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Review

Prolyl isomerase, Pin1: new findings of post-translational modifications and physiological substrates in cancer, asthma and Alzheimer's disease

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Abstract. The peptidyl prolyl *cis*/*trans* isomerase Pin1 specifically binds phosphorylated Ser/Thr-Pro protein motifs and catalyzes the *cis/trans* isomerization of the peptide bond. Accumulating studies have revealed that Pin1 isomerase activity is regulated by its post-translational modifications, including phosphorylation and oxidation. Various transcription factors and regulators have been identified as substrates for Pin1. It enhances AP-1 activity via isomerization of both c-Jun and c-Fos for cellular proliferation and stabilizes the oncosuppressors p53 and p73 against DNA damage at the checkpoint. We demonstrated the association between the intracellular form of Notch1 (NIC) and Pin1 by analyzing Pin1/p53 double-knock-out mice. Pin1 also regulates the post-transcriptional level of some cytokines, associated with asthma, that possess 3′ untranslated region AU-rich elements (AREs) via interaction with AUF1, the nucleoprotein in the ARE-binding complex. Pin1 has been identified as the molecular partner of tau and amyloid precursor protein (APP), the key factors of Alzheimer's disease (AD). It interacts with the phosphorylated Thr-231 of tau and regulates its activity to bind microtubules. It further interacts with the phosphorylated Thr-668 of APP and affects its metabolism. Thus, Pin1 is probably involved in the pathogenesis of human diseases, including cancer, asthma, and AD, presenting an attractive target for future therapeutical drugs.

Keywords. Pin1, posttranslational modification, pathogenesis, cancer, asthma, Alzheimer's disease.

Introduction

Peptidyl prolyl *cis/trans* isomerases (PPIases) catalyze the *cis/trans* isomerization of the peptidyl prolyl (X-Pro) bonds, and this *cis/trans* rotation of the peptide bond affects the spatial arrangement of the backbone

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segments in the proteins. The three phylogenetically conserved PPIase families involved in the catalysis associated with protein folding are the cyclophilins, FK506-binding proteins (FKBPs) and parvulins. The PPIase activity of the cyclophilins is inhibited by the immunosuppressant drug cyclosporin A, whereas that of the FKBPs is inhibited by FK506 and rapamycin. The parvulins are not inhibited by these immunosuppressant drugs [1–3].

Pin1 belongs to the parvulin family of PPIases and is inhibited neither by cyclosporin A nor by FK506. Among the PPIases, Pin1 is the only enzyme known to be highly specific for substrates with phosphorylated Ser or Thr, followed by a Pro, and it is structurally and functionally related to Essl/Ptfl, which is an essential protein in budding yeast. Pin1 consists of the PPIase domain and group IV WW domains, and it specifically associates with a phosphorylated Ser or Thr residue, followed by a Pro residue (pSer/pThr-Pro) in order to catalyze both *in vivo* and *in vitro cis/trans* isomerization of the peptidyl prolyl bonds in peptides and proteins. Interestingly, both the PPIase and WW domains recognize the pSer/pThr-Pro motifs (Fig. 1).

Isomerization by Pin1 modulates protein folding, biological activity, stability, and subcellular localization of its substrates, and also regulates the factors involved in the control of cell growth and apoptosis [4–6]. Depletion of Pinl/Essl from yeast or HeLa cells induces mitotic arrest, whereas HeLa cells that overexpress Pinl are arrested in the G2 phase of the cell cycle [4]. Hence, Pinl is an essential PPIase that presumably regulates mitosis by interacting with mitotic phosphorylated proteins (MPM-2 antigens), including Cdc25, NIMA, and Wee-1, and by attenuating its mitosis-promoting activity [5]. Analysis of Pin1-deficient mice generated in our laboratories has provided evidence that Pin1 is involved in the pathology of cancer and Alzheimer's disease (AD) [7–11]. Pin1-deficient mice display immature germ cells, mammary gland impairment and retinal dystrophy, that is, decreased levels of cyclin D1 that result in cyclin D1-null phenotypes [8, 12–14]. And when Pin1 was depleted from the transgenic mice with MMTV-c-Neu/HER2/ErbB2 or -v-Ha-Ras, the rate of incidence of breast cancer in there mice was reduced [15]. Therefore, Pin1 physiologically controls cyclin D1 levels, and Pin1 overexpression promotes tumor growth via stabilization of cyclin D1 [8, 12–15]. Furthermore, *Pin1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) do not respond to serum stimulation after serum depletion-induced G0 arrest. These observations also suggest that Pin1 is required for cell cycle progression subsequent to G0 arrest and mitotic progression in normal mammalian cells [7]. Suizu et al. showed that transgenic overexpression of Pin1 in mammary glands induced centrosome amplification, eventually leading to mammary hyperplasia and malignant mammary tumors with overamplified centrosomes [16]. The phenotypes of both Pin1 knockout mice and Pin1 overexpressing transgenic mice indicate that Pin1 can effectively induce the progression and malignancy of tumors. The molecular mechanism in Pin1 expression can be associated with tumorigenesis under Neu/Ras signaling [15,17]. The *Pin1* promoter sequence possesses neither a TATA nor a CAAT box but has two putative GC boxes and three putative E2F-binding sites [17]. Indeed, the E2F family proteins activate the *Pin1* promoter via these E2F-binding sites. The E2F proteins also bind the *Pin1* promoter *in vitro* and *in vivo*, and increased Pin1 levels in breast cancer cell lines correlate with an increase in the binding of E2F to the *Pin1* promoter. The oncosuppressor BRCA1 represses the expression of Pin1 transcripts [18]. Moreover, E2F overexpression enhances *Pin1* promoter activity and messenger RNA (mRNA) levels in breast cancer cells. As commonly noted in many other E2F-target genes, *Pin1* transcription and protein levels fluctuate during cell cycle progression in non-neoplastic cells [17].

PPIase activity of Pin1 may undergo physiological regulation in a manner specific to each organ. Recently, it was discovered that Pin1 activity is modified by phosphorylation or oxidation [19–21]. These modifications might be associated with human pathology. Lu's cohort reported that Pin1 phosphorylated by cAMP (cyclic AMP)-dependent protein kinase A is an inactive form [19]. Phosphorylation of Ser-16 in the WW domain impairs nuclear localization and inhibits its binding to substrates. The dominant-negative mutants induce a mitotic block and apoptosis and increase the number of multinucleated cells with 8N DNA content [19]. It has been reported that the altered enzymatic activity of Pin1 is involved in the pathogenesis of human diseases, including not only cancer, but also AD and asthma [19–24]. The Pin1 expression level increased in various tumor tissues [25]. Polo-like kinase 1 (Plk-1) phosphorylated the PPIase domain of Pin1; this does not affect PPIase activity and enhances Pin1 stability by regulating its ubiquitination [26]. On the other hand, the down-regulation of Pin1-induced hyperphosphorylation of tau, one of the hallmarks of AD [9]. Actually, the aged *Pin1*-deficient mice displayed tauopathy and neurodegeneration similar to that observed in patients with AD, and reduced Pin1 levels increased the risks associated with late-onset AD [9–11]. Interestingly, oxidative modification of Pin1 was observed in the hippocampi of patients with mild cognitive impairment (MCI). Butterfield's cohort indicated that

