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Pin1 Protects Against Alzheimer's Disease: One Goal, Multiple Mechanisms

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1. Introduction

1.1. Plaque and tangle pathology in AD

Alzheimer's disease (AD) is the most common form of dementia, and it accounts for more than 60% of all cases of dementia. Although many factors may increase the risk for AD, the only cause so far known is aging [1]. Most of the cases are sporadic, as only less than 0.1% of the cases occur because of inherited mutations on genes directly involved in the disease (familial AD, FAD) [2].

AD is caused by progressive and irreversible neurodegeneration. At the moment, there is no cure for AD. Therapies available are only aimed at lessening the progression of the cognitive decline and neurodegeneration and do not target pathways directly causative of the disease [3]. These include the acetylcholinesterase inhibitors (Aricept) [4] or inhibitors of the glutamatergic NMDA receptor (Namenda) [5] and were shown to be mostly effective when administered at early stages [6-8]. However, a proper diagnostic approach able to identify AD early in the development is still missing, and this reduces the efficacy of the treatments available. Therefore, there is the need to develop both diagnostic tools able to detect early stages of the disease, and to generate effective treatments targeting the early pathogenic events in AD. This is becoming increasingly important also considering that the population affected by AD will dramatically increase in the years to come. Numbers are in fact dramatic: 10 million baby boomers may develop AD within the next 10-20 years [9]. Currently, in the United States alone there are more than 5 million AD patients, and the costs to the US government exceeds the 200 billion/year. These numbers are expected to quadruple in the next 40 years, causing unsustainable costs for the care of these patients and their caregivers, who could not receive support and care and would then have to face undignified life conditions.

Studying the molecular mechanisms responsible for the neurodegeneration in AD can help identify new effective therapeutic targets. Two main pathways are identified in AD. They involve two proteins, the amyloid precursor protein APP and the microtubule-associated protein tau, as they are responsible for the formation of the two characteristic lesions, the extracellular plaques and the intracellular neurofibrillary tangles (NFTs), respectively [10, 11]. Both plaques and tangles are considered causative of the disease; they deposit following the progression of the disease, and they could contribute to alter neuronal morphology leading to neuronal death [12-16].

The origin and composition of plaques and tangles are quite different. Plaques are forms of aggregated, fibrillar material called amyloid, insoluble fibrous protein aggregations organized in β -sheet strands that deposits in the outer part of the brain [17-19]. Their core is mainly composed of Abeta (beta-amyloid), a peptide of small molecular weight deriving from APP, which tends to form small size aggregates called oligomers with known toxic properties [20]. Oligomers are found intracellularly, but can be secreted to the extracellular space, where they will aggregate into larger structures called fibrils, forming the core of the plaque [18, 21, 22].

Similarly, tangles are formed by insoluble structures organized into fibrils, the pair helical filaments (PHFs), which eventually organize and aggregate into larger structures, the tangles [19]. The main component is hyperphosphorylated protein tau, which in this form becomes insoluble and tends to form aggregates [13, 23].

The biological functions of APP and tau are very different [13, 24], but during the disease both the beta amyloid product and the hyperphosphorylated tau become toxic to the neuron, causing neurodegeneration. However, the mechanisms by which tau and Abeta may be toxic differ. In fact, as a microtubule stabilizing protein, tau can become toxic to the cytoskeleton when hyperphosphorylated, as in this form it would detach from the microtubules destabilizing them. Hyperphosphorylated tau would also tend to aggregate into NFTs, impairing cellular functions [23]. As to the plaques, their mechanism of toxicity is still under debate. Although they cause the formation of dystrophic neuritis [18], it is still unclear whether they are really toxic or rather protecting, by sequestering Abeta oligomers from the environment. In fact, Abeta is sequestered from the extracellular space to form the plaque [25]. Indeed, oligomers are considered toxic: they form early in the pathology [26], associate with impaired cognitive functions in mice [27] and in AD patients [28], and impair neurotransmission [29-33]. Therefore, identifying the pathways that lead to both increased Abeta production and/or tau hyperphosphorylation and also regulate their aggregation into organized insoluble structure may dramatically help find a cure to treat AD.

1.2. Pin1-regulated protein isomerization as a mechanism to control tangle and plaque pathologies

Protein phosphorylation seems to be a common feature of both plaque and tangle pathologies. In fact, changes in the levels of phosphorylated APP seems to influence APP function and toxicity in the pathology, as increased phosphorylation of APP at specific domains positively regulates Abeta production [34-36]. Of note, both APP and tau can be phosphorylated by the same kinases, such as cdc2, CDK5 and GSK3, and such kinases seem to be particularly active

during the disease [23, 37-40]. Hence, the identification of molecular pathways that can control non physiologic phosphorylation of both tau and APP in the disease could help identify targets to tackle at the same time both tangle and plaque pathologies.

We found that the enzyme Pin1 protects from both tangle and Abeta pathology, since a genetically modified animal model lacking Pin1 (Pin1KO) developed age-dependent tauopathy and was characterized by increased production of Abeta, deposited in form of intracellular aggregates [41, 42]. This seems to be due to the capability of Pin1 to regulate the conformation of cis and trans isomers of both phosphorylated tau and APP, as shown using conformation specific antibodies for tau, and by means of NMR.

Pin1 (Protein interacting with NIMA (never in mitosis A)-1) is a prolyl isomerase, which regulates the function of phosphorylated protein substrates by regulating their cis/trans isomerization [43, 44]. Pin1 belongs to the family of PPIase (peptidyl prolyl cis trans isomerase), enzymes that are evolutionary conserved. Unlike other PPIases, Pin1 specifically regulates the conformation of substrates phosphorylated at specific serine or threonine residues preceding a proline (S/T-P motifs) [45-47]. The stereochemistry of Proline allows the protein to undergo two different conformations (cis and trans), which could be determined by the presence of a phospho group on the S or T residue [43, 48]. Since Proline-directed phosphorylation regulates key cellular mechanisms, by maintaining the equilibrium between the two conformations, Pin1 may dramatically contribute to the maintenance of vital cellular functions.

The structure of Pin1 consists of two domains, an N-terminal WW domain comprised of the first 40 aminoacids which is responsible for Pin1 binding to its substrates, and a larger PPIase domain that spans the remaining part of the protein and catalyzes the substrate's isomerization [49]. Of note, although mostly in the nucleus, Pin1 subcellular localization is driven by the presence of its substrates [50], to extranuclear compartments, with obvious expression in the plasma membrane, cytosol and cytosolic organelles involved in endocytosis [41, 51]. The ubiquitous expression of Pin1 allows the protein to control the isomerization of multiple substrates in different cellular compartments, including cytosolic proteins like NF-KappaB [52], p53 [53], beta-catenin [54], IRAK1 [55] and others [46], or protein that localize at different compartments like APP [41, 51] and tau [42, 56]. This determines a crucial role for Pin1 in controlling the physiological activity of proteins involved in diverse functions, such as protein transcription and stability, and protein interaction, by regulating the aforementioned substrates [43].

