Pinning down proline-directed phosphorylation signaling

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The reversible phosphorylation of proteins on serine or threonine residues preceding proline (Ser/Thr-Pro) is a major cellular signaling mechanism. Although it is proposed that phosphorylation regulates the function of proteins by inducing a conformational change, there are few clues about the actual conformational changes and their importance. Recent identification of the novel prolyl isomerase Pin1 that specifically isomerizes only the phosphorylated Ser/Thr-Pro bonds in certain proteins led us to propose a new signaling mechanism, whereby prolyl isomerization catalytically induces conformational changes in proteins following phosphorylation to regulate protein function. Emerging data indicate that such conformational changes have profound effects on catalytic activity, dephosphorylation, protein–protein interactions, subcellular location and/or turnover. Furthermore, this post-phosphorylation mechanism might play an important role in cell growth control and diseases such as cancer and Alzheimer's.

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Serine or threonine residues preceding proline (Ser/Thr-Pro) are the major regulatory phosphorylation motifs that function in diverse cellular processes. Enzymes responsible for such phosphorylation belong to a large family of so-called Pro-directed protein kinases, including cyclin-dependent protein kinases (CDKs), mitogen-activated protein kinases (MAPKs), Jun N-terminal protein kinases (JNKs) and glycogen synthase kinase-3 (GSK-3). Enzymes that reverse the phosphorylation step are Ser/Thr phosphatases, including phosphatase 2A (PP2A), RNA polymerase II C-terminal domain (RNA Pol II CTD) phosphatase (FCP1) and calcineurin. These kinases and phosphatases play a crucial role in diverse cellular processes such as the cell cycle, transcription and various signal-transduction pathways, as well as in human diseases such as cancer and Alzheimer's disease (AD) [1–5]. Ser/Thr phosphorylation has long been believed to regulate the function of proteins by inducing conformational changes. However, until recently, little was known about how the phosphorylation actually regulates protein function. It has become evident that, like phosphotyrosine (pTyr), phosphorylated Ser/Thr (pSer/Thr) residues can also function as binding motifs for recruiting proteins into signaling networks or placing enzymes close to their substrates [6–8]. Importantly, the conformational change following phosphorylation seems to play a pivotal role in regulating the function of a subset of pSer/Thr-Pro-containing proteins [9,10].

A key breakthrough in appreciating the post-phosphorylation regulatory mechanism was the characterization of the peptidyl–prolyl *cis*/*trans* isomerase (PPIase) Pin1 [11–13]. The human *Pin1* gene was originally identified in 1996 in a yeast genetic screen for proteins involved in mitotic regulation and shown to be the first PPIase that is crucial for cell growth [11]. One year later, Pin1 was shown to target to a defined subset of pSer/Thr-Pro-containing proteins and isomerize only the pSer/Thr-Pro bonds, a change that cannot be effectively catalyzed by other known PPIases [12,13]. These studies led us to hypothesize that phosphorylation-specific prolyl isomerization is a signaling mechanism that might catalytically change the conformation of phosphorylated substrates and thereby regulate their function [9,12]. This hypothesis has now been supported by recent biochemical, biological and structural studies, which also suggest that phosphorylation-specific prolyl isomerization is an important regulatory mechanism under physiological and pathological conditions, although it remains to be determined why the *Pin1* gene is essential in some systems but not in others. Here, we review our current understanding of the role of this mechanism in phosphorylation signaling.

A unique conformational switch in the pSer/Thr-Pro bonds

Proline residues exist in two completely distinct *cis* and *trans* conformations and can provide a 'backbone switch' in proteins controlled by peptidyl-prolyl isomerization (Box 1). This intrinsically rather slow conversion is catalyzed by PPIases, which play an important role in protein folding or refolding [10,14]. There are two well-known families of PPIases – cyclophilins and FK506 binding proteins (FKBPs). However, they can be deleted entirely in yeast, and the biological importance of their enzymatic activity remains to be determined [10,14,15]. A third family of PPIases has been identified recently and includes parvulin and Pin1 [11,16]. Although this family of PPIases is sometimes referred to as the parvulins, bacterial and mouse parvulins have no activity towards pSer/Thr-Pro bonds, and their function remains unknown [16,17]. By contrast, Pin1 and its homologs are extremely specific for pSer/Thr-Pro bonds and have well-defined biochemical functions. For the purposes of this review, we divide this new family of PPIases into Pin1-type and parvulin-type subfamilies and here focus on the former enzymes.

