulation in the cytsosol and aggregation. Figure 3A summarizes several aspects discussed in this paragraph and emphasizes the proposed kiss-and-hop mechanism of the tau-MT interaction[167]. Figure 3B illustrates potential patho-mechanisms of AD, FTD and other tauopathies in the context of impaired tau-MT interaction.

3C. Lack of strong evidence for physiological multimeric forms of tau

The tau literature does not provide ample evidence for the existence of native tau higher-order assemblies. One study[172] employed a surface force apparatus to determine the force profile for each of six tau isoforms physisorbed through self-assembly from solution to negatively charged mica surfaces. The authors interpreted their data as incompatible with tau acting on MTs as a monomer and speculated that two tau molecules may associate in an antiparallel configuration. This structure was suggested to be held together by an electrostatic 'zipper' of complementary salt bridges composed of the N-terminal and central regions of each tau monomer, with the C-terminal MT-binding regions extending outward

from each end of the dimeric backbone. This tau dimer would then determine the length and strength of the linker holding two MTs together and to be the fundamental structural unit of tau, underlying both its normal and pathological action. Independent confirmation of such a mechanism is lacking.

3D. Molecular determinants of the transient nature of tau

Tau is typically characterized as consisting of an N-terminal-, a proline-rich, a MT-binding domain (MBD) and a C-terminal domain (Fig. 4). As it is evident from their names, the MT-binding domains are important for MT interactions[139,173]. Each isoform of tau has three to four repeats ('R-repeats') located in the C-terminus.[174] Using synthetic tau fragments, it has been shown that these repeats form MT binding units.[175,176] Positively charged repeat sequences of the MBD were proposed to interact with negatively charged residues in tubulin and hence to facilitate the tau-tubulin interaction[155,162]. Recent studies based on NMR[177] and cryo-EM[178] provide detailed insight into a complex mode of interaction. Both studies reiterate the previous notion that MT-bound tau does not become helical - in contrast to vesicle-bound αS that forms membrane-induced amphipathic 3-11 helices. Instead, tau adopts an 'extended' secondary structure, a state that is both different from unfolded and from helically or beta-sheet folded. In the extended structure, each of tau's R-repeats was observed to span both intra- and inter-dimer interfaces, centered on α-tubulin and connecting three tubulin monomers (Fig. 4). In each individual interaction site, key tau residues engage in specific interactions with key MT residues. Examples from the cryo-EM study[178] are: Ser258 and Ser262 in tau form hydrogen bonds with α-tubulin Glu434. Tau's conserved Lys259 interacts with an acidic α-tubulin patch formed by Asp424, Glu420, and Glu423. Ile260 is buried within a hydrophobic pocket formed by α-tubulin's Ile265, Val435, and Tyr262 at the interdimer interface. Lys267 may be positioned to interact with the acidic α-tubulin C-terminal tail. The universally conserved Ser262, was proposed to be critically involved in tight contacts with tubulin near a polymerization interface. The new structure thus now offers a detailed explanation for how Ser262 phosphorylation may disrupt tau-tubulin interactions.

What these new studies leave open, however, is the question why tau binding to MTs is actually dynamic and tau's dwell-time on MTs is so short. On the contrary, the studies should not be misinterpreted as evidence for the presence of stable MT-tau assemblies within cells. The authors do not point to specific amino acids that are 'sub-optimal' for achieving energetic minima (such as the central threonine in the hydrophobic half of αS membrane-associated helices; see Fig. 2). The rapid switch of tau between soluble and MT-bound tau suggests that the energetic minimum of the soluble unfolded state is similar to that of MT-bound state(s). Though it may contribute to it, Ser262 phosphorylation is not expected to be the sole reason for tau's dynamic behavior, for reasons discussed in section 3B.