Notably, Pin1 function is highly regulated and its aberration affects Pin1's ability to isomerize its substrates with consequences on their function, contributing to an increasing number of pathological conditions, including Alzheimer's disease, cancer and immunologic disorders and aging. Lack of Pin1 function was found to impair immune responses in Pin1KO animal models [55], due to lack of activation of IRAK1, which is involved in the regulation of the TLR signaling [57]. In cancer, Pin1 levels are increased due to transcriptional activation and loss of inhibitory phosphorylation and other mechanisms [45, 58]. This leads to up-regulated isomerization of substrates involved in hyperproliferative processes, activating two dozens of oncogenes and inactivating a dozen of tumor suppressors [46, 59, 60]. On the contrary, in AD brain Pin1 activity is reduced due to decreased protein level and to oxidation [56, 61, 62]. Some

genetic polymorphisms on the Pin1 gene were found to associate with forms of late onset AD [63-65]. Interestingly, a polymorphism that associated with increased Pin1 levels by regulating AP-4 mediated transcription, was found to be protective as it correlates with delayed disease onset in a Chinese cohort [66]. In AD, the changes in Pin1 levels and activity prevent from an effective isomerization of the phosphorylated APP and tau [41, 56]. As a consequence, the equilibrium between the cis and trans conformation is not maintained and the proteins exist in the pathogenic cis conformation: APP will generate more Abeta and tau will lose normal microtubule function and become toxic, leading to both plaque and tangle pathologies.

In this book chapter we will discuss findings from our and other labs that point to a crucial role of Pin1 in protecting against AD by regulating diverse cellular pathways using multiple mechanisms. We will specifically highlight how Pin1 regulates protein conformation of APP and tau to control APP trafficking, APP stability and Abeta production as well as tau phosphorylation, microtubule function, stabilization and aggregation *in vivo* and *in vitro*. We will also emphasize the importance of Pin1-mediated regulation of APP and tau conformation as a modulator of pathogenic mechanisms that might occur early in the development of the disease. Finally, we will also discuss how Pin1 is emerging as a novel diagnostic and therapeutic tool for early intervention to tackle both tau and Abeta pathologies in AD.

2. Pin1 as a crucial regulator of APP trafficking and stabilization to protect from Abeta pathology in AD

Although both tau and Abeta pathologies define AD, only Abeta is the characteristic feature that distinguishes AD from other forms of dementia. In fact, only the presence of plaques containing Abeta peptide allows a definite AD diagnosis [10, 67-69], whereas the presence of PHF alone could be related also to other forms of tauopathies, like FTPD, Pick disease and others [13]. The specificity of Abeta pathology to AD makes of Abeta and its precursor APP ideal therapeutic targets. Here we will review the role of APP in AD, the molecular mechanisms that regulate Abeta formation, focusing on the role of Pin1 as a post-phosphorylative event to regulate both APP intracellular localization and trafficking, and also turnover, preventing Abeta formation. These topics are of particular relevance for the understanding of the mechanisms underlying Abeta production in AD. In fact, the intracellular localization of APP will determine whether APP will be toxic influencing the production of beta-amyloid peptides. Moreover, impaired APP turnover will cause APP stabilization, which will lead to increased levels of both APP and beta-amyloid peptides. This phenomenon is particularly consistent with pathologies associated with higher levels of APP and development of AD, such as Down syndrome.

2.1. APP trafficking and processing pathways

APP is a type 1 transmembrane protein that is ubiquitously expressed. APP is characterized by a long extracellular domain, a short transmembrane domain and a small intracellular domain that regulates APP phosphorylation and trafficking [68, 70]. The domain that contains the sequence for Abeta spans a region of approximately 40 aminoacids across the N-terminal

portion of the transmembrane domain [71]. Three isoforms of APP exist characterized by different molecular weight, the result of alternative RNA splicing, APP751, APP750 and APP695 [72]. Since the splicing occurs in the most N-terminal region of the protein, all the three isoforms express the domains for both Abeta and the intracellular domain [72]. APP isoforms may be differently expressed in the various organs. APP770 is for example mostly present in the heart and in peripheral cells, whereas APP695 is the only form expressed in the brain and therefore linked to Abeta generation in AD [72]. For this reason, the APP isoform considered in AD studies is APP695, and the numbering of the aminoacids follows this sequence.

Within the cell, APP localization is not limited to a single part, as it undergoes trafficking through different compartments. Upon synthesis in the ER, APP travels through the Golgi compartment where it undergoes glycosylation, to finally reach the plasma membrane. It eventually will recycle to the Golgi, following internalization from the plasma membrane and trafficking through the endosomal pathway [70, 73, 74]. Of note, the significance of APP physiological function may depend on the compartment where APP localizes during the life of the cell. In fact, depending on whether APP is retained at the plasma membrane or it is internalized to the endosomes, it will generate different metabolites with diverse function, either neurotrophic and therefore protective from AD, or toxic. More in details, at the plasma membrane APP will undergo a processing pathway called non-amyloidogenic [75, 76], in which metalloproteases of the ADAMs family and others (ADAM10, ADAM17 and TACE [77-81]), called alpha secretase, will cleave APP in the middle of the sequence for Abeta, generating the extracellular stub alphaAPPs with known neurotrophic properties [82], and a C-terminal stub called C83. C83 will be further cleaved in the late endosomes by a complex of four proteins called gamma-secretase, to generate a small fragment called p3 with no amyloidogenic properties. This pathway is called non-amyloidogenic, as it prevents the formation of intact Abeta peptides. The amount of APP at the plasma membrane that does not undergo alpha-secretase cleavage will internalize in the cell through the endocytic pathway [70, 73, 74]. This occurs thanks to the binding of proteins such as Fe65 to the 682YNPTY687 motif at the intracellular, C-terminal domain of APP [83-85]. Once in the early endosomes, full length APP is cleaved by BACE or beta secretase [86, 87], an aspartyl protease that cuts APP at the beginning of the sequence for Abeta. Such cleavage generates a soluble stub called betaAPPs with known apoptotic properties in the neuron [88], and a C-terminal stub called C99 which still contains the intact sequence for Abeta. C99 will traffic to the late endosomes, where it will be cleaved by gamma-secretase to generate intact Abeta [17, 89]. This pathway is called amyloidogenic as it produces Abeta peptides, and is increased in AD [90, 91].

It is clear that the intracellular localization of APP will determine whether APP will be amyloidogenic or not. Therefore, any mechanism that may help APP stay retained at the plasma membrane will protect from Abeta production and AD, whereas those that help APP internalize to the endosomes will favor the amyloidogenic processing and Abeta formation.

2.2. APP phosphorylation and conformation to regulate APP processing

One such mechanism is APP phosphorylation. In fact, it was shown that the Y682 residue can be phosphorylated by different kinases such as abl and TrkA [92, 93]. Phosphorylation at this

level can regulate the association of APP to binding partners such as Fe65, X11/MINTs and Shc [94-97], ultimately controlling APP trafficking, processing and function also associated with cell movement and axonal branching [98, 99], and with NGF activity [100]. Tyr phosphorylation at Y682 motif has also been associated with increased Abeta production and amyloidogenic processing in vitro [101], in vivo [102] and in AD [103].

Interestingly, APP can be phosphorylated at a further N-terminal part of the intracellular domain, the 668Thr-669Pro residue [104], and phosphorylation at this domain has been associated with increased amyloidogenic processing of APP, both in vivo [39, 40] and in vitro [34]. The kinases involved in such phosphorylation are GSK3beta, CDK5, cdc2, known to be overactive in AD and responsible also for tau phosphorylation [23, 38, 39, 104, 105]. Of note, T668 phosphorylation was found to be elevated in AD brain [34], suggesting that it might induce toxic mechanisms linked to Abeta production. Such mechanisms seemed to relate to conformational changes affecting the 682YNPTY687 motif and therefore its ability to interact with binding partners [106, 107]. In support of this hypothesis, T668 has been linked to specific isomer formation. In fact, by means of NMR studies, it was observed that phosphorylation at the Thr668 residue causes an isomerization of APP from trans to cis. In fact, non-phosphorylated APP retains 100% trans conformation, and upon phosphorylation at T668 approximately 10% of the population turns to cis [108, 109].