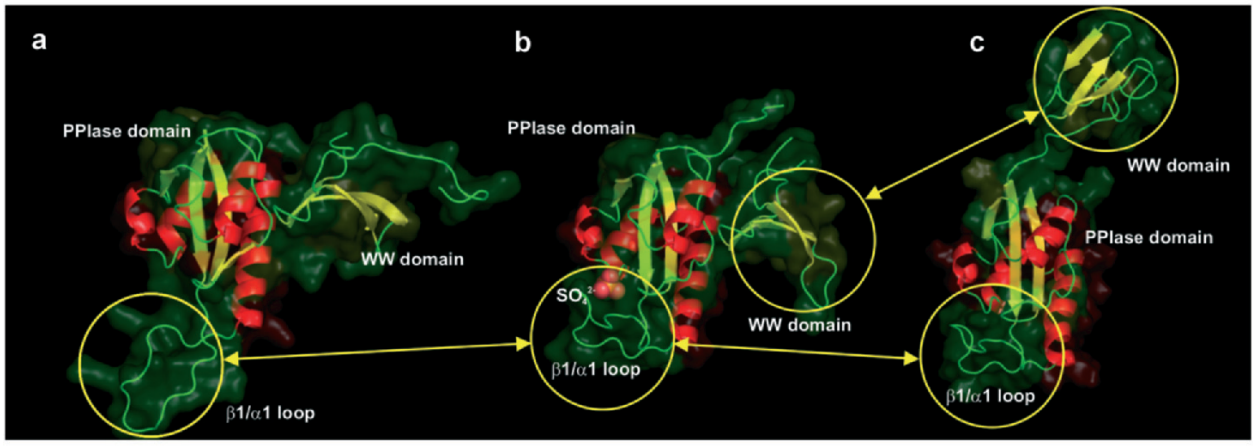

Figure 1. Flexible Pin1 structure, (*a*) and (*b*) show two possible β1/α1 loop structures. β1/α1 loop, where phosporylated peptides bind, moves flexibly when it binds the substrates. (*c*) shows the flexible structure of WW domain. The WW domain also binds phosporylated peptides and moves flexibly when it binds substrates. The structures were obtained from protein data bank: (*a*) 1F8A, (*b*) 1PIN and (*c*) 1NMV.

oxidative modification inhibits Pin1 isomerization activity [20, 21].

Shen et al. reported that Pin1 might be associated with the pathogenesis and malignancy of allergenic inflammation in asthma via stabilization of AU-rich element (ARE) mRNAs, including various cytokine mRNAs, and cytokine secretion [22]. Pin1 controls the post-translational levels of some cytokines that possess the 3′ untranslated region of AREs via interaction with the nucleoprotein in the ARE-binding complex AUF1 [22, 23]. In resting cells, phosphorylated Pin1 interacted to stabilize AUF1 and thereby degraded GM-CSF (granulocyte-macrophage colony-stimulating factor) mRNA. Dephosphorylated Pin1 exhibits enzymatic activity in activated eosinophils and may isomerize AUF1 to release ARE-mRNAs, resulting in the accumulation of ARE-mRNAs. Pin1 is a critical regulator of cytokine mRNA turnover, which in turn controls the survival of activated eosinophils in the lungs of asthmatics. Since AUF1 controls the decay of many ARE-mRNAs, the Pin1-AUF1 interaction might be important in cytokine production for inflammatory reactions and immune responses [22, 23].

As mentioned above, the post-translational modifications of Pin1 influence its isomerase activity, and associate it with certain human pathologies. The post-translational modified form of Pin1 controls the phosphorylated transcription factors and transcriptional regulators, which alter the levels of some transcripts required in cell proliferation, differentiation, and apoptosis. Many transcription factors and their regulators that are involved in a variety of physiological processes, such as reproduction, development, and cellular homeostasis, have been discovered to be substrates for Pin1. The components of activating protein-1 (AP-1); steroid receptor coactivator 3(SRC3), p53, p73; and the intracellular form of Notch1 (NIC) are physiologically associated with Pin1 [14, 27–33].

These new findings suggest that Pin1 plays an important role in the pathogenesis and therapeutics of human diseases such as cancer, asthma, and AD.

Post-translational modification of Pin1

Phosphorylation. Protein phosphorylation plays an important role in the regulation of various intracellular signal transduction pathways. Recently, several reports have indicated the presence of post-translational modification systems for Pin1's phosphorylation [19, 26, 34–36]. Phosphorylated Pin1 displays impaired isomerase activity, substrate interaction, cellular localization, and stabilization, all of which depend on Pin1 phosphorylated sites.

The phosphorylation at Ser-16 in the human Pin1 WW domain by cAMP-dependent protein kinase A (PKA) impairs its ability to function as a substrate-binding molecule [19]. The WW domain mainly serves as a pSer/Thr-Pro-binding module; the binding pocket includes Ser-16, Arg-17, Tyr-23, and Trp-34 side chains. The phosphorylation of Ser-16 by cAMP-dependent PKA inhibits the interaction with Pin1 substrates in mitotic cell extracts. The dephosphorylated form (hypophosphorylated) of Pin1 is specifically observed during the mitotic phase, and this modification results in diffusion of Pin1 in the entire cell from the nuclear speckle. Several reports revealed that both cAMP and PKA activities oscillate during

proliferation, with their lowest and highest levels during the G2/M transition and at exit from mitosis, respectively [37, 38]. Ser-16 is not the only site phosphorylated by PKA; however, phosphorylation of Ser-16 is sufficient to inactivate the WW domain [19].

Phosphorylated sites were also identified within the PPIase domain of Pin1. Eckerdt et al. (2005) were the first to report the regulation of Pin1 by phosphorylation of its PPIase domain [26]. They showed that Plk-1 functioned as the kinase responsible for phosphorylating the PPIase domain of Pin1. Plk-1 is a Ser/Thr kinase that represents a key regulator of the late stage of mitosis and is overexpressed in many human tumors [36, 39–41]. During the G2/M transition, Plk-1 contributes to the control of centrosome maturation, bipolar spindle formation, mitotic checkpoints, and activation of the maturation-promoting factor by phosphorylation of Cdc25C and cyclin B1. Furthermore, Plk-1 positively regulates the ubiquitination of two M-phase inhibitors: Wee1 and the early mitotic inhibitor 1 Emi1 [42–44]. Plk-1 mainly phosphorylated Ser-65 within the Pin1 catalytic domain. Ser-65 phosphorylation does not alter the enzymatic activity of Pin1 but enhances its stability by regulating its ubiquitination. The Pin1 proteins stabilize in the presence of the proteasome inhibitor, and this indicates that the degradation of Pin1 is catalyzed by the proteasome. Plk-1 kinase activity is linked to the regulation of the Pin1 protein stability. In mitotic cells, Pin1 Ser-65 appears to be important for the regulation of Pin1 protein levels by Plk-1. Eckerdt et al. used mutational analysis to demonstrate the prolonged half-life of the mutant $Pin1^{S67E}$ as compared to that of wild-type (WT) Pin1 [26]. These findings suggest that Plk-1 overexpression might contribute to tumorigenesis via the stabilization of Pin1, followed by upregulation of the oncogenic molecules that serve as substrates for Pin1. Further, Min et al. (2005) demonstrated that the dephosphorylation of tau and GSK3β induces Pin1 phosphorylation at Ser-16. Hence, Pin1 phosphorylated at Ser-16 might confer protection against the progression of AD [35].