Isomerization of pSer/Thr-Pro motifs is particularly important because they are the only phosphorylation motifs known for almost all Pro-directed protein kinases [1–3]. MAPKs and CDK2 appear to preferentially phosphorylate the *trans* isomer [18,19]. However, Ser/Thr phosphorylation

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Box 1. Peptidyl-prolyl *cis*/*trans* isomerization and PPIases

Proline residues have the unique property of existing in two isomers (Box Fig. I) and can provide a potential backbone switch in the polypeptide chain controlled by *cis–trans* isomerization about the peptidyl–prolyl bond (a). Although these two isomers can be converted into each other spontaneously in peptides, it is an intrinsically rather slow process and can be rate limiting in protein folding or refolding. However, it can be catalyzed by ubiquitous enzymes called peptidyl–prolyl *cis*/*trans* isomerases (PPIases). There are two extensively characterized families of conventional PPIases – cyclophilins and FKBPs, and a recently identified third family of PPIases, which can be divided into two subfamilies based on their substrate specificity: parvulin-type and Pin1-type PPIases. Cyclophilins and FKBPs play an important role in regulating a wide range of cellular processes; their best-known function is in the immune system, where they act as cellular receptors for clinically important immunosuppressive drugs and are important for immune responses. When the cyclophilins and FKBPs bind to the immunosuppressive drugs cyclosporin A and FK506, respectively, there are two common outcomes: inhibition of the prolyl isomerase activity and inhibition of the common target calcineurin. Interestingly, it is the inhibition of the calcineurin phosphatase activity that prevents lymphocytes from responding to antigen-induced mitogenic signals, thus resulting in immunosuppression. Surprisingly, the inhibition of the prolyl isomerase activity apparently is unrelated to the immunosuppressive property of the drug–isomerase complexes. In addition, all the known members of the cyclophilin and FKBP family genes can be entirely disrupted without any significant phenotype. In addition, little is known about the substrates and biological function of parvulin-type PPIases. Therefore, evidence for the biological importance of the enzymatic activity in these PPIases remains elusive.

By contrast, Pin1 and Pin1-type PPIases have a well-defined function in phosphorylation signaling by specifically isomerizing pSer/Thr-Pro bonds (b), an activity that cannot be effectively catalyzed by cyclophilin, FKBP or parvulin-type PPIases.

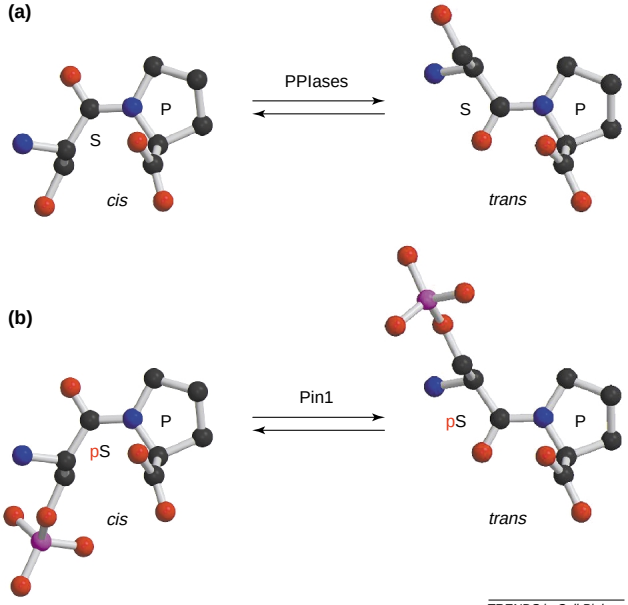

Fig. I.

significantly restrains the spontaneous isomerization of pSer/Thr-Pro bonds, which contrasts with Tyr phosphorylation, which has little effect [12,20]. Furthermore, phosphorylation also renders the peptide bond resistant to the catalytic action of cyclophilin-18, FKBP-12 or mouse parvulin [12,17]. These striking differences might be related to the fact that the side chain of Tyr is longer than that of Ser or Thr, which could relieve the effects of the phosphate on the backbone rotation. These results point to a need for different PPIases for isomerizing pSer/Thr-Pro bonds.