Altogether, these findings draw attention to the role of T668 phosphorylation as an initiator of molecular pathways that lead to Abeta production by regulating APP conformation, trafficking and processing. They also suggest that different cis and trans APP isomers may contribute to shift the processing of APP towards either the amyloidogenic or the non-amyloidogenic processing, and therefore T668 phosphorylation may emerge as a potential target to halt amyloidogenic pathways in AD.

2.3. Pin1 to protect from Abeta pathology in animal models

Based on these findings and on the capability of Pin1 to protect from tau pathology by regulating tau conformation [42, 56], we hypothesized that Pin1 might regulate also the conformation of APP protecting from Abeta pathology. We found that Pin1 can bind to phosphorylated APP at T668, maintains the equilibrium between cis and trans conformation, ultimately shifting the processing of APP from the toxic amyloidogenic to the protective non-amyloidogenic [41].

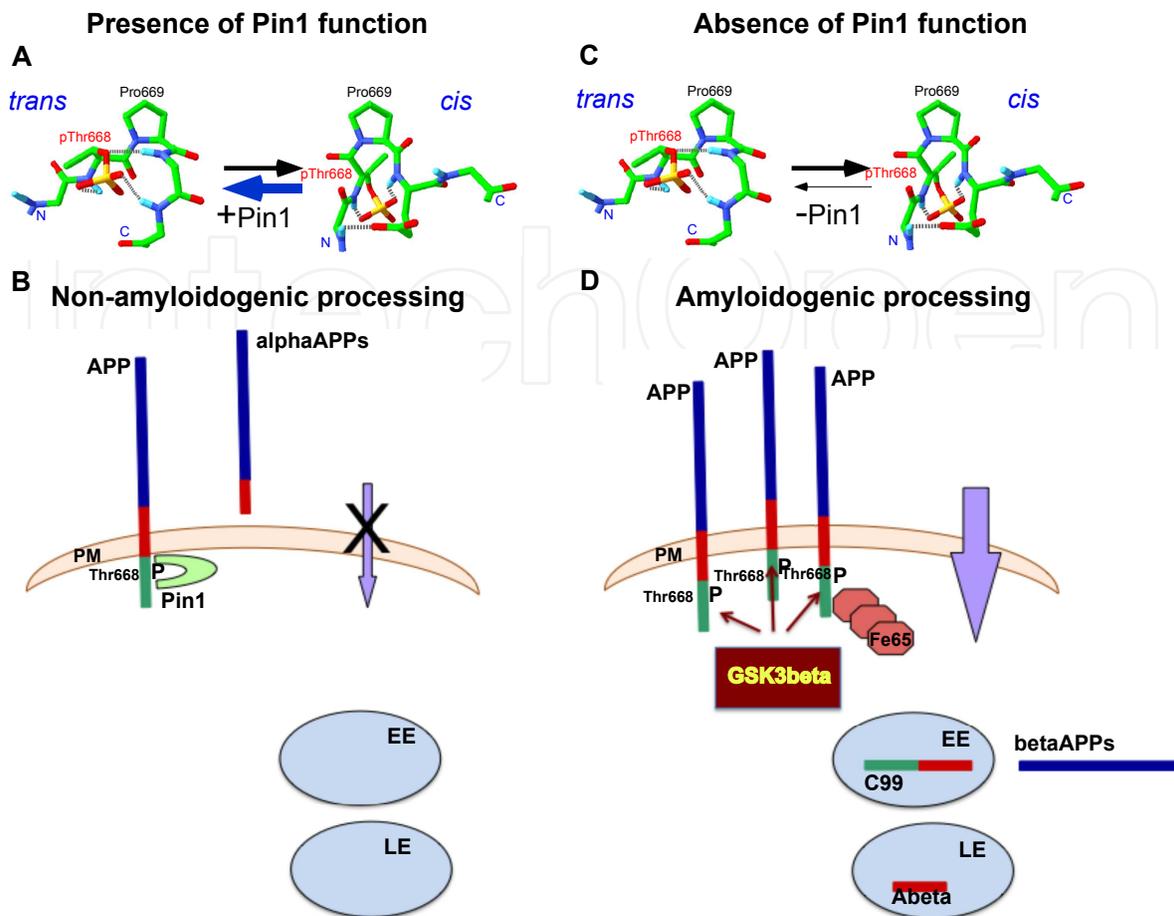
More in detail, by means of pull down experiments, we observed that Pin1 can bind to APP only if phosphorylated at T668 [41]. Such interaction regulates APP isomerization. In fact, using a pentapeptide containing part of the C-terminal domain and the T668-P motif, we observed that Pin1 isomerizes the conformation of this peptide from cis to trans 1000 times faster than the reversed equilibrium, suggesting that shifting the isomerization towards the trans conformation may be crucial for APP function, and that Pin1 might be key to regulate APP physiologic activity. We then tested whether altering the equilibrium between cis and trans conformation might result in changes of APP functions. For this purpose, we manipulated Pin1 cellular levels either by knocking Pin1 out in genetically modified animals (Pin1KO), or by overexpressing Pin1 in cultured cells. Our in vitro experiments showed that when levels of

Pin1 were elevated beyond physiologic, APP amyloidogenic processing would be reduced, as Abeta levels were decreased in the media of the cultured cells. On the contrary, lack of Pin1 expression in cultured Pin1KO breast cancer cells resulted in decreased alphaAPPs secretion and increased Abeta production. Similarly, in the brain of Pin1KO mice we could observe age-dependent increase of Abeta production, since levels of aggregated insoluble Abeta were elevated in 18 months old mice when compared to 5 months old mice.

We then crossed Pin1KO animals to APP^{Tg2576} and studied the processing of APP. We observed an age-dependent shift in the processing of APP that would result in an increase of the amyloidogenic versus the non-amyloidogenic, paralleled by the accumulation of Abeta42 deposits in multivesicular bodies, a form of deposited Abeta associated with early stages of AD [32, 33]. This led us to hypothesize a model in which Pin1 would protect against neurodegeneration possibly by maintaining the equilibrium between the cis and the trans conformation of APP. In particular, in physiological conditions, Pin1 would favor the trans conformation of APP, increasing the non-amyloidogenic processing. Vice versa in the absence of Pin1, the cis form would accumulate as the isomerization between the two forms would be lost, ultimately favoring the amyloidogenic processing.