Ser-67 might serve as another site for phosphorylating Pin1 [34]. The Ser-67 residue precedes the two Arg residues (Arg-68 and Arg-69) involved in binding the targeted p-Ser/p-Thr motif in the substrate proteins and are essential for recognizing the catalytic transition state of Pin1-substrate complexes. Since the mutant $Pin1^{S67E}$, which mimics phosphorylation of Ser-67, induces complete loss of Pin1 activity, Ser-67 is important for regulating Pin1 activity [34]. Although PKA might be responsible for phosphorylation on Ser-67, it remains unidentified.

With regard to human Pin1, the A-kinase-dependent phosphorylation of Ser-16 inhibits the Pin1 and substrate interaction and influences Pin1 enzymatic activity; further, Ser-65 phosphorylation by Plk-1 increases Pin1 stability. The regulation of Pin1 phosphorylation might proceed via a complicated mechanism, and the modification depends on the enzymatic activity level and amount of Pin1. These findings demonstrate that phosphorylation of the Pin1 WW domain might inhibit tumorigenesis and enhance AD progression; however, phosphorylation of the PPIase domain might be essential for accumulation of Pin1 in the proliferating cells.

Oxidation. Protein carbonyls, including aldehydes and ketones, are the most commonly used markers for protein oxidation; they can be generated using direct oxidation of amino acid side chains, by oxidative cleavage of the proteins via the alpha-amidation pathway, or by the Michael addition reaction of alpha- and beta-unsaturated aldehydes such as acrolein, derived by lipid peroxidation. Generally, increased levels of protein carbonyls result in disease associated with protein dysfunction [45–47].

The hippocampus of the brain is intimately involved in memory processing and is one of the sites of the brain that is most vulnerable to pathological lesions specific to AD. Brains with AD undergo oxidative stress caused by protein oxidation, lipid peroxidation, DNA oxidation, advanced glycation-end products, and ROS (reactive oxygen species) formation; all of these might play important roles in the pathogenesis and progression of AD [21, 47–51]. Butterfield's cohort formulated a novel method for analyzing oxidative proteins; this method, named 2D Oxyblot analysis, uses the anti-2,4-dinitrophenylhydrazone (DNP) polyclonal antibody [20, 21, 52, 53].

Using 2D Oxyblot analysis, Sultana et al. (2006) demonstrated that Pin1 is subjected to oxidative modification in the AD hippocampus [20]. In an attempt to identify specific targets for protein oxidation relevant to oxidative stress in the AD hippocampus by using proteomic analysis, they discovered that Pin1 is subjected to oxidative modification, suggesting that oxidative modification is related to the reported loss of Pin1 isomerase activity crucial for the neurofibrillary pathology of AD. Notably, the authors demonstrated that Pin1 enzymatic activity decreased (approximately 60%) in the AD brain as compared to that in the control brain, and that *in vitro* oxidation of the purified Pin1 protein with Fe^{2+} and H_2O_2 resulted in a decrease (approximately 60%) in Pin1 activity. Hence, the loss of Pin1 activity due to oxidative damage could be crucial in the neurofibrillary pathology of AD [20].

Table 1. Substrates upregulated by Pin1.

	Consequence	Effect of Pin1	References
APP	amyloid precursor protein	reduction of cleavage for amyloid beta	11
CyclinD1	cell cycle control	stabilization	8
Plk1	G2/M transition	phosphorylation and stabilization of Pin1	26
CK2	G2/M transition, checkpoint control	inhibition of kinase activity	107, 108
Emi1	G2/M transition, checkpoint control	accumulation of cyclins	153
Raf-1	signal transduction	dephosphorylation and recycling	154
c-Jun	transcription factor	transactivation	14
c-Fos	transcription factor	transactivation	27
p73	transcription factor	transactivation	29
p53	transcription factor	transactivation	30–32
p65/Rel	transcription factor	transactivation	130
β-catenin	transactivator	nuclear localization	155
AUF	AU-rich element RNA binding protein	stabilization of AU-rich mRNA	22, 23
Synphilin-1	α-synuclein aggregation	Lewy bodies of Parkinson disease	156
Rab4GTP	membrane recycling	inhibition of endocytic membrane transport	157
Gephyrin	microtubule binding	binding the beta subunit of glycine receptors	158
BIM_{EL}	apoptotic inducer	activation of neronal apoptosis	159
p66Shc	growth factor adaptor, apoptotic inducer	mitochondrial accumulation, apoptosis inducing	160
Neurofilament-H	neurofilament	neurodegeneration	161
Virus molecule			
HBx	promotion of hepatocarcinogenesis	transactivation	162

Table 2. Substrates downregulated by Pin1.

	Consequence	Effect of Pin1	References
APP	amyloid precursor protein	facilitating C99 turnover	10
Tau	microtubule association	regulation of microtubule assembly	9
SRC3	transcription factor	transactivation	28
c-Myc	transcription factor	dephosphorylation	163
RARα	transcription factor	transactivation	164
IRF3	transcription factor	controlling antiviral responses	165
RNA polymerase II	transcription of mRNA precursors	inhibition of ongoing transcription	54, 55
NIC	transactivator	transactivation	33
Btk	signal transduction (nonreceptor tyrosine kinase)	inhibition of kinase activity	166
Cyclin E	activator of CDKs	multiubiquitylation of cyclin E by SCFhCdc4	167, 168
Che-1	G2/M transition	inhibition of apoptosis	169
Survivin	inhibitor of apoptosis	unknown	170

Butterfield et al. (2006) examined redox proteomics and identified oxidatively modified proteins associated with MCI that refers to the transitional zone between normal cognitive function and early dementia or clinically probable AD [21]. The oxidatively modified proteins identified include Pin1, whose enzymatic activity reduced (approximately 90 %) in the brain due to MCI as compared to that in the control brain [21].

The oxidative inactivation of Pin1 in the hippocampus may be one of the initial events that triggers disease progression from MCI to AD, including tangle formation, oxidative damage, cell cycle alteration, and eventually neuronal death. In contrast, Pin1 activation might promote tumorigenesis and tumor malignancy. Since Pin1 oxidation might serve as a target for chemotherapy against cancer, identification of amino acid residues in Pin1 that are oxidatively modified is required.