While the 2015 and 2018 structural studies mainly focused on the R-repeats, the proline-rich regions in tau are also believed to influence the tau-MT interaction[179,180]. The proline regions line the repeat domain of tau and may initiate the formation of the tau-MT interaction by binding to the MT-binding repeats of tubulin, a 'green light' to trigger tau-MT interactions[152,181]. Notably, there are many phosphorylation sites on the proline-rich region

which may affect interactions with other proteins as well as tau-MT interactions[182,183]. Mutagenesis studies on human tau isoforms revealed that loss of the proline-rich domains reduces MT binding[180]. Tau mutants with a deletion of the repeat domain, but with an intact proline-rich domain, were found to still bind to MTs (see below)[181,184]. Combining the proline-rich regions with the adjacent repeat regions increased the binding affinity of tau 10-fold compared to mutants lacking the proline-rich region[185]. Taken together, the data show that the proline-rich domain and the repeat regions in tau synergistically promote its MT binding. Accordingly, Mandelkow and colleagues proposed the 'jaws' model of MT-tau interaction to explain these observations[184,186]. Mutants of tau with deletions or multiplications of different domains were generated and tested for tau-MT interactions in cells. The authors found evidence for the binding of tau with MTs in the absence of the repeat regions. However, these mutants were unable to stabilize MTs. Increasing the number of repeats in the repeat region enhanced the interaction and effect of tau on MTs. On the other hand, mutants with deletion of the proline-rich regions flanking the repeat regions showed only low affinity to MTs and did not induce reorganization of the MTs. The authors concluded that the proline-rich regions flanking the binding repeats serve as targeting domains to position tau on the MTs and that the repeat domain act as the primary interacting domains, hence the 'jaw' model of tau-MT interaction[184]. According to the authors, stabilization of MTs is only achieved if both domains of tau act together.

4. Therapeutic intervention via correcting transient behaviors of αS and tau?

The transient behavior and context-specific folding of αS and tau is not a contradiction to their frequent characterization as'intrinsically disordered proteins' because this term can simply be interpreted as lack of a sole and fixed three-dimensional structure. However, all current major protein classes considered 'druggable' are stably folded in their native states. Proteins like αS and tau on the contrary seem to exist as heterogeneous conformational ensembles, which renders them unsuitable for standard rational drug design approaches.[187] Nonetheless, several different small molecule strategies are currently under investigation, including: (1) stabilizing the proteins in their natively disordered states, (2) inhibiting interactions with binding partners, and (3) inducing allosteric inhibition.[187]

In agreement with scenario (1), Collier et al.[188] proposed that the tricyclic antidepressant compound nortriptyline can inhibit the aggregation of αS by directly binding to the soluble, monomeric form. The authors further proposed that nortriptyline-mediated 'reconfiguration of the monomer' can inhibit the formation of toxic conformations of the protein. Similarly, a recent study reported that stabilizing αS monomers with the polyphenol Oleuropein aglycone (OleA) reduced the likelihood for αS to aggregate into toxic molecules[189] (see Singh et al.'s review for further comment on phenol as therapeutic targets[190]). More in line with scenario (2), Perni et al.[191] demonstrated that squalamine (a natural product with proposed anticancer and antiviral activity that can be extracted from shark liver) can prevent αS aggregation by displacing αS from the surfaces of vesicles. This was suggested to block a first, membrane-assisted, step in the αS aggregation process. The same group then reported that the related compound trodusquemine not only inhibits αS-membrane

interactions, but also blocks the fibril-dependent secondary pathways in the aggregation reaction, thereby effectively suppressing the toxicity of αS oligomers in neuronal cells.[192] However, other groups had suggested that pharmacological stabilization of monomeric αS in its helical form at membranes may inhibit pathogenic misfolding and aggregation.[193] And yet, both approaches could eventually turn out to be problematic since αS monomers both in membrane-associated[96] and soluble conformation[194,195] have been reported to be aggregation-prone. Thus, another attractive option of preventing αS aggregation would be stabilizing native multimeric/tetrameric states of αS that have been reported to be aggregation-resistant.[92,196] An example of the direct stabilization of a tetrameric protein via a small molecule, thereby preventing amyloidosis, is the drug tafamidis that stabilizes transthyretin.[197] While the design of direct stabilizers of αS tetramers will likely depend on the successful generation of αS tetramer/multimer high-resolution structural data, it was recently shown that stabilizing native αS self-assembly can be achieved in an indirect fashion: the inhibition of stearoyl-CoA-desaturase (SCD) can increase αS multimer: monomer ratios in the multimerization-deficient familial-PD-linked mutant E46K.[198] The exact mechanism is not clear, but it seems likely that blocking the SCD-catalyzed production of oleic acid (18:1) causes a reduced occurrence of this fatty acid in membrane lipids, leading to higher levels of saturated fatty acids. A higher degree of saturation has been reported to interfere with αS-membrane interaction because 'membrane defects' that favor αS binding are less likely to occur.[199] Thus, the correction of excess αS E46K membrane binding and the correction of E46K's lack of multimerization seem to go hand in hand. The target SCD was confirmed in a different study that was based on αS (yeast) toxicity.[200]