2.4. Pin1 inhibits APP trafficking and internalization

Because Pin1 was found to localize with full length APP at the plasma membrane, we speculated that Pin1 may be involved in APP trafficking and internalization, regulating the amount of APP that undergoes amyloidogenic processing. We therefore tested the hypothesis whether Pin1 protects from Abeta formation by inhibiting APP internalization to amyloidogenic compartments [51]. For this purpose we used brain derived human H4 neuroglioma cells expressing APP either at endogenous level or stably overexpressing it, and Pin1 expression was knocked down by RNAi. We found that lower Pin1 levels associated with i) decreased levels of APP at the plasma membrane, ii) increased levels of betaAPPs and decreased alphaAPPs and iii) increased kinetic of internalization, as evidenced by means of immunocytochemistry in both fixed and living cells [51]. Levels of APP phosphorylated at T668 seemed to be elevated too. These data are in agreement with data from other groups that propose a toxic role of T688 phosphorylated APP [34], and may suggest that reduced Pin1 levels could be toxic in the same pathways. Interestingly, Ando and colleagues suggested that phosphorylation at T668 may affect APP conformation to ultimately alter the capability of APP to bind to partners such as Fe65 regulating APP trafficking, even if such interaction occurs at the 682YNPTY687 domain, further C-terminal than T668 [106]. This effect could be related to Pin1-mediated changes in APP conformation that could change the 682YNPTY687 stereochemistry. Of note, in Pin1 KD treated cells that were also overexpressing Fe65, we found that higher amounts of Fe65 associated with APP and that C99 accumulated, as compared to wild type cells. This was probably due to stabilization of Fe65 under these conditions, since Fe65 levels were elevated at the steady state in Pin1KD cells. Together with our immunocytochemistry data, under conditions that promote Fe65/APP interaction, these results suggest that reduced Pin1 expression may be linked to fastened internalization of APP to amyloidogenic compartments, where C99 is produced and accumulates (Fig. 1).



Our hypothesis is that Pin1 binds to and isomerizes the phosphorylated T668-Pro motif in full length APP, resulting in protein conformational changes that ultimately affect APP intracellular trafficking. (A, B). In the presence of proper Pin1 function, the equilibrium between the *cis* and the *trans* form of phosphorylated APP is maintained [41] (A), and this may help APP stay anchored at the plasma membrane where it will undergo the non-amyloidogenic processing (B). On the contrary, when Pin1 function is reduced, as in AD, the equilibrium between the *cis* and the *trans* form of phosphorylated APP will be disrupted, as the *cis* form of phosphorylated APP will not be isomerized to *trans* in a timely manner (C), and the levels of Fe65 will be stabilized. Moreover, reduced Pin1 function will enhance GSK3beta activity, leading to overall increase of phosphorylated APP at T668 and inhibiting APP turnover. These effects may lead to overall increased levels of APP undergoing internalization, trafficking and amyloidogenic processing (D). PM: Plasma membrane. EE: Early Endosomes. LE: Late Endosomes.

Figure 1. A model for the role of Pin1 in inhibiting APP accumulation and amyloidogenic processing.

We had previously observed that reduced Pin1 expression is linked to Abeta production [41], and we found that Pin1KD may increase gamma-secretase cleavage of APP to generate AICD [51]. Hence, we could assume that AD-associated reduced Pin1 expression is linked to increased amyloidogenic processing, promoting both internalization and gamma-secretase dependent cleavage of APP.

2.5. Pin1 increases APP protein turnover

Recent findings from our lab link Pin1 deficit and amyloidogenic processing in AD to increased APP stabilization [110]. This is particularly relevant to a role of increased APP in the disease,

as it is known that higher APP levels correlate with AD. In fact, genetic modifications causing either duplication of the APP gene [111] or increased expression [112] were found to cause familial early onset AD. In addition, in Down syndrome patients, the triplication of the APP gene associates with the development of AD after age 40 [113], with the exception of those individuals affected by partial trisomy excluding the APP region [114]. In our experimental paradigm, such APP stabilization is caused by the lack of GSK3beta inhibition under conditions of impaired Pin1 activity. This may suggest that lack of Pin1 in AD impacts Abeta pathology by targeting multiple pathways, from APP trafficking to APP stabilization via GSK3beta activation.

More in details, we found that GSK3beta inhibitory mechanism was decreased in Pin1KO mice [110], since phosphorylation at S9, a mechanism that inhibits the kinase's activity, was decreased in these mice. We speculated that GSK3beta, which contains several Ser-Pro motifs, might serve as a substrate of Pin1 and that, by regulating GSK3beta conformation, Pin1 could control GSK3beta activity. This mechanism would contribute to the understanding of a link between loss of Pin1 activity and APP and tau pathologies in AD. GSK3beta in fact is responsible for the phosphorylation of both T668 in APP and T231 in tau, crucial in determining toxic conformations of both proteins in the disease. We found that Pin1 binds to GSK3beta at the T330 residue, and that lack of phosphorylation at this site using a T330A mutant would prevent such interaction. Of note, changes in Pin1 levels would affect GSK3beta activity, *in vivo* and *in vitro*. In fact, in crude brain lysates from Pin1Tg mice, GSK3beta activity was decreased, whereas it increased in Pin1KO mice. Similarly, overexpression of a wild type form of Pin1 reduced GSK3beta activity in H4 cells, whereas overexpression of mutants in regulatory regions of Pin1 such as the WW (W34A) or at the PPIase (K63A) domains, or at the Pin1 binding site (T330A) would not induce any change in the kinase activity, suggesting that by binding to T330, Pin1 is a crucial negative regulator of GSK3beta activity.

We then tested the hypothesis whether the Pin1-mediated control of GSK3beta activity could help prevent APP from entering the amyloidogenic processing. We found that lack of Pin1-mediated regulation of GSK3beta activity in T330A mutant expressing H4 cells reduced the levels of the non-amyloidogenic alphaAPPs, whereas it increased overall T668 APP phosphorylation. Of note, under these conditions, levels of APP were elevated at the steady state in cells, as well as in mice Pin1 concentration regulated APP levels. In fact, APP was reduced in Pin1Tg mice, whereas it accumulated in the brain of Pin1KO mice, similarly to what we had previously observed [41]. We found that APP accumulation in Pin1KO mice or in Pin1KD cells is the result of a failed physiologic degradation of APP, which is stabilized under these conditions. In fact, by means of cycloheximide treatment, we tested APP turnover under conditions of either reduced Pin1 expression (Pin1KD cells) or in absence of Pin1-mediated regulation of GSK3beta activity, in cells overexpressing the T330A mutant. We found not only that APP was stabilized in Pin1 KD cells as compared to wild type cells, also such stabilization seemed to depend on Pin1-mediated regulation of GSK3beta activity, since APP was stabilized in cells overexpressing the GSK3beta mutant T330A as compared to cells GSK3beta wild type.

Altogether, these data suggest that Pin1 regulates APP turnover by inhibiting GSK3 β activation and therefore contributes to lower T668 phosphorylation, which is responsible for toxic conformations of APP, as previously discussed in this chapter. These evidences suggest that in the pathology, mechanisms that favor the accumulation of APP will be toxic by increasing the amount of APP that will undergo amyloidogenic processing, and lack of Pin1 function could be one of these (Fig.1). Therefore, Pin1-mediated GSK3 β activity is an additional mechanism that Pin1 uses to protect from Abeta pathology, and strengthen the possibility to consider Pin1 as a valid tool to target Abeta pathologies in AD.

3. Role of T668 phosphorylation and APP conformation in AD: Pin1 as a molecular switch to regulate APP function

The question how increased APP phosphorylation at T668 is linked to AD has raised a real debate in the field. Many evidences point to a role in the disease. In fact, not only phospho-T668 levels were increased in AD brains [34], also many studies in vitro and in vivo point to a role of T668 phosphorylation by different kinases in altered protein transport in neurons [115, 116], also associated with increased amyloidogenic processing and Abeta production in cellular and animal models [34, 36, 39, 117]. In these studies, a non-phosphorylatable mutant T668A was used as an experimental paradigm to compare the effects of phosphorylated endogenous APP to the non-phosphorylated T668A form. Interestingly, a knock-in T668A animal model did not show any age-dependent alteration in APP processing [118], since in this study levels of Abeta, alphaAPPs and betaAPPs were comparable in both T668A and wild type mice during aging. Although at a first glance these data seem to challenge the hypothesis that regulation of T668 phosphorylation might be involved in AD, from these data it cannot be concluded that such phosphorylation is irrelevant to AD progression. It is not by abolishing APP phosphorylation at T668 by either knocking out APP or knocking in a non-phosphorylatable version of it that we can exclude a role for such pathway in the disease. APP KO mice are viable and their development and aging does not rely on alterations of APP processing [119, 120], and yet a role for APP in the development of AD is not disputed. Similarly, tau KO mice develop properly, reach adulthood and age normally [121-123], however a role for hyperphosphorylated tau in AD is quite clear.