Substrates

The PPIase Pin1 is specifically needed for normal mitotic progression, and a subset of mitosis-specific phosphoproteins have been identified as substrates for Pin1 [5].

Pin1 interacts with its substrates via recognition of the specific phosphorylated Ser/Thr-Pro bond and promotes conformational changes by facilitating the *cis/trans* isomerization of these peptide bonds. Pin1 substrates might represent target proteins, including mitosis-specific phosphoproteins, for Pro-directed protein kinases such as those belonging to the MAPK and CDK family [5]. We have created up-to-date tables listing Pin1 substrates to facilitate easy searching (Tables 1–3).

Among Pin1 substrates, RNA polymerase II is responsible for transcription of mRNA-encoding genes, and takes part in post-transcriptional events [54, 55]. The C-terminal domain of RNAP II is

Table 3. Substrates of Pin1 (the effects of Pin1 on their cellular levels are unknown).

	Consequence	Effect of Pin1	References
Topoisomerase II	G2/M transition	inhibition of CK2-dependent phosphorylation	107, 108
Nek6	G2/M transition	unknown	171
Myt1	G2/M transition	inhibition of mitotic function, not its catalytic activity	172
NIMA	G2/M transition	inhibition of mitotic function, not its catalytic activity	172
Cdc27	G2/M transition	unknown	172
Cdc25	G2/M transition	inhibition of mitotic function, not its catalytic activity	173
Plx1	G2/M transition	unknown	173
Wee	G2/M transition	inhibition of mitosis by inactivation of catalytic function	172, 174
KRMP1	G2/M transition	inhibition of mitotic function	175
NFAT	transcription factor	inhibition of NFAT activation	176
p54nrb	transcriptional control, RNA splicing	unknown	177
Bcl-2	suppressor of apoptosis	unknown	178
Sil	positive regulator of the sonic hedgehog pathway	spindle checkpoint arrest	179
Stat3	transcription factor	promotion of Stat3 transcription activity	180

essential for cell viability and gene transcription. The CTD can be extensively phosphorylated, and undergoes dynamic changes in phosphorylation during the transcription cycle. Pin1 influences the phosphorylation status of the CTD by inhibiting CTD phosphatase and stimulating CTD phosphorylation by cdc2/cyclinB. A hyper-hyperphosphorylated form of RNAP II accumulates in M-phase cells in a Pin1-dependent manner. Including the CTD of RNAP II, the elements of the transcription machinery are one of the key targets of Pin1 [54, 55]. Recently, several reports have indicated that Pin1 regulates the activity of transcriptional factors, namely, AP-1, p53, p73, and SRC3 [14, 27–32]. And in this context, we also discovered that Pin1 downregulates the transcription regulator, an intracellular form of Notch 1 called NIC [33]. Pin1's isomerase activities on these transcriptional regulators affect oncogenesis. Pin1 also regulates the post-transcriptional level of some cytokines associated with asthma that possess 3′ untranslated region AREs via interaction with AUF1, the nucleoprotein in the ARE-binding complex [22, 23]. Pin1 has been identified as the molecular partner of tau and amyloid precursor protein (APP), the key factors of Alzheimer's disease (AD) [9–11]. Thus, Pin1 is probably involved in the pathogenesis of human diseases, including cancer, asthma, and AD. We are interested in these molecules as the Pin1's substrates that are associated with the post-translational modification of Pin1 and human pathologies.

AP-1. The transcription complex AP-1 is a heterodimer comprising Jun and Fos family members whose expression and activity are strongly regulated by the MAPK family of Ser-Thr kinases. These kinases regulate AP-1 expression and activity by post-translational processing of the pre-existing or newly synthesized AP-1 proteins [56, 57].

Pin1 binds c-Jun that is phosphorylated on the Ser-63/73-Pro motif by activated JNK or oncogenic Ras. Wulf et al. (2001) indicated that Pin1 increased c-Jun transcriptional activity toward the cyclin D1 promoter that was activated via the AP-1 site [14].

With regard to the other AP-1 component c-Fos, ERK activation leads to increased expression of c-fos mRNA. In turn, Gutkind's cohort showed that ERK phosphorylated multiple residues within the carboxyl-terminal transactivation domain (TAD) of c-Fos and increased AP-1 transcriptional activity [27]. Monje et al. (2005) reported that Pin1 binds c-Fos via specific pSer/Thr-Pro bonds within the c-Fos TAD, and that this association results in an enhanced transcriptional response of c-Fos to polypeptide growth factors that stimulate ERK [27]. Specifically, AP-1-dependent transcription is promoted by the phosphorylation of c-Fos by ERK, and function of the phosphorylated c-Fos is directly regulated by Pin1 [27].

Pin1 might facilitate regulation of AP-1-dependent gene transcription upon phosphorylation by MAPKs via the isomerization of both phosphorylated c-Jun and c-Fos. These findings suggest that Pin1 exerts an oncogenic activity through AP-1 activation.

Steroid receptor coactivator 3. The steroid receptor coactivator 3 (SRC3/AIB1/ACTR/pCIP/RAC3/TRAM1) interacts with steroid receptors in a ligand-dependent manner to activate receptor-mediated transcription [58–65]. This includes various physiological processes, including reproductive function, cytokine signaling, cell proliferation, and somatic growth [65–67]. Since SRC3 is overexpressed in breast and ovarian cancers, it is an oncogene candidate [68, 69]. SRC3-deficient mice display delayed mammary gland development, growth retardation, and impaired vasoprotection [66, 67, 70–72]. Their phenotypes resemble those of *Pin1*-deficient mice [7, 8] and *cyclin D1*-deficient mice [73, 74]. Particularly,

Pin1 deficiency induces defects in germ cell maturation [12, 13]. Hence, Pin1 might play a role in the function of sex steroid, estrogen, and progesterone receptors by influencing the activities of their coactivators. Pin1 selectively interacts with phosphorylated SRC3 and synergistically activates nuclear hormone-regulated transcription [28]. This effect depends on Pin1 isomerase activity. The nuclear hormone-dependent transcriptional activities might depend on the Pin1 dephosphorylated form. Interestingly, Pin1 overexpression enhanced SRC3 cellular turnover, and Pin1 depletion induced SRC3 stabilization [28].

Pin1 functions as a transcriptional coactivator for nuclear receptors by modulating SRC3 coactivator protein-protein complex formation and also by promoting turnover of the activated SRC3 oncoprotein candidate [28]. If Pin1 action on SRC 3 depended on Pin1 phosphorylation, the phosphorylated Pin1 would inhibit SRC 3 transcriptional activity by blocking Pin1 isomerization activity and enhance SRC 3 degradation by promoting self-turnover.