In the case of tau, a recent study investigated the structural basis of small molecule druggability of native tau monomers.[201] As an example, the authors showed that methylene blue binds to monomeric full-length tau selectively with high affinity (K_d = 86.6 nM). The authors interpret their study as evidence that tau can be a viable drug target for small molecules that bind to monomeric tau and influence the way in which the protein interacts among itself and with other proteins.[201] Clinical trials based on methylene blue have thus far turned out to be rather disappointing[202–204]. Authors' claims of some efficacy after a post-hoc subgroup secondary analysis have been controversial. [205] A 2013 review[206] lists several classes of potential inhibitors/modulators of tau aggregation such as polyphenols, rhodanines, phenyl-thiazolylhydrazide, N-phenylamines, benzothiazoles and aminothienopyridazines. Only few of these structures are expected to actually bind to and stabilize monomeric soluble tau; most inhibitors/modulators likely redirect the self-assembly toward 'off-pathway' oligomeric forms.[206] In light of the topic of this review, such compounds are not considered modulators of *normal dynamic* behavior of tau. Moreover, many putative aggregation inhibitors have demonstrated to be potent in *in vitro* assays, but evidence of inhibiting tau aggregation *in vivo* and, even more importantly, cognitive improvement is still lacking.[205] It is also worth mentioning that the specificity of tau aggregation inhibitors, including methylene blue, is limited and pleiotropic effects can be expected.[205] Harnessing the hyper-phosphorylation of aggregated tau[207] for therapy has been considered a viable approach for a long time. However, tau phosphorylation cannot solely be considered a pathological event because the protein's transient binding to MTs is likely to be regulated at least in part via phosphorylation as well (see above).[208] The kinases

and phosphatases that have been demonstrated to be involved in tau phosphorylation comprise GSK3β, Cdk5 and p25[209,210]. Interfering with tau phosphorylation promises to affect protein homeostasis upstream of proteinaceous aggregation and remains a valuable strategy. However, the exact regulation as well as physiological and/or pathological significance of tau phosphorylation at all its different phosphorylation sites remains a question to be answered.

Lastly, while modifying the composition of the vesicles that αS binds to (e.g., via SCD inhibition) has emerged only recently as a new potential strategy for treating synucleinopathies[198,200], tubulin-stabilizing compound have already been tested in clinical trials[211,212], albeit unsuccessfully in phase III[212]. MT imbalance is typically considered a downstream effect of tau dyshomeostasis. The interplay between αS and fatty acid/lipid/vesicles, however, may be more complicated. For example, a previous study demonstrated that expressing αS alters fatty acid composition of dopaminergic neurons in a way that reflects alterations in human brains with synucleinopathies.[213] Genetic evidence, on the other hand, suggests that impaired glucocerebrosidase activity may be upstream of αS dyshomeostasis in PD pathogenesis[214,215]. Mazzuli et al. proposed a 'bidirectional pathogenic loop' between glucocerebrosidase and αS.[216] Other lipid-related genes that emerged from GWAS as PD risk factors comprise a diacylglycerol kinase, DGKQ, which controls cellular diglyceride content[217–221], and fatty acid elongase 7, a determinant of acyl-chain length and hence lipid composition/membrane fluidity[222]. Both indicate that changes in lipid metabolism can be upstream of αS dyshomeostasis (which does not exclude additional effects on lipids downstream of αS). In turn, modifying cellular lipid composition could indeed help keep cellular αS folding homeostasis intact or correct it back to normal. A similar approach for correcting tau homeostasis via modification of MT biology may be less evident from genetics or the literature in general.