Catalysis of the conformational switch by Pin1 and Pin1-type PPIases

Pin1-type PPIases are enzymes that specifically isomerize pSer/Thr-Pro bonds [12]. Pin1 homologs are highly conserved in eukaryotes [9,22–24]. Interestingly, the budding yeast homolog was isolated a long time ago but was of unknown activity [25,26]. With the exception of plant enzymes, most other Pin1-type PPIases also contain a WW domain, a protein-interacting module present in many different proteins [8] (Fig. 1a). Various studies indicate that the WW domain targets the enzyme to its substrates, where the PPIase domain is both sufficient and necessary to induce the conformational changes and to carry out the essential function of the enzyme.

Structural basis for enzymatic specificity

The striking substrate specificity of Pin1-type PPIases towards pSer/Thr-Pro bonds results from the unique organization of their active sites [12,13,27]. In the Pin1 crystal structure, the side chains of conserved Arg68 and Arg69 residues form a basic patch at the entrance to the Pro-binding pocket and sequester a sulfate ion, an analog of phosphate (Fig. 1a) [13]. Mutation of these two residues completely abolishes the phosphorylation-specificity but has little effect on the basic enzymatic activity [12,27]. Importantly, this basic patch is present in the catalytic active site of all Pin1-type PPIases but not any other known PPIases [9,22–24]. Therefore, this basic patch is the signature motif for Pin1-type PPIases and is responsible for their phosphorylation specificity (Fig. 1b).

Substrate recognition

Unlike many other WW domains [8], the WW domain in Pin1 binds to specific pSer/Thr-Pro motifs in a defined subset of phosphoproteins [28] (Table 1). Under normal expression conditions, both the WW domain and PPIase domain are required to rescue the yeast *pin1* mutant, but, under overexpression conditions, only the PPIase domain, but not the WW domain, can carry out the Pin1 essential function [27,28]. These results indicate that the WW domain functions to target Pin1 to its substrates. This is supported by structural analyses of Pin1 or its WW domain complexed with peptides [29,30]. The pSer/Thr-Pro peptides all bind to the WW domain in the *trans* conformation, with multiple sequence-specific interactions contributing to the binding specificity (Fig. 2a,b). However, these

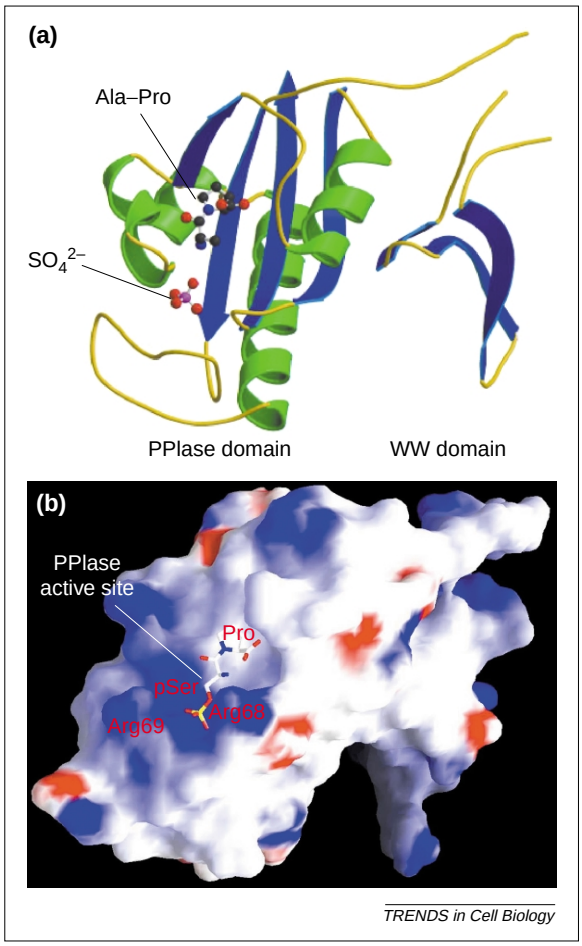

Fig. 1. Pin1 structure and its specificity towards pSer/Thr-Pro bonds. (a) Ribbon representation of the X-ray structure of human Pin1 in a complex with an Ala–Pro dipeptide. The N-terminal WW domain is connected to the C-terminal peptidyl–prolyl *cis*/*trans* isomerase (PPIase) domain by a disordered linker. A sulfate ion is sequestered by a conserved basic cluster consisting of Arg68 and Arg69 in close proximity to the β methyl group of the Ala residue in the bound Ala–Pro dipeptide. (b) A model for a pSer-Pro dipeptide bound to the active site of Pin1 in a molecular surface representation. The pSer has been modeled on a co-crystallized Ala ligand and sulfate ion. The acidic and basic residues are colored red and blue, respectively. The basic patch consisting of Arg68 and Arg69 in the active site is the signature motif for the Pin1-type PPIases and is responsible for the phosphorylation specificity.