The steroid hormone precursor cholesterol is rich in brain [75–77]. Cholesterol metabolites are known to be key indicators of AD progression, and may induce the pathogenesis of sporadic AD via SRC3 activation.

p73. p73 belongs to the tumor suppressor p53 gene family. The p53 family proteins, namely, p53, p63, and p73, share similar architecture, including an N-terminal transactivating domain and a central DNA-binding domain; they can bind certain p53-responsive elements involved in cell cycle arrest and apoptosis [78–80]. The full-length p73 and the N-terminal-truncated ΔNp73 are generated from the *p73* gene by activating two distinct promoter domains. These two *p73* forms have opposing effects: the full-length p73 exhibits pro-apoptotic effects, while the ΔNp73 protein possesses an evident antiapoptotic function. Interestingly, the ΔNp73 protein shows selective degradation in response to DNA damage, while the full-length p73 level increases due to DNA damage [81, 82].

Several mechanisms such as phosphorylation by c-Abl, p38MAPK, and c-Jun; acetylation by CBP/p300; and sumoylation have been shown to stabilize p73 [83]. A subset of DNA-damaging stimuli trigger p73 phosphorylation by c-Abl, thereby increasing proapoptotc activity [84–88]. Mantovani et al. (2004) reported that Pin1 is necessary for the acetylation of phosphorylated p73 by p300 and for it to function as a transcription factor [29]. Among the p73 isoforms, Pin1 interacts with p73α and p73β. These two isoforms possess the C-terminal polyproline domain containing the Pin1 consensus sites at residues Ser-412, Thr-442, and Thr-448, which may be responsible for regulation by Pin1. Phosphorylation of these residues may be required for p73 stabilization by binding and isomerization with Pin1 [29].

In order to block tumorigenesis, the dephosphorylated Pin1 probably facilitates p73 activation.

p53. The *p53* tumor suppressor gene plays a complex and critical role in maintaining genome integrity [89–91]. Loss of p53 function increases susceptibility to malignant transformation, and mutations in the *p53* tumor suppressor gene are the most common genetic abnormalities in oncology: they are found in $> 50\,\%$ of all human cancers [92]. The level of active p53 in the cells is rapidly elevated via acetylation and phosphorylation in response to DNA damage caused by various types of stress such as UV irradiation, DNA-damaging chemicals, hypoxia, and activated oncogenes [93]. Transcriptional activity of the *p53* gene is also physiologically elevated during organogenesis [94]. Subsequently, the high levels of activated *p53* drive transcription of a variety of genes that mediate the protein's biological functions in its decision to arrest cells for facilitating DNA repair or to destroy the cells by apoptosis [95, 96]. Even though some p53-deficient mice do succeed in being born, they rapidly succumb to the tumors, particularly those belonging to the thymic lymphoid lineage [97–99].

Further, studies have also reported on the high-frequency embryonic lethality due to female-specific developmental abnormalities, namely, neural tube-closure failure leading to exencephaly and subsequent anencephaly [100, 101].

Recently, Pin1 was shown to be involved in controlling p53 accumulation and its activity in cells exposed to DNA-damaging conditions [30–32]. These reports indicated that p53 was one of the substrates for Pin1, and that the effects on p53 were the result of isomerization by Pin1. The interaction between p53 and Pin1 depends strictly on p53 phosphorylation, and it requires the p53 residues Ser-33, Thr-81, and Ser-315. On binding, Pin1 induces a conformational change in p53, enhancing its transactivation activity. Pin1-mediated p53 activation requires the WW domain and Pin1 isomerase activity. Stabilization of p53 is impaired in UV-treated *Pin1*$^{-/-}$ cells due to its inability to efficiently dissociate from Mdm2, a regulatory partner of p53. Pin1 might influence the interaction of Mdm2 with p53, resulting in the stabilization and transactivation of p53 [30–32]. Pro-82 of p53 was identified to be essential for its interaction with the checkpoint kinase 2 (Chk2) and consequent phosphorylation of p53 on Ser-20, following DNA damage. These physical and functional

interactions are regulated by Pin1 through *cis/trans* isomerization of Pro-82. A study by Berger et al. (2005) unraveled the pathway by which Pin1 activates p53 in response to DNA damage and explained how Pin1 protects p53 from Mdm2. This proposed a role for Pin1-dependent induction of p53 conformational change as a mechanism responsible for the enhanced interaction between p53 and Chk2 following DNA damage [102].

Interestingly, overexpression of the oncogenes that activate p53 promote the interaction of p53 with recombinant Pin1. The oncogene products are required for p53-dependent caspase activation during the apoptotic response as a result of certain DNA-damaging stresses [103]. Hence, caspase activation during the apoptotic response might be induced by interaction between Pin1 and p53 in the event of oncogene overexpression. In fact, we observed that the primary MEFs of both WT and *Pin1*$^{-/-}$ did not undergo apoptosis in response to DNA-damaging stimuli, including UV radiation and chemotherapeutic agents. Deficiency of p53 in the primary MEFs enhanced apoptotic cell death in response to the DNA-damaging agents [Shimazaki et al., unpublished data]. Therefore, Pin1 possibly stabilizes p53 and p73 and plays a critical role in the integration of the checkpoint induced by DNA damage [29–32].

The protein kinase CK2 participates in checkpoint control and p53 phosphorylation involved in DNA damage responses [104–106]. Messenger et al. indicated that CK2 also serves as a substrate for Pin1, and that Pin1 influences CK2 stabilization (Table 1) [107]. Olsten et al. investigated the effects of Pin1 on the phosphorylation of recombinant p53 by CK2, following UV stimulation. With regard to the human osteosarcoma cell line U2-OS, UV radiation failed to enhance CK2 phosphorylation, and Pin1 did not exhibit any effect on the ability of CK2 to phosphorylate p53; however, it is important that the interactions between p53 and Pin1 be phosphorylation-directed. Hence, Pin1 may influence the CK2-catalyzed phosphorylation of p53 when p53 is phosphorylated in a manner that promotes Pin1 interaction [108]. Messenger et al. reported that Pin1 inhibited the phosphorylation activity of CK2 toward topoisomerase IIα, the phosphorylation of which is essential for the G2/M transition [107]. Hence, topoisomerase IIα might undergo phosphorylation to facilitate progression of G2/M transition by Pin1 deficiency. The topoisomerase II inhibitor etoposide influences *Pin1*-deficient MEFs to a lesser degree than WT MEFs in response to G1 arrest [K. Shimazaki et al., unpublished data]. The interaction of Pin1 with p53 might depend on the topoisomerase II-CK2 system with regard to cellular growth or checkpoint control.

Further, since we have been interested in the physiological link between Pin1 and p53, we recently generated *Pin1*$^{-/-}$*p53*$^{-/-}$ mice [33]. Pin1 knockout in mice completely prevented malignant tumor development induced by p53 knockout. Although these mice could develop, the proportion of males (80%) was significantly higher than that of females (20%). The *Pin1*$^{-/-}$*p53*$^{-/-}$ mice [median survival time ($t_{1/2}$) 141 days] died earlier than the *p53*$^{-/-}$ mice ($t_{1/2}$ 195 days), and the immature thymocytes, i.e., the CD4–CD8– (double-negative) cells, displayed thymic hyperplasia. The thymocytes obtained from the *Pin1*$^{-/-}$*p53*$^{-/-}$ mice possessed a substantial amount of NIC1, and both Pin1 and p53 might regulate Notch1 cleavage by presenilin1. It is clear that within the thymus, p53 is essential for elimination of DNA-damaged cells via apoptosis, and it appears that the loss of apoptotic pathways is critical to tumor formation within this cell type. The *Pin1*$^{-/-}$*p53*$^{-/-}$ mice phenotypes indicated that the physiological network between Pin1 and p53 might facilitate the elimination of DNA-damaged cells, partially by controlling the NIC level [33]. In order to block tumorigenesis, isomerase activity of dephosphorylated Pin1 probably facilitates p53 activation.