It is possible that the controversy around the role of T668 phosphorylation in AD lies in the fact that physiologic phosphorylation at this domain is low, and it is only elevated in AD. This would explain why the T668A knock in mice did not show any difference from the wild type [118], because basal levels of phosphorylated T668 APP are very low, and phosphorylation-mediated regulation of APP activity in wild type mice was therefore comparable to the T668A mutant mice in this study.

Based on our data in vivo in Pin1KO mice and in vitro in Pin1KD cells [41, 51], we could hypothesize that the relevance of physiological T668 phosphorylation could be to maintain allow the equilibrium between cis and trans conformation. We could also assume that reduced Pin1 levels or increased T668 phosphorylation, both conditions associated with AD [34, 56],

may disturb such equilibrium leading to increased Abeta production. Of note, the overexpression in Pin1KO breast cancer cells of a T668A APP mutant, which retains 100% trans conformation, rescued the amount of APP anchored at the plasma membrane, and also the levels of alphaAPPs [51]. These data may suggest that the protective non-amyloidogenic processing of APP is maintained only if APP is in the trans conformation, a conditions that associates with physiologic low levels of phosphorylated T668 and to physiologic levels of Pin1. This poses attention on protein isomerization and Pin1 as a fine post-phosphorylative tool to regulate a protein function, bypassing the regulation of the kinases. Targeting abnormal protein isomerization and Pin1 function may therefore offer a preferred approach in AD to halt the toxic effects of hyperphosphorylated proteins, such as phosphorylated T668 APP and T231 tau, instead of the pharmacological inhibition of the many kinases responsible for their phosphorylation.

Altogether, the data discussed here emphasize a role for Pin1-mediated isomerization of APP and GSK3beta as a mechanism to control APP physiologic function, shifting APP processing toward the non-amyloidogenic pathway (Fig.1). Such regulation prevents the formation of toxic species produced downstream the amyloidogenic pathway, such as Abeta and betaAPPs, by regulating both APP trafficking and stabilization, and occurs as a post-phosphorylative event to maintain the equilibrium between cis and trans conformations. Therefore, Pin1 and regulation of APP conformation emerge as ideal candidates in the search of therapeutic targets for AD.

4. Pin1 and tau pathology in animal models and in AD

Tau-mediated neurodegeneration may result from the combination of toxic gains-of-function acquired by the aggregates or their precursors and the detrimental effects that arise from the loss of the normal function(s) of tau in the disease state [15]. The toxic gains-of-function includes sequestration of normal tau function by NFTs made of hyperphosphorylated tau. NFTs also become physical obstacles to the transport of vesicles and other cargos[15]. The loss of the normal function of tau includes detachment of tau from microtubules that causes loss of microtubule-stabilizing function [124]. Although dynamic tau phosphorylation occurs during embryonic development [125], aberrant tau phosphorylation in mature neurons is harmful to the neuron [126]. Tau hyperphosphorylation is a key regulatory mechanism that leads to both such toxic gains-of-function and the loss of the normal function(s) of tau [15].

4.1. Prolyl isomerization of tau

A high proportion of prolines residues are common to intrinsically disordered proteins, and tau is no exception [127]. Nearly 10% of full-length tau is composed of proline residues and around 20% of the residues between I151 and Q244 are proline. Many functions of tau are mediated through microtubule (MT) binding domains distal to this proline-rich domain. Interestingly, many disease-associated phosphorylation events that seed tau tangle formation occur at proline-directed serine (S) and threonine (T) residues in this proline-rich region. This indicates that important structural changes in the proline-rich region of tau are regulating

tangle formation. In particular, cis-trans isomerization around these prolines modulates protein phosphatase binding and activity at specific S/T sites. We discovered that Pin1 regulates tau phosphorylation in concert with protein phosphatase 2A (PP2A) especially at T231 [42, 56, 128-130]. Findings by Dickey's group suggest that FKBP51 has a similar activity to Pin1; but unlike Pin1, FKBP51 coordinates with Hsp90 to isomerize tau [131].

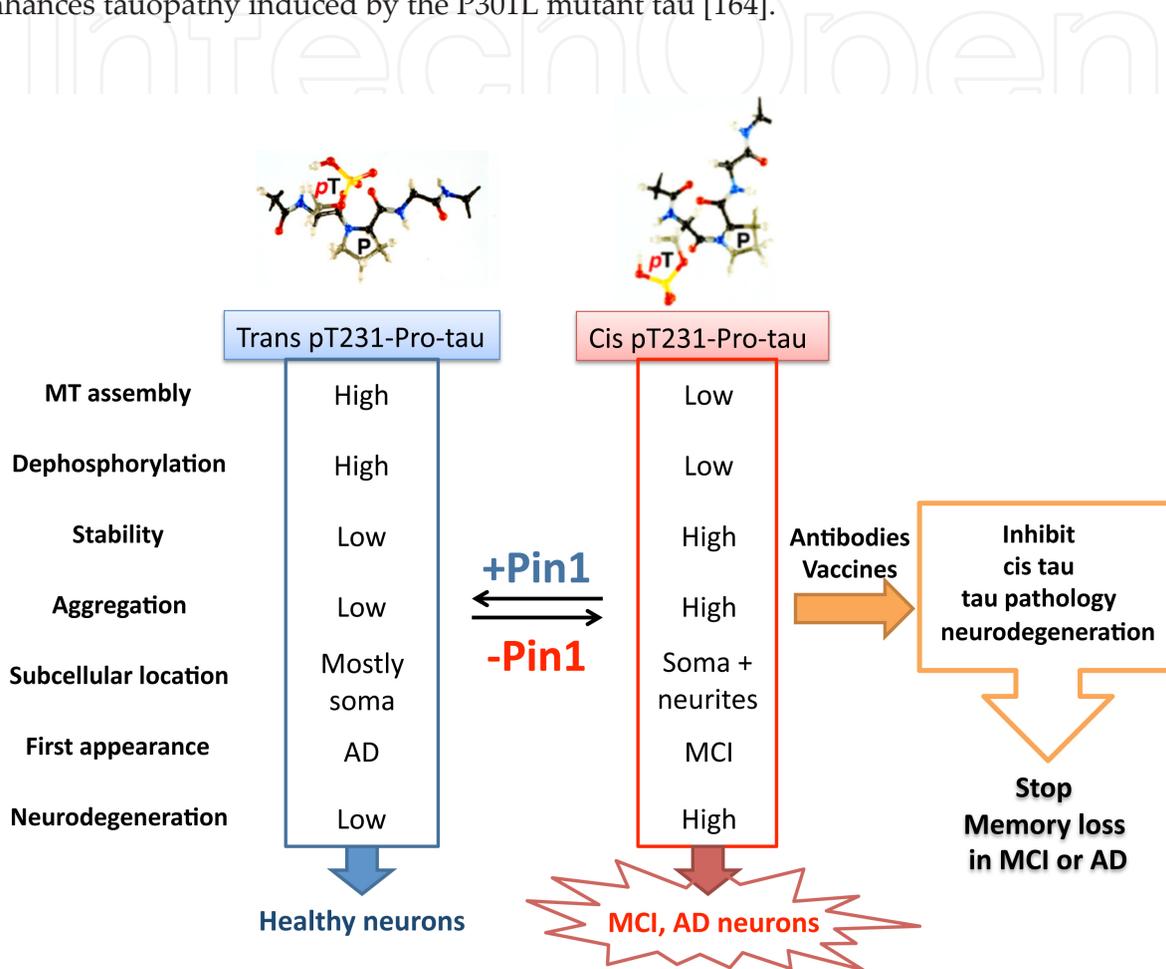
4.2. Deletion of Pin1 causes tauopathy in mice