structures do not yet clearly explain the high specificity of Pin1 substrate recognition (Table 1) [28,31–34]. Furthermore, it remains to be determined how the WW domain and the PPIase domain of Pin1 bind to and isomerize, respectively, the substrates, although there is evidence that these domains might act on the same pSer-Pro motifs. For example, the optimal binding peptide for the WW domain is the best available substrate for the PPIase activity of Pin1 [12,28]. WW domain binding sites often are crucial regulatory phosphorylation sites, and mutating them abolishes the effects of Pin1 on its substrates (Fig. 3) [27,28,31–33,35]. These results imply that the WW domain has to dissociate from, and then the catalytic domain isomerizes, the same pSer/Thr-Pro, or that the catalytic domain acts on another pSer/Thr-Pro in the same protein molecule.

Pin1 biological functions

Human Pin1 was originally identified by its ability to interact with the *Aspergillus* mitotic kinase NIMA and suppress the ability of NIMA to induce mitotic catastrophe [11]. This physical and functional interaction has been confirmed in *Aspergillus* ([36]; A.R. Means, pers. commun.). Various studies have supported a role for Pin1 in mitotic regulation and also suggested important roles for Pin1 in many other cellular processes under normal and pathological conditions.

Mitotic regulation

Inhibition of Pin1 through various approaches, such as mutations, deletions or expression of antisense or dominant-negative constructs, induces mitotic arrest and apoptosis in budding yeast and tumor cell lines [11,37–40]. Depletion of Pin1 also affects the DNA replication mitotic checkpoint and the G2–M transition in *Xenopus* extracts [41,42]. Conversely, overexpression of Pin1 prevents entry into mitosis in HeLa cells and *Xenopus* extracts [11,35,36]. Although deletion of Pin1 homologs in some other organisms appears to lead to no obvious mitotic defects [24,56], the above results suggest a crucial role for Pin1 in mitotic regulation in some cells.

Identification of Pin1 substrates further supports its role in mitotic regulation. Pin1 interacts with a number of mitotic phosphoproteins (Table 1) [12,35,36,43–47]. Many of these proteins are also known as MPM-2 antigens because of their recognition by the phosphospecific mitosis marker antibody MPM-2 [12,35], and, in fact, Pin1 and MPM-2 bind to similar motifs [12]. Pin1 regulates the activity of and/or has genetic interactions with several mitotic regulators (Table 1). The phosphatase Cdc25C is probably the most extensively studied Pin1 mitotic substrate [12,27,28,35,36,42,43]. Pin1 can directly affect the phosphatase activity of Cdc25C that has been phosphorylated by *Xenopus* extracts [35,42], but does not affect Cdc25C that has been expressed in and purified from insect cells [36], suggesting that Cdc25C proteins might be phosphorylated on different pSer-Pro sites or exist in different conformations. In addition, Pin1 could antagonize the effect of the Cdc2-associated Suc1/Cks1 protein on the phosphorylation of Cdc25 by Cdc2 [43], and Pin1 and Suc1/Cks1 appear to bind to the same pSer/Thr-Pro sequences in Cdc25C *in vitro* [21]. Pin1 can induce conformational changes in Cdc25C, as detected by the sensitivity to phosphatase or proteases, or by MPM-2 recognition [27,42]. Pin1 binds to pThr48-Pro and pThr67-Pro motifs in Cdc25C, which are crucial for Cdc25C to activate Cdc2 and to trigger the G2–M transition [28]. Mutating these two sites abolishes the characteristic mitosis-specific gel-mobility shift, Pin1 binding and the G2/M activity of Cdc25C [27,28]. These results suggest that isomerization of pThr48-Pro and/or