The roles of Pin1 played in cancer appear controversial. In general, Pin1 protects against cancer. Yeh et al. reported that embryonic fibroblasts derived from Pin1 knockout mice in C57BL6 genetic background exhibit increased genomic instability and are more prone to Ras-dependent transformation after being immortalized by p53 inactivation, leading to a hypothesis that Pin1 is a conditional tumor suppressor [24]. We found that ablation of both *Pin1* and *p53* accelerates thymic hyperplasia, whereas the thymocytes in the *pin1*$^{-/-}$*p53*$^{-/-}$ do not infiltrate other organs. These results suggest that the role of Pin1 in cancer may vary depending on organ, tissue, age, and genetic background. We speculate that post-translational modifications and levels of functional homologues of Pin1 affect the function of Pin1.

NIC. Notch1 is a transmembrane receptor that is crucial for T-cell development [109, 110]. The intracellular domain of Notch1 (NIC), which is the activated form, translocates from the membrane to the nucleus and induces transcription of downstream genes such as HES1 and cyclin D1 [111–113]. p53 downregulates presenilin1, which is a component of the γ-secretase complex that activates Notch1 by cleaving it, and thereby negatively regulates Notch1 activation [114–116]. Notch1 is expressed at varying levels in developing and mature T cells, including

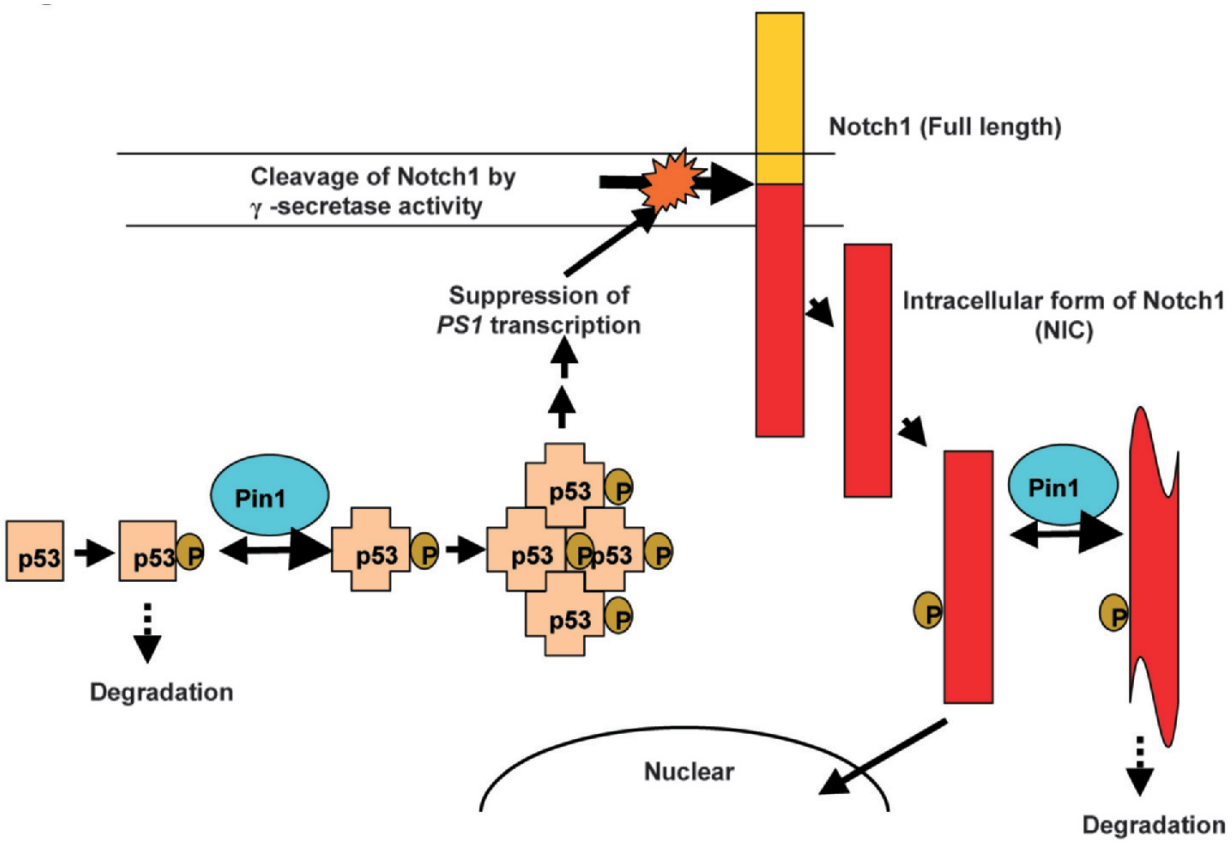

Figure 2. The intracellular level of NIC, the active form of Notch 1, is regulated by p53 and Pin1. Transcriptional activities of p53 repress γ-secretase activity through inhibition of presenilin-1 transcripts, and suppress cleavage of Notch 1. Pin1 may bind NIC and induce its degradation [33].

hematopoietic progenitors of peripheral lymphocytes [117]. Both p53 and Notch1 are linked to lymphoid malignancies [118]. Pears et al. demonstrated that mice transplanted with bone marrow cells expressing NIC resulted in T-cell leukemia and presented evidence that Notch1 plays a role in lymphoid oncogenesis in mice [119]. Overactivation of Notch1 might lead to tumorigenesis via loss of functional p53. In order to identify the physiological relationship between Pin1 and p53, we generated *Pin-1*$^{-/-}$*p53*$^{-/-}$ mice. NIC levels significantly increased in the thymocytes obtained from 12-week-old *Pin1*$^{-/-}$*p53*$^{-/-}$ mice, and the upregulated NIC may cause hyperplasia in the thymocytes [33]. The presenilin1 level concomitantly increased with the NIC level in the thymocytes of the *Pin1*$^{-/-}$*p53*$^{-/-}$ mice. We also discovered that Pin1 could bind with NIC and induce NIC degradation, and that both Pin1 and p53 were key regulators of thymocyte proliferation via Notch1 activation [33].

p53 deficiency increases the expression of presenilin1 transcripts [120]. Presenilin-1 is a component of the γ-secretase complex that acts as a cleavage protease, releasing NIC from Notch-1 and amyloid-β (Aβ) from the APP [121, 122]. Actually, p53-deficient mice exhibited accumulation of NIC in the thymus and Aβ in the brain [123, 124]. We could not detect the increase in NIC in Pin1-deficient mice because cleavage of NIC from Notch1 was not affected by Pin1 deficiency. Our examinations revealed that NIC was upregulated in the thymocytes in the *p53*$^{-/-}$ and *Pin1*$^{-/-}$*p53*$^{-/-}$ mice, and that the *Pin1*$^{-/-}$*p53*$^{-/-}$ mice exhibited NIC levels higher than those of *p53*$^{-/-}$ mice. p53 deficiency induced presenilin1 expression and thereby increased NIC production. Therefore, we speculated that Pin1 deficiency might lead to NIC stabilization, which is facilitated by the cleaving activity of presenilin1 [33].