It is also important to note that in both tauopathies and synucleinopathies the pathway toward fibrillary assembly may be quite different from one disease to another. As far as the endpoints are concerned, there is growing evidence for disease-specific aggregate formation. For example, the single protofilaments with an elongated and loosely arranged structure found in Pick's Disease are markedly different from the compact bundles of C-shaped paired helical tau filaments found in AD.[223,224] Similarly, PD, DLB and MSA have been reported to be characterized by distinct αS aggregate 'strains' that exhibit different 'seeding' characteristics[225–227], and the notion of non-fibrillar, membranous αS aggregation[98] adds further complexity. These differences in advanced pathology may point at differences in disease initiation. As a consequence, drugs targeting certain transient forms within tau and αS dynamic equilibria could be disease-specific and inefficient toward another assembly pathway.

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- tau and α-synuclein are central in the pathogenesis of a variety of neurologic diseases
- both engage in transient interactions with cellular components
- both constantly undergo structural changes and context-specific folding in the cell
- new insights on regulation and exact nature of this dynamic biology are emerging
- a deeper understanding of transient conformations may lead to new therapies

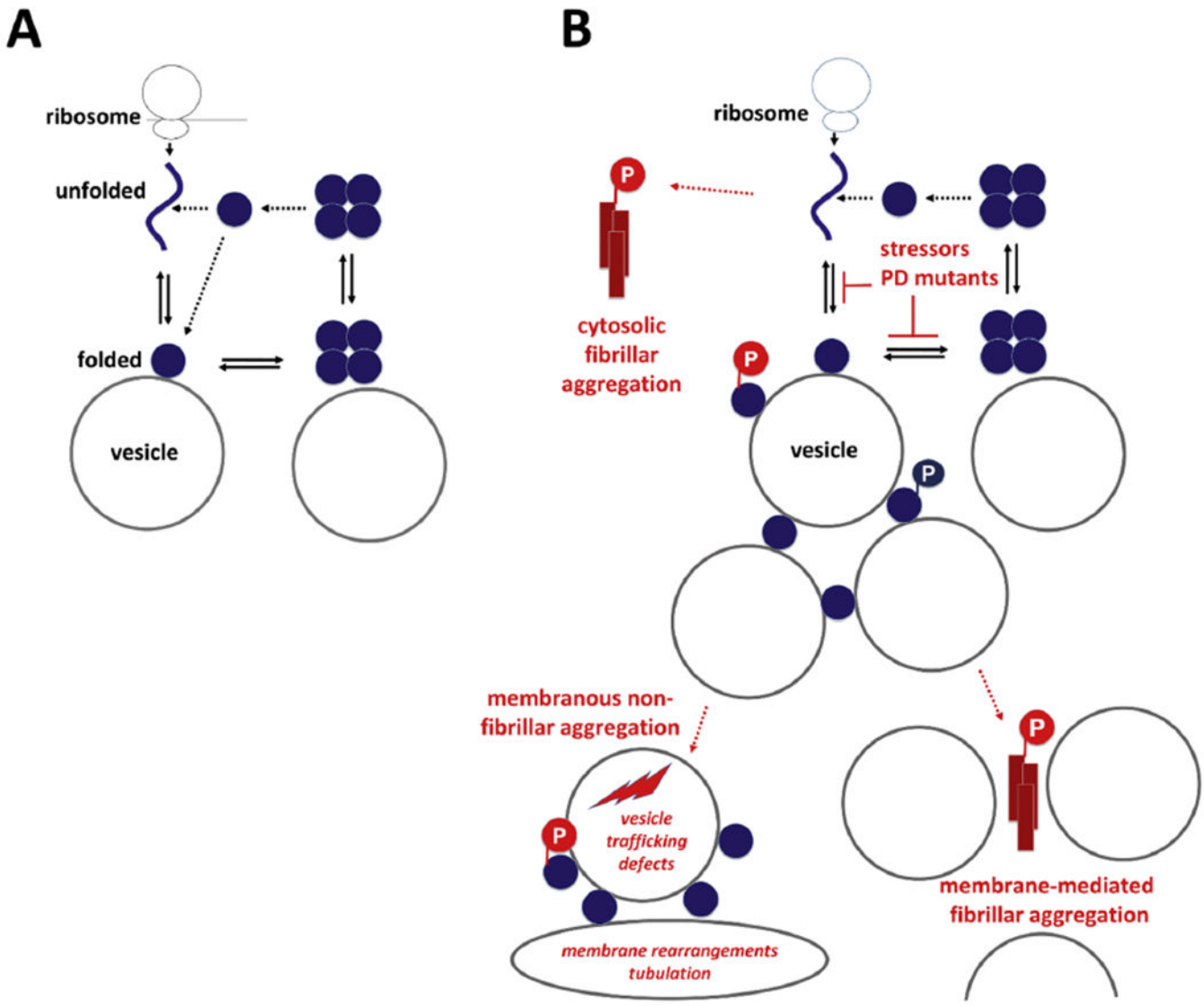

Figure 1. Transient cellular behavior of αS.