Table 1. Pin1 substrates and their regulation by Pin1

Proteins	Substrate activity	Targeting sites for Pin1[a]	What Pin1 does to the proteins	Refs
Mitotic proteins				
NIMA	Mitotic kinase		Genetic interaction	[11,35,36]
Cdc25	Mitotic phosphatase	pThr48-Pro; pThr67-Pro	Phosphatase activity, protein dephosphorylation, genetic interaction	[12,27,28,35, 36,42,43]
Wee1	Mitotic kinase		Genetic interaction	
Plk1	Mitotic kinase			
Myt1	Mitotic kinase			
Cdc27	Anaphase-promoting complex			
CENP-F	Kinetochore protein			
Incenp	Inner centromere protein			
Rab4	G protein			
Bcl-2	Anti-apoptotic protein			
NHERF-1	Na^+/H^+ exchanger regulatory factor			
KRMP1	Kinesin-related protein			
Cytoskeleton proteins				
Tau	Microtubule-binding protein	pThr231-Pro	Protein–protein interaction, protein dephosphorylation	[27,31]
Transcription factors or proteins				
Pol II	RNA polymerase II	pSer5-Pro (in the repeat)		[29,38,50]
Sin3–Rpd3	Histone deacetylase		Genetic interaction	[49]
c-Jun	Transcriptional activator	pSer63-Pro; pSer73-Pro	Transcriptional activity	[32]
β-Catenin	Transcriptional activator	pSer246-Pro	Protein stability, localization, transcriptional activity	[33]
Cf-2	Transcriptional repressor		Protein stability, transcriptional activity	[52]
G1/S proteins				
Cyclin D1	G1/S cyclin	pThr286-Pro	Protein expression, protein stability, localization	[32–34]

[a]Abbreviations: p, phospho-; Pro, proline; Ser, serine; Thr, threonine.

pThr67-Pro motifs probably induces conformational changes in Cdc25C, which could regulate the catalytic activity of Cdc25C directly [35,42] or indirectly through other mechanisms.

One such indirect mechanism is through dephosphorylation [27]. PP2A dephosphorylates only the *trans* pSer/Thr-Pro motif, and its activity to dephosphorylate Cdc25C and tau is increased by Pin1-induced prolyl isomerization [27]. Furthermore, Pin1 and PP2A display reciprocal genetic interactions in budding yeast [27]. In fission yeast, disruption of the *Pin1* gene results in hypersensitivity to inhibition of Ser/Thr phosphatases [24]. In addition, FCP1 is a suppressor of the budding yeast Pin1 homolog, and its activity towards the C-terminal domain is enhanced by Pin1 [38,48]. Therefore, Pin1 plays an important role in controlling the accessibility of pSer/Thr-Pro motifs to phosphatases. Given that Pin1 target proteins are distributed at