NIC might be one of the target substrates for Pin1 because NIC associates with and is downregulated by Pin1, and this downregulation is inhibited by the addition of the proteasome inhibitor MG132 [33]. It has been shown that NIC is degraded via the ubiquitin-proteasome pathway [125]. Pin1 might isomerize phosphorylated NIC and render it susceptible to degradation via the ubiquitin-proteasome pathway (Fig. 2). The Pin1-p53-NIC system might be essential for preventing the pathogenesis and progression of AD and cancer.

Overactivation of Notch1 might play a role in tumorigenesis resulting from the loss of functional p53 [126]. NIC is abundant in human T-cell acute lymphoblastic leukemia (T-ALL), and high levels of NIC are associated with the attendant pathogenesis [127]. Vilimas et al. reported that NF-κB is one of the major mediators of Notch-1-induced transformation, and that the NF-κB pathway is highly active in established human T-ALL [128]. NF-κB has been established as a target for Pin1. NF-κB function is regulated in a manner by which Pin1 binds to the pThr-254/Pro motif in p65/RelA and inhibits p65/RelA binding to IκBα, resulting in an increase in nuclear accumulation and protein stability of p65/RelA and enhanced NF-κB activity [129]. Phosphorylation of p65/RelA at Ser-536 contributes to the inhibition of p53 transcriptional activity, thereby mediating the association of p53 and p65/RelA [130, 131]. Together with these findings, it can be deduced that Notch-1 activation is controlled by the Pin1/p53 network. With regard to *Pin1*$^{-/-}$*p53*$^{-/-}$ mice, activated NF-κB was not regulated by Pin1 under p53-deficient conditions, and leukemia development might have partly resulted from NF-κB activation based on NIC accumulation.

ARE-binding proteins. Shen et al. reported that Pin1 acts as an essential component of the ribonucleoprotein complex responsible for GM-CSF mRNA stabilization, cytokine secretion, and survival of activated eosinophils [22].

The 3′ untranslated region AREs are found in many unstable cytokine and proto-oncogene mRNAs, including GM-CSF mRNA [132, 133]. It was suggested that AUF1, an ARE-binding protein with four isoforms of 37, 40, 42, and 44 kD (p37, p40, p42, and p44, respectively), both stabilizes and destabilizes ARE-containing mRNA [132–138]. Pin1 regulates the association of AUF-1 and hnRNP C with GM-CSF mRNA in response to external stimuli, including hyaluronic acid.

Hyaluronic acid enhanced cell viability of the purified peripheral blood eosinophils [139]. Specifically, hyaluronic acid causes stabilization of GM-CSF mRNA, resulting in cytokine secretion via the Erk-mediated pathway and culminating in the prevention of caspase3-mediated apoptosis. Treatment with the specific and irreversible Pin1 inhibitor juglone suppressed this enhancement of cell viability and induced caspase3-mediated apoptosis via downregulation of GM-CSF mRNA. These results demonstrated that Pin1 is required for eosinophil survival by GM-CSF secretion resulting from the stabilized GM-CSF mRNA [22].

With regard to the resting eosinophils, GM-CSF mRNA is mainly present in complex with the AUF1-Pin1 ribonucleoproteins; however, after cell activation, it binds to hnRNP C. Cytoplasmic extracts obtained from the resting eosinophils included the complex composed with Pin1 and the ARE-binding proteins AUF1 and HuR. This complex forms independent of the mRNA. Hyaluronic acid stimulation has no effect on the complex and cytoplasmic Pin1 levels; however, the GM-CSF mRNA binds hnRNP, which does not complex with Pin1. Inhibition of Pin1 decreases the association of GM-CSF mRNA with hnRNP C and increases association with AUF1. This switching of the GM-CSF mRNA-associating protein might depend on Pin1 enzymatic activity. In fact, Shen et al. indicated that hyaluronic acid stimulation reproducibly dephosphorylates Pin1 in eosinophils, and Pin1 activity increases due to hyaluronic acid. Further, juglone reduces both Pin1 and AUF1, suggesting that the combined post-translational modification of Pin1 and AUF1 induced by hyaluronic acid resultes in AUF1 isomerization with loss of binding to GM-CSF mRNA [22].

Furthermore, the Pin1-AUF1 complex present in resting cells interacts with the exosome-associated protein PM-scl75. Among the AUF1 isoforms, p37 associates with PM-scl75 independent of mRNA. When the ribonucleoprotein contains AUF1, rapid GM-CSF mRNA decay is ensured by targeting of p37-PM scl 75 to the exosome. Pin1 stabilizes the AUF1 proteins and Pin1 itself, and AUF1 is degraded in the proteasome. In the resting eosinophils, the moderately serine-phosphorylated p40 and p45 AUF1 physically associate with serine-phosphorylated Pin1. Association with hnRNP C protein caused by external stimuli increases GM-CSF mRNA stability. *In vitro* and *in vivo* activation considerably reduces p37-PM-Scl75 interaction and triggers Pin1 dephosphorylation and activation by isomerization of hyperphosphorylated p45 and p40 AUF isoforms [22].

AUF1 controls the decay of many ARE mRNAs other than GM-CSF. Hence, Pin1 might be important in cytokine production via other activated cells as well. Esnault et al. reported that the activated T lymphocytes also display Pin1-dependent GM-CSF mRNA stabilization [23]. Malter's cohort reports indicated that Pin1 can regulate allergic inflammation in asthma via cytokine secretion from the eosinophils [22, 23].

Tau and APP. AD is characterized by the presence of massive senile plaques (SPs) and NFTs, the two hallmark pathological features of AD, in the brain [140]. Both lesions result from the abnormal deposition of proteins that are normally distributed throughout the brain. SPs are extracellular deposits of the fibrillar amyloid β-peptide (Aβ), which is a cleavage product of the membrane-associated APP. NFTs are intracellular aggregated bundles of the microtubule-

associated protein tau. Although the mechanism for AD pathogenesis remains elusive, accumulating data have indicated that tau and APP/Aβ are among the most important factors in the events leading to AD onset and progression. Notably, hyperphosphorylation of the phosphoprotein tau is linked to its aggregation into filamentous structures, and the phosphorylation of APP at its cytoplasmic Thr-668 is linked to Aβ generation [141, 142]. These observations suggest that the phosphorylation events are critical for understanding AD pathogenesis. Pin1 has received increasing attention as a new regulator of the above-mentioned phosphorylated proteins. Pin1 is a partner molecule that performs phosphorylation-dependent binding on tau and APP. Among the known binding ligands for tau or APP, Pin1 is uniquely situated such that it can bind both tau and APP.