A, Physiological situation: Coming off the ribosome, αS is soluble, unfolded and monomeric. Upon binding to vesicular membranes, it adopts helical fold. Folded monomers transiently assemble to form metastable multimers/tetramers on membranes. Multimers/tetramers are only weakly membrane-associated and likely in an equilibrium with cytosolic multimers/tetramers. Cytosolic tetramers/multimers may have an intrinsic propensity to disassemble - and eventually unfold, initiating a new cycle. **B,** Pathological situation (pathological states are in red): perturbed cellular αS homeostasis increases i) the levels of aggregation-prone unfolded monomers in the cytosol or ii) the level of membrane-associated monomeric αS. Excess soluble αS monomers may be the starting point of cytosolic fibrillar αS aggregation (top left), while excess membrane association may either cause fibrillar αS aggregation via 'nucleatin'[96] (bottom right) or membranous non-fibrillar αS aggregation (bottom left). The role of phosphorylation atSerl29 is unclear. While primarily considered a

pathological event before or in response to fibrillar aggregation (red), there may also be physiological aspects of this post-translational modification (blue).

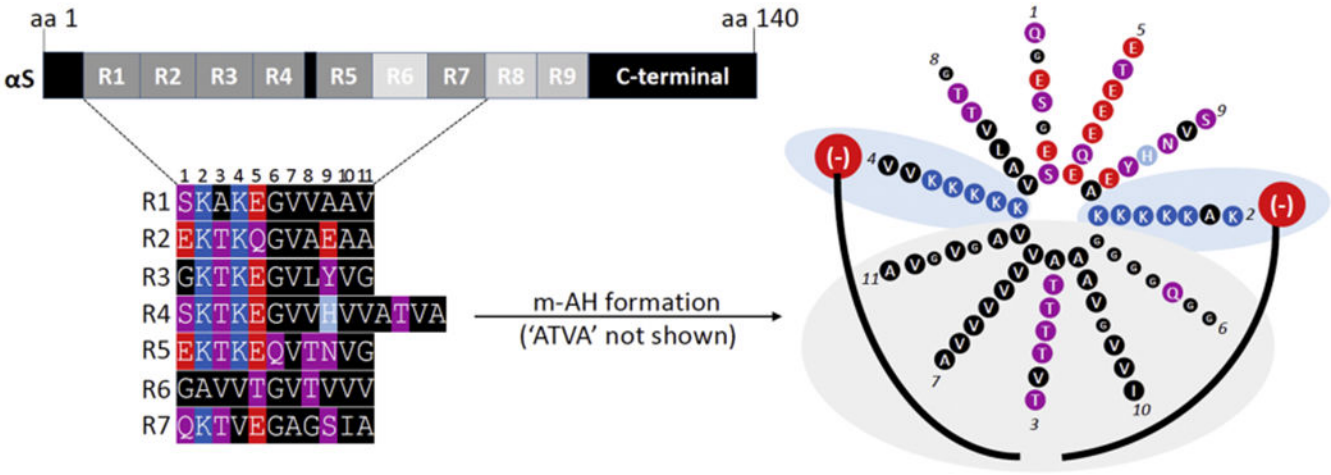

Figure 2. Molecular determinants of the transient nature of αS.

Top left: the 140-aa sequence of human wildtype αS contains 9 semi-conserved 11-aa repeats with the core consensus motif 'KTKEGV'. Highly conserved repeats are indicated in dark grey, poorly conserved repeats in light grey. Bottom left: color-coded schematic of repeats 1-7 of human αS by aligning the aa sequence via the KTKEGV motifs. Blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, and black: non-polar residues. In addition to KTKEGV, the polar character of positions 1 and 9 as well as the non-polar, hydrophobic character of positions 8, 10, and 11 are relatively well conserved. Repeats 1–9 are interrupted only once: by 'ATVA' between repeat 4 and 5. Right: Color-coded schematic of αS repeats 1-7 (omitting 'ATVA' between repeats 4 and 5) in an 11/3 helical wheel, embedded in the outer leaflet of a curved vesicle membrane (negatively charged lipid head-groups in red, fatty acid 'tails' in black). The helix is stabilized by hydrophobic interactions (gray area) and electrostatic interactions (blue area). Certain structural aspects such as the presence of polar threonines as well as the small and 'helix-breaking' glycine prevent the formation of a highly stable helix.