Pin1 knockout (KO) mice [42] serve as a good model to investigate the effect of Pin1 on endogenous tau *in vivo*. Pin1-deficient mice showed progressive age-dependent motor and behavioral deficits including abnormal limb-clasping reflexes, hunched postures and reduced mobility [42] similar to tau transgenic mice [132, 133]. These phenotypes in Pin1 mutant mice are significant because the total level of NFTs correlates with the degree of cognitive impairment [134, 135]. Pioneering studies that used immunohistochemical techniques to determine the level of both NFTs and senile plaques in different brain regions of AD patients, as well as non-demented elderly individuals, demonstrated that the number of NFTs, but not the numbers of senile plaques, correlates with the degree of cognitive impairment [134, 135].

4.3. Pin1 acts on the pT231-P motif in p-tau to protect against tauopathy in AD

It is increasingly evident that tauopathy in AD may result from the combination of toxic gains-of-function acquired by phosphorylated tau (p-tau) aggregates, and the malignant effects from loss of tau normal function, including the failure of p-tau to bind and promote microtubule (MT) assembly [15]. Interestingly, a common characteristic event that disrupts tau MT function and precedes tangle formation is increased phosphorylation of tau especially on S/T-P motifs [11, 126, 136-141]. Indeed, tau kinases, such as mitogen-activated protein (MAP) kinases, cyclin-dependent protein kinase 5 (CDK5) and glycogen synthase 3 (GSK3) [142-144] or phosphatases, such as protein phosphatase 2A (PP2A) [145, 146] are deregulated in AD and modulating these enzymes can affect tauopathy [23, 39, 147-155]. Moreover, recent GWAS studies identify several new AD genes that might modulate tau phosphorylation and/or cytoskeleton, including MARK4 (pro-directed tau kinase) and BIN1 [156], and CD2AP [157, 158]. Notably, T231 phosphorylation in tau is reported to be the first of a number of tau phosphoepitopes appearing sequentially during early stages in pretangle AD neurons: pT231 → TG3 → AT8 → AT100 → Alz50 → → NFTs. However, it is still unclear how this phosphorylation leads to tau misfolding, aggregation and tangle formation [130, 159-161]. The results from our group and others support a critical role for Pin1 in protecting against tauopathy in AD by acting on the phosphorylated T231-P (pT231-P) motif (Fig.2). We discovered that pT231-P motif in tau exists in distinct cis and trans conformations, and that the cis to trans conversion is accelerated by a unique isomerase, Pin1 [44, 56, 128, 162], thereby protecting against tangle formation in AD [42, 48, 56, 163, 164]. pT231 is the first of sequential tau phosphoepitopes appearing in pretangle neurons in prodromal AD [159, 160] and its cerebrospinal fluid (CSF) level is an early biomarker that tracks MCI (mild cognitive impairment) conversion to AD, albeit with wide interindividual variations [165, 166]. Pin1 acts on the pT231-P motif in tau (i) to restore the ability of p-tau to promote MT assembly [56], (ii) to facilitate p-

tau dephosphorylation because PP2A is trans-specific [42, 128], and (iii) to promote p-tau degradation [164] (Fig.2). Indeed, Pin1 overexpression promotes tau dephosphorylation selectively on pT231 in neurons and mouse brains [167, 168]. Pin1 has no effect on T231A mutant tau in vitro or vivo [56, 128, 139, 164]. Furthermore, Pin1 deficiency (Pin1-KO) mice display age-dependent tauopathy [42], whereas Pin1 overexpression in postnatal neurons effectively suppresses tauopathy induced by human wild-type (WT) tau in mice, albeit it enhances tauopathy induced by the P301L mutant tau [164].



pT231-tau protein exists in the two completely different cis and trans conformations, as depicted in cartoons of the primary backbone structures. Cis, but not trans, pT231-tau loses normal function and also gains toxic function. Pin1 inhibits the accumulation of the pathogenic cis pT231-tau conformation in AD by converting it to the nonpathogenic trans form. Conformation-specific antibody and/or vaccines against the pathogenic cis pT231-tau might be developed for the diagnosis and treatment of AD, especially during its early stages.

Figure 2. Pin1 inhibits the accumulation of the pathogenic cis pT231-tau conformation in AD by converting it to the nonpathogenic trans form

These findings are highly relevant to human AD. We found that Pin1 localize to tangle and is depleted in AD brains [56], which has been confirmed and expanded to other tauopathies including FTD [61, 62, 169-172]. Furthermore, Pin1 is induced by neuron differentiation and highly expressed in most normal neurons, but inhibited by various mechanisms in AD neurons [44, 54, 56, 59, 62, 167, 169, 171-175]. For example, Pin1 expression is especially low in AD

vulnerable neurons or actual degenerated neurons in AD [42]. Pin1 can be inactivated by oxidation in mild cognitive impairment (MCI) and AD neurons [61, 169, 170]. Pin1 is sequestered into tangles [56] or tangle or Ab-free granulovacuolar bodies with increasing tauopathy [176]. The human Pin1 gene is located at 19p13.2, a new late onset AD locus distinct from ApoE4 [173] and Pin1 promoter SNPs that reduce Pin1 expression [177] are associated with increased risk for AD in some cohorts [174], although not in others [65, 178]. A different Pin1 promoter SNP that prevents its suppression by AP4 is associated with delayed onset of AD [178]. Our findings of the opposite effects of Pin1 on cancer and AD [43, 45] have been supported by genetic association studies [174, 177, 179] and epidemiological studies [180-182]. Our analysis of the Framingham Study has further shown that cancer patients have decreased risk of AD, that AD patients have reduced cancer risk, and importantly, that this inverse relationship is not due to selective mortality or underdiagnosis [183]. Thus, we recently proposed that lack of sufficient Pin1 to catalyze cis to trans isomerization of pT231-tau might be a pathogenic mechanism leading to tauopathy in AD [48, 163].

5. Regulation of tau conformation as an early change in AD pathology: Implications for Pin1-catalyzed protein conformational regulation as a therapeutic and diagnostic tool

5.1. Cis pT231-tau is extremely early pathogenic conformation that is accumulated in MCI and AD

To analyze Pin1-catalyzed cis/trans protein conformational regulation and conformation-specific function or regulation, we have developed a novel technology to generate the first cis and trans-specific antibodies. We discovered that cis, but not trans, pT231-tau is extremely early pathogenic conformation in AD due to lack of Pin1 convert it to the physiological trans [129].