The involvement of Pin1 in AD was first described in association with tau. Pin1 is predominantly a nuclear and mitotic regulator [4, 143, 144]. It binds and regulates the function of a subset of mitotic phosphoproteins, most of which are recognized by the monoclonal antibody MPM-2 [143]. MPM-2 also recognizes tau, which is phosphorylated at a number of Ser/Thr-Pro sites during mitosis. These observations prompted Lu and colleagues [145] to examine whether the phosphorylated tau interacts with Pin1, and they discovered that tau indeed binds to the WW domain of Pin1. The longest isoform of human tau possesses 17 Ser/Thr-Pro sites. Of these, Thr-231 is required for interacting with Pin1 when the site is phosphorylated. Further, Thr-231 plays a critical regulatory role because its phosphorylation greatly diminishes the ability of tau to bind and stabilize microtubules in the cell [146–148]. The Thr-231 residue is hyperphosphorylated in AD, and the authors discovered that Pin1 binds hyperphosphorylated tau obtained from the brain of AD patients. Surprisingly, the functional effect of the interaction between Pin1 and tau phosphorylated at Thr-231 was restoration of the biological activity of phosphorylated tau. The tau functions regarding binding and stabilization of microtubules are regulated by its phosphorylation; tau binds the microtubules on dephosphorylation and does not bind the microtubules on phosphorylation. In the presence of Pin1, the impaired activity of hyperphosphorylated tau regarding promotion of the microtubule assembly is restored. The conformation of the Ser/Thr-Pro bonds is crucial for the action of the phosphatases. PP2A is able to dephosphorylate Ser/Thr-Pro only when it is in the *trans* conformation and not in the *cis* conformation [34]. Thus, the restoring effect appears to depend on the prolyl isomerase activity of Pin1. Indeed, *in vitro* experiments demonstrated that Pin1 binds phosphorylated Thr-231 and promotes dephosphorylation of tau via PP2A [34, 149]. Finally, Lu and colleagues [145] demonstrated that the levels of soluble Pin1 are decreased in the brains of AD patients. This decrease might impair dephosphorylation of tau and restoration of its biological activity and have harmful effects on the balanced phosphorylation state of tau, leading to tau hyperphosphorylation and abnormal tau pathology. This assumption is supported by a study that used Pin1 knockout mice that we generated [7]. Pin1 knockout in mice induces age-dependent abnormalities, including hyperphosphorylation of endogenous tau, tau filament formation, and neuronal degeneration [8], similar to that observed in the brains of patients with AD and tauropathy [140]. The deficits observed in the Pin1-null mice suggest that Pin1 might protect against tau phosphorylation and thus inhibit the formation of NFTs.

Pin1 was further implicated in AD with its association to APP. APP undergoes proteolytic cleavage during and after its transport via the secretory pathway to the cell surface. In the major secretory pathway, some APP molecules are cleaved within the Aβ sequence by α-secretase, releasing the large soluble ectodomain fragment. With regard to the alternative amyloidogenic pathway, processing of the APP fraction by β-secretase secretes a slightly shorter ectodomain fragment. The membrane-bound proteolytic fragments generated by the α-secretase C83 and β-secretase C99 are further converted by the γ-secretase cleavage within the transmembrane domain to form p3 (a truncated form of Aβ) and Aβ, respectively. Both these peptides terminate at either residue 40 or 42 of the Aβ sequence. The Thr-668 residue (human APP695 isoform numbering) preceding the Pro residue in the cytoplasmic APP domain is a known *in vivo* phosphorylation site, and its phosphorylation at Thr-668 is believed to regulate APP metabolism; this facilitates Aβ production [141, 142]. The Thr-668-Pro motif might serve as a potential binding site for Pin1, and we presume that Pin1 binds APP and influences its metabolism. In order to verify this hypothesis, we investigated the *in vitro* and *in vivo* effects of Pin1 on APP metabolism [10]. We discovered that Pin1 binds the phosphorylated Thr-668-Pro of APP in the HEK293 cells after it is cleaved at the β-secretase site, and this promotes its turnover, resulting in increased Aβ production. In the brain of the Pin1-deficient mice, Aβ production was decreased as compared to that in their control littermates, indicating that Pin1 facilitates an increase in Aβ production. Subsequent to our study, Pastorino et al. (2006) also studied the interaction of Pin1 and phosphorylated Thr-668 of APP [11]. In contrast to our study, the authors showed that Pin1 binds to full-length APP at

phosphorylated Thr-668, and the effect of Pin1 on Aβ generation was not stimulatory but inhibitory. Further studies are required to resolve the discrepancy between these two studies. Pin1 is likely to interact with APP via recognition of the phosphorylated Thr-668 in its cytoplasmic tail and to influence APP metabolism.

Conclusions

The functions of Pin1 are complicated in that Pin1 generally protects cancer and neurodegenerative diseases while it occasionally accelerates their disease progression. We believe that the profile of phosphorylated proteins in cells determines the direction of Pin1 functions. Ageing is one of the key conditions for determining the profile of phosphorylated proteins. The condition of cancer cells is related to ageing because the risk of cancer increases with age. Pin1 is strongly related to the time-dependent functions of cells. It maintains appropriate timing for signal transduction in cells as noted from the functions of p53 [30, 31]. Hence, if Pin1 does not work well in the cells due to ageing or cancer, it may worsen cell conditions.

We do not consider Pin1 to be the only enzyme that controls the functions of phosphorylated proteins. We attempted to find molecules containing sequences similar to that of Pin1 and identified Par14 as a molecule containing sequences homologous to those of the Pin1 PPIase domain; however, its substrate specificity differed from that of Pin1 [150, 151]. We also found that PPIase is upregulated in the Pin1-knockout mouse brain as compared to the WT brain; here, Pin1 activity was found to be high, and only FKBP6 was upregulated [152]. However, the functional homologue of Pin1 remains unclear. We believe that homologues of the WW domain in Pin1 exist and that they might function at the pSer-Pro sites of proteins similar to Pin1. As mentioned in this review, it is also important to analyze the changes in the activity and localization of Pin1 after post-translational modifications. Recently, prevention of oxidation of proteins such as lipoproteins has been one of the strategies used in drug discovery because modification of protein oxidation might be a candidate for the malignant factor in various diseases related to the metabolic syndrome. With regard to therapeutic targets for AD, prevention of Pin1 oxidation appears to be an attractive strategy.

The main function of Pin1 is to alter the function of the target phosphorylated proteins by changing their conformation. However, the details of the change in the structure of the target molecules remain a mystery. Similar to the breakthrough obtained from previous studies for the functions of Pin1 using Pin1-knockout mice [7], further innovative studies are required aimed at the structure of Pin1 and the target phosphorylated proteins.

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