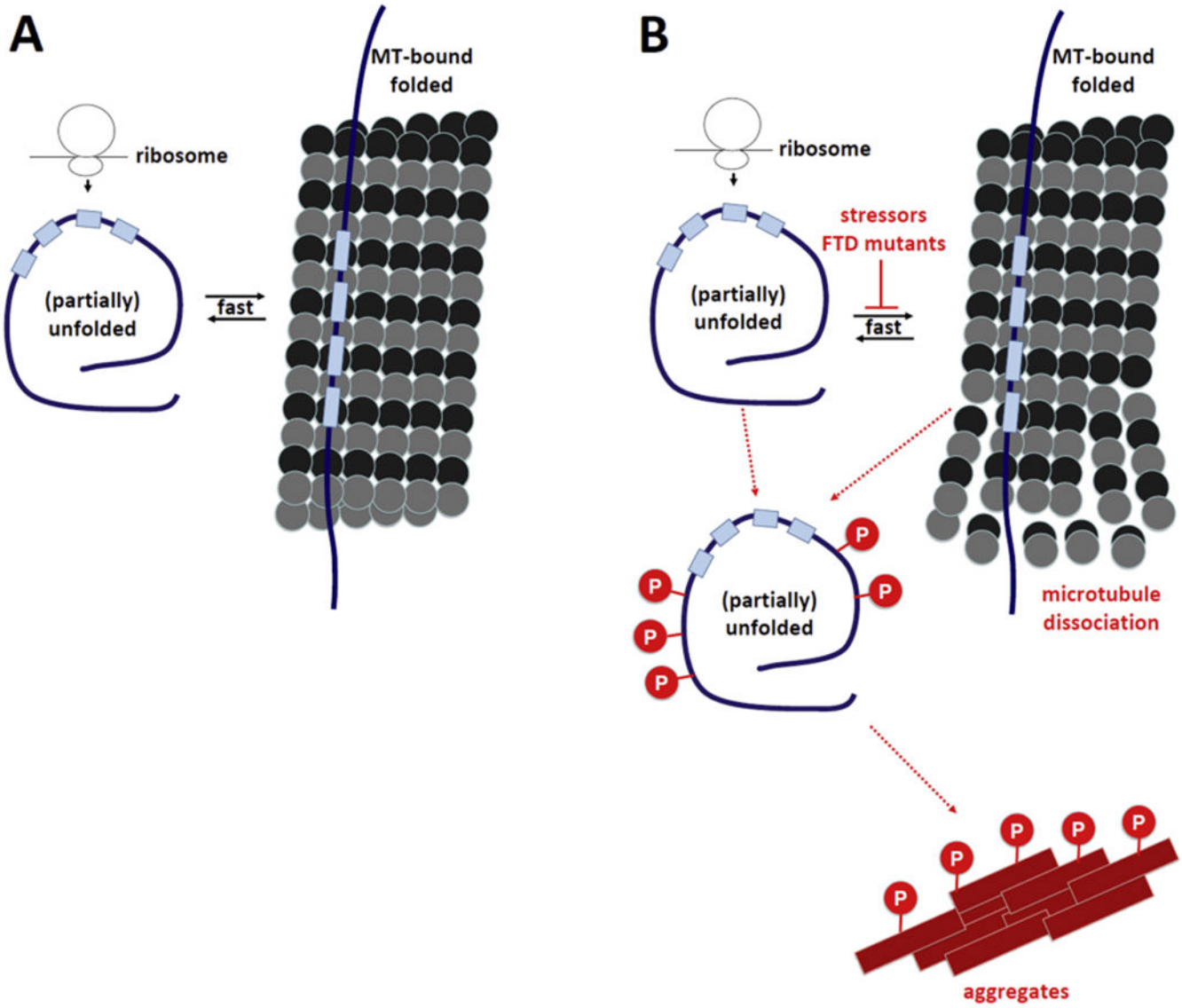

Figure 3. Transient cellular behavior of tau.