We found that replacing the five membered ring of Pro232 with the six membered ring homoproline (Pip) increases cis to 74%, since ~90% of pT231-P motif in a synthetic peptide is in trans. Polyclonal antibodies raised against a pT231-Pip tau peptide recognize human and mouse p-tau. Resulting cis-specific antibody recognize regular pT231-Pro tau and cis pT231-5,5-demethylproline (Dmp) tau peptide, whereas trans-specific antibody recognizes regular pT231-Pro and trans pT231-Ala tau peptide. Neither antibody recognizes the non-phosphopeptide. Furthermore, both antibodies recognize tau, but not its T231A mutant expressed in SY5Y cells. Thus, cis- or trans- antibodies are highly specific [129]. Antibodies against cis pT231-tau might provide opportunity for efficient immunotherapy and diagnostic tools against early pathogenic tau conformation, raising the possibility of preventing tauopathy in AD patients at early stages.

5.2. Cis pT231-tau not only loses normal MT functions, but also gains toxic functions

As we and other groups had been shown [56, 184-186], phosphorylation of tau on T231 by Ccd2 abolishes its ability to promote MT assembly, which can be restored after dephosphor-

ylation by PP2A or Pin1 [129]. Importantly, the ability of Pin1 to restore p-tau MT function is fully blocked by incubation of Pin1-treated p-tau with trans antibodies, but not cis antibodies. Furthermore, trans pT231-tau peptide is readily dephosphorylated by the tau phosphatase PP2A, which dephosphorylates on trans pS/T-P motifs [128]. Moreover, cis pT231-tau is much more stable than the trans both in cells and in Tau-transgenic (Tg) mouse brain slice cultures. Finally cis pT231-tau is much more prone to aggregation than the trans in Tau-Tg mouse brains and human MCI brains, as detected by sarcosyl fractionation experiments [42, 164, 187]. Thus, cis, but not trans, p-tau loses normal MT functions, and gains toxic functions.

5.3. Pin1 overexpression increases cis to trans pT231-P conversion in WT tau-Tg mice

To detect the ability of Pin1 to increase cis to trans isomerization, we showed that Pin1 reduces cis, but increases trans pT231-tau in vitro [129]. Moreover, Pin1 overexpression in Tau-Tg mice (Tau+Pin1) reduces the cis content, but increases the trans content, whereas Pin1 deficiency in Tau-Tg mice (Tau+Pin1-KO) has the opposite effects, as shown by immunoblots and immunostains [129]. In contrast to Wt tau-Tg mice, Pin1 overexpression increased cis, but decreased trans pT231-tau in P301L tau Tau-Tg mice because the P301L tau mutation greatly reduced cis, but not trans, pT231-tau, as we hypothesized [164]. These results explain why Pin1 has the opposite effects on WT tau and P301L tau [164] and are consistent with that CSF pT231-tau can differentiate AD from frontotemporal dementia [165, 166].

5.4. Cis, but not trans, pT231-tau is significantly elevated and localized to dystrophic neurites in MCI and further accumulated in AD

To examine p-tau conformational changes during the development of AD, we analyzed different Braak brain tissues with *cis* or *trans*-specific tau antibodies. There is little *cis* or *trans* pT231-tau in normal brains [129]. In AD cortex, *trans* pT231-tau is very low, but the *cis* is readily detected, even at Braak stages III and IV (MCI), and further accumulates as the Braak stage increases [129]. Notably, *cis*, but not *trans*, is localized to the dystrophic neurites [129], an early hallmark change highly correlating with synaptic and cognitive loss in AD [188-194]. Given Pin1 inhibition by oxidation in MCI brains [169, 170, 195], Pin1 might act at a very early step to inhibit tauopathy in AD.

5.5. Cis pT231-tau fully overlaps with neurofibrillary degeneration and correlates with reduced Pin1 levels in the AD hippocampus

As shown [42, 134, 196, 197], Pin1 is highly expressed in the CA2 region of the hippocampus, but dramatically reduced in the CA1 region, whereas PHF-1, a solid neurofibrillary neurodegeneration marker [161], is prevalent in the CA1, but not in CA2 region [129]. Importantly, trans-positive neurons are dominant in the CA2 region. However, in the CA1 region, cis-positive neurons are greatly increased. All cis-positive neurons are also positive for PHF-1 in both CA2 and CA1 regions. However, 74% of trans-positive cells in the CA2 region are negative for PHF-1. Thus, cis, but not trans, pT231-tau is fully overlapped with neurofibrillary degeneration.

5.6. Potential novel *cis* and *trans* conformation-specific disease diagnoses and therapies

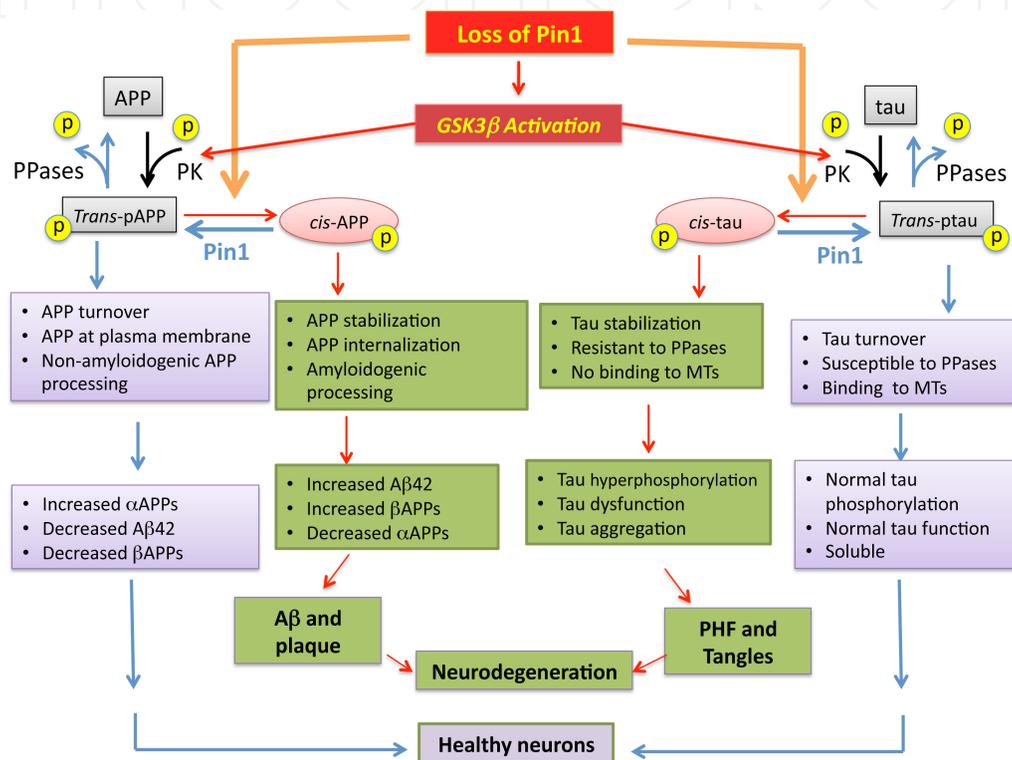
Our exciting new insight into the role and regulation of p-tau conformations in AD might have important and novel therapeutic implications. For example, it has been shown that Thr231 phosphorylation is the earliest detectable tau phosphorylation event in human AD [130, 159, 160, 198] and its levels are elevated in cerebrospinal fluids and tracks AD progression, but with large individual variations [199, 200], making it difficult to become a standardized test. Our findings that the *cis* conformation appears earlier in MCI and is pathologically more relevant suggest that *cis* pThr231-tau and especially its ratio with *trans* might be a better and easier standardized diagnostic marker, especially for early diagnosis and patient comparison. Furthermore, the findings that Pin1 overexpression converts *cis* to *trans*, promotes tau degradation and inhibits tau pathology and neurodegeneration in AD mouse models [201] and that Pin1 SNPs preventing its inhibition by brain-specific transcription factor AP-4 is associated with delayed onset of AD [202] suggest that overexpressing Pin1 or preventing Pin1 inhibition might be a new approach to reduce the *cis* to *trans* pThr231-tau ratio to block tau pathology at early stages. Finally, active or passive immunization against some pSer/Thr-Pro motifs in tau including the pThr231-Pro motif has been shown to reduce tau aggregates and improve memory deficits in mouse models [203-209]. However, we have here shown that only ~10% of regular synthetic pThr231-tau peptides is in the pathologically relevant *cis* conformation and the remaining 90% is in *trans*, which can still promote MT assembly and is not related to neurofibrillary degeneration. Therefore, immunotherapies either using conformation-specific vaccines or antibodies specifically against the pathologically relevant *cis* pT231-tau conformation might be more specific and effective and safer in treating AD. Given the critical role of Pin1 and other isomerases in controlling the function of many other key regulators in the pathogenesis of human disease, notable Alzheimer's disease, cancer, viral infection, inflammation and autoimmune disorders [210-213], it would be interesting to determine whether prolyl isomerization regulates the cellular function of these proteins and whether these conformational switches might be explored for developing novel diagnoses and therapies.