A, Physiological situation: Coming off the ribosome, tau is soluble, unfolded and monomeric. Upon binding to MTs, tau adopts a different fold, but almost immediately becomes soluble again. Tau phosphorylation may contribute to regulating the transient tau-MT interaction. **B,** Pathological situation (pathological states are in red): perturbed cellular tau homeostasis is characterized by tau hyper-phosphorylation, lack of tau-MT interaction, disintegration of MTs and the formation of proteinaceous tau aggregates.

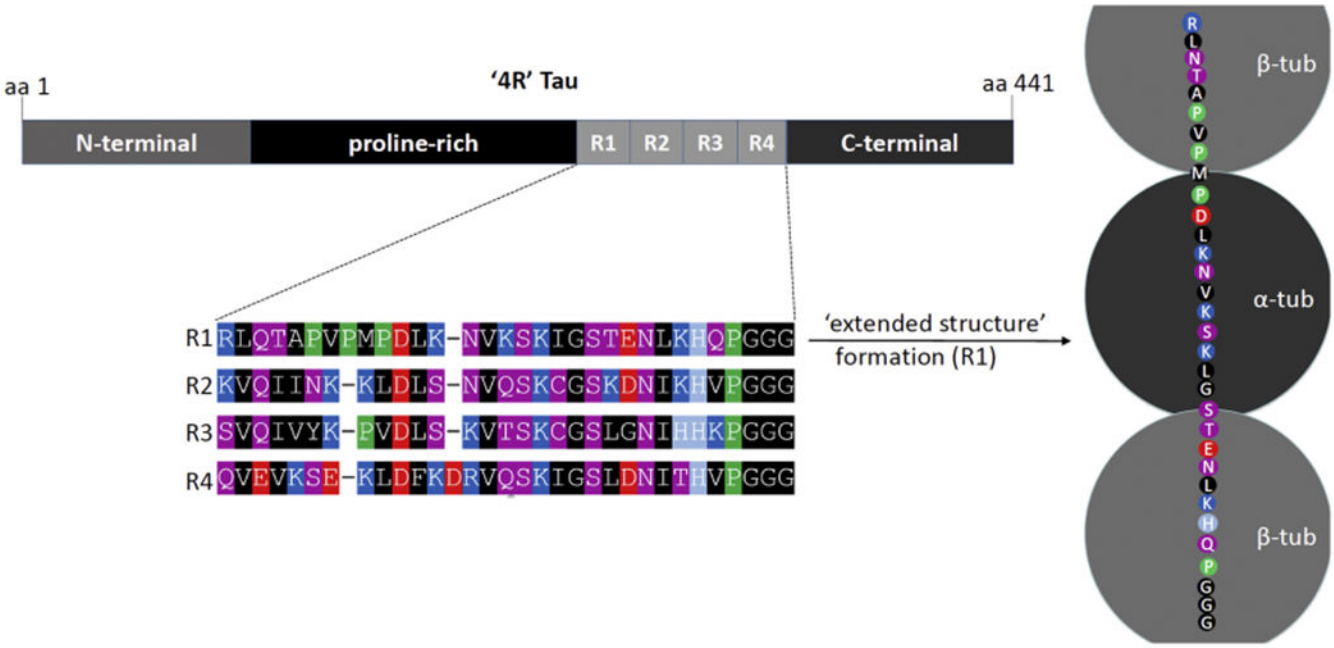

Figure 4. Molecular determinants of the transient nature of tau.

Top left: the 441-aa sequence of human wildtype '4R' tau contains 4 semi-conserved 31 or 32-aa 'R repeats', plus proline-rich, N-terminal and C-terminal domains. Bottom left: color-coded schematic of the 4 R repeats. Blue indicates basic (light blue: histidine), red: acidic, purple: polar uncharged, black: non-polar, green: proline residues. Right: Color-coded schematic of R repeat 1 bound to MTs via 3 tubulin subunits (2β, 1α).The proposed structure is neither unfolded nor helically or β-sheet folded. Instead, it is characterized as 'extended'. The exact molecular basis fora highly transient, instead of a strong binding to MTs will require further research.