6. Finding a proper animal model to study AD: A lesson from the pin1KO mice

One of the biggest challenges when studying a disease is to develop the proper animal model that would reproduce the main features of that disease within the animal's biological environment. In the case of AD, this is not an easy goal, since mice do not spontaneously develop the features characteristic of this disease.

The only way to induce AD-like pathology with plaques and/or tangles in mice is by generating genetically altered animals. These may either overexpress aggressive mutants of APP linked to familial forms of AD (FAD) [214-218] and hence produce higher amounts of Aβ peptide, or express either wild type or aggressive mutants of tau [217, 218], leading to sustained tau hyperphosphorylation and tangle formation or may express both [217, 218]. These models may recapitulate plaque (APPTg) or tangle (tauTg) pathologies, or both (APPTg crossed to tauTg) [217, 218], and are extremely useful to understand the molecular pathways involved in AD,

however they may [216] or may not [219] undergo neurodegeneration, which is a feature of AD. Moreover, they may not be representative of the way the disease progresses in sporadic AD, which affects the vast majority of AD patients, as they may represent only those familial cases of AD caused by those same mutations. Furthermore, these models may not be all specific for AD, since tau hyperphosphorylation and tangle formation occur also in other neurodegenerative diseases, and some of the tau mutations used to generate animal models for AD do not associate with AD, but with other neurodegenerative diseases, such as frontotemporal dementia associated with parkinsonism FTDP [17, 220, 221].



Both APP and tau are phosphorylated by protein kinase (PKs) as part of their normal function. The trans-conformation of phosphorylated APP and tau may present the physiological conformation that promotes their normal function (green boxes). Pin1 expression is induced during neuron differentiation and necessary to maintain normal neuronal function by preventing the unscheduled activation of mitotic events and/or controlling the function of phosphoproteins in the event that they become abnormally phosphorylated. For example, by catalyzing isomerization of the cis to trans conformation, Pin1 might promote non-amyloidogenic APP processing reducing Abeta production, as well as promote tau dephosphorylation and restore tau function. However in AD, a loss of Pin1 function, either through downregulation of Pin1 function, oxidative inactivation, phosphorylation or possible genetic alterations, can lead to build-up of cis-pS/T -P motifs. Cis-p-tau and cis-p-APP are proposed to represent pathological conformations (red ovals). Cis-p-APP is processed by the amyloidogenic pathway, which lead to a build-up of amyloid beta-42 (Abeta42), decreased levels of neurotrophic alphaAPPs and the resultant formation of amyloid plaques. Cis-p-tau is resistant to protein phosphatases, which leads to a loss of MT binding, hyperphosphorylated tau and the formation of neurofibrillary tangles. The formation of tangles and plaques might further reduce Pin1 function by sequestering Pin1 and inducing oxidative modifications, respectively, in a positive feedback loop. In addition, a lack of proper Pin1 function leads to activation of kinases such as GSK3beta, which further increases both phosphorylation of tau and APP and also inhibits APP turnover, contributing to both tau and Abeta pathologies and causing neuronal death. Therefore, Pin1 deregulation might act on multiple pathways to contribute to AD development.

Figure 3. The regulation of APP processing and tau function by Pin1 in healthy and Alzheimer's neuron.

In addition, also animal models developed to understand a specific pathway even in absence of plaques [118], show limitations in the interpretation of the results.

In contrast, the model offered by knocking out the Pin1 gene in our Pin1KO model may recapitulate some of the features characteristic of both tau and Abeta pathology in sporadic AD, and therefore could serve as a valid tool to investigate the pathways that can be targeted to prevent or halt the disease progression. In fact, Pin1KO mice 1) develop age-dependent Abeta pathology associated with early neuronal deficit that leads to neurodegeneration (elevated Abeta levels associated with increased intracellular deposition) [41], 2) are characterized by age-dependent tau hyperphosphorylation, stabilization and PHF formation [42], and 3) show age-dependent neurodegeneration in selected areas [42]. Because genetic and proteomic findings link decreased Pin1 levels and/or activity to AD [56,61], we could speculate that the Pin1KO animal model be very close to recapitulating the features that characterize AD in humans, and therefore may serve as a valid model to study the molecular pathways involved in AD.

7. Conclusion

We have here reviewed studies showing how Pin1 is an essential regulator of APP, tau and GSK3beta conformations, maintaining their physiological functions, and how loss of Pin1 in AD contributes to the accumulation of toxic conformations that turn the proteins' function pathologic. Moreover, the data here discussed present Pin1 as a link between both Abeta and tau pathology that could be exploited to tackle both pathologies in AD, even at early stages. The emerging new concept is that protein conformation might be a key regulatory element in toxic pathways in AD, and that Pin1 regulation of protein conformations might be a promising avenue to fight AD.

The debate about Abeta and tau pathology, which occurs first, which causes the other, is still unsolved, and clarifying it would help identify the correct therapeutic target to successfully prevent AD progression. Although studies in animal models *in vivo* showed that Abeta pathology occurs first and may be causative of tau pathology [222, 223], they were performed in animal models genetically modified to develop both tau and Abeta pathologies, and therefore may not be representative of the molecular mechanisms underlying sporadic forms of AD. In fact, it is still unclear which pathology occurs first in human AD, and only fine diagnostic tools able to identify early modification on both APP and tau that may render the proteins toxic would be of help.

As appropriate early diagnostic tools are still missing, the evidences here presented highlight Pin1 as an ideal therapeutic target to block the toxicity of both APP and tau in AD. In fact, the data here discussed show that equilibrium between cis and trans conformation of APP and tau is crucial to maintain their physiological function, and that this is disrupted either by hyperphosphorylation at S/T-P or by lower Pin1 levels or both in AD. In addition, we show here evidences that, in tau, alteration in the equilibrium between cis and trans conformation is an event that precedes massive cognitive decline, since the cis form of phosphorylated tau accumulates in MCI patients [129]. These results directly link conformational changes to

pathologic protein functions, and highlight Pin1 as a successful regulator of such toxic conformations, opening new avenues in the medical field of AD. In fact, if a therapeutic target, Pin1 could block both tau and Abeta pathologies early in the disease, also resolving the eternal and unsolvable conflict: What happens first?

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