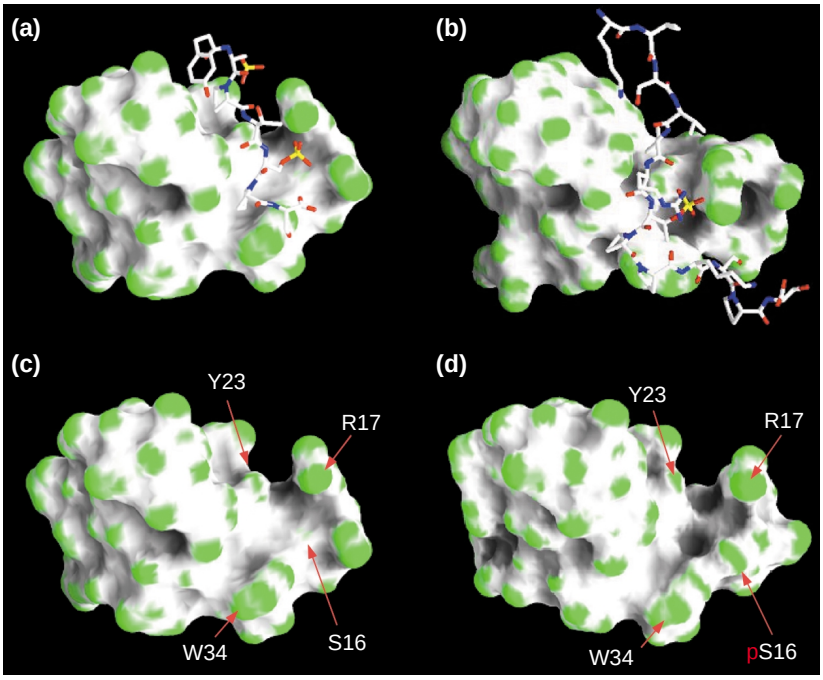

Fig. 2. Structural basis for WW domain binding and its regulation by phosphorylation. (a, b) Molecular surface representation of the binding interface between Pin1 and the carboxy-terminal domain (CTD) peptide (YpSPTpSPS) or between the Pin1 WW domain alone and the tau peptide (KVSVVRpTPPKSPS), as determined by x-ray (a) and NMR (b), respectively. The coloring is in accord with the curvature of the surface (green, convex; gray, concave), and peptides are shown as ball-and-stick representations. Both phosphopeptides bind to the WW domain, with an overall similar three-dimensional structure. The residues responsible for binding to phosphate are Ser16, Arg17 and Tyr23. The aromatic rings of Pin1Tyr23 and Trp34 form an aromatic clamp, which accommodates the ring atoms of Pro in peptides. (c, d) Molecular surface representation of the binding surface of the WW domain and Ser16-phosphorylated WW domain in Pin1. The core pSer/Thr-Pro-binding pocket (c) includes the side chains of Ser16, Arg17, Tyr23 and Trp34 in the WW domain, with Ser16 at the center. Ser16 phosphorylation (d) introduces a negatively charged phosphate group and sterically blocks the pocket from interacting with pSer/Thr-Pro motifs in target proteins.

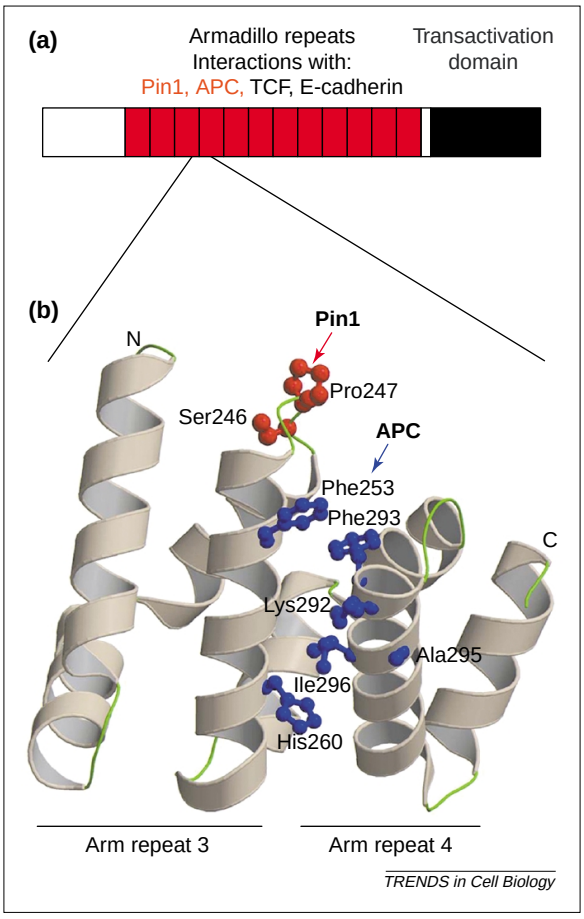

Fig. 3. A model for inhibition of the APC–β-catenin interaction by Pin1. (a) Schematic representation of the domain structure of β-catenin, with a closed-up representation of Pin1- and APC-binding sites in the third and fourth armadillo (Arm) repeats in β-catenin (b). The Pin1-binding motif, Ser246–Pro (labeled in red), is located at the loop between the two helices of β-catenin, which is in the vicinity of the crucial APC-binding residues in β-catenin (labeled in blue). When Pin1 binds to and isomerizes the pSer246–Pro motif in β-catenin, this can change the spatial arrangement between the third and fourth Arm repeats in β-catenin and thereby inhibits the interaction between β-catenin and APC (which would otherwise lead to β-catenin degradation). This might explain how Pin1 regulates subcellular localization and turnover of β-catenin.

different mitotic structures at various phases of mitosis, Pin1 might help coordinate mitotic phosphorylation events into an abrupt wave of signaling that proceeds in a synchronous manner.

Transcription and the G1–S transition

An alternative model for Pin1 function in mitosis has also been proposed in which Pin1 acts as a regulator of general transcription [38,49]. This model is based on the findings that some high-copy suppressors of the yeast Pin1 homolog Ess1 bind to DNA, modify chromatin structure or are regulatory subunits of RNA Pol II and that *ess1* mutant yeast cells have defective 3′ end formation of pre-mRNA [37,38,49]. However, because hyperphosphorylation of the RNA Pol II CTD on Ser-Pro motifs helps to turn off transcription during mitosis [50], Pin1 might help cells turn off transcription at mitosis. Furthermore, Pin1 does not have obvious effects on general transcription in mammalian cells [32,33] or even in yeast [51]. Thus, it is unlikely that the major function of Pin1 is to regulate general transcription.

However, Pin1 has been shown to play an important role in the expression of some specific genes. Overexpression of Pin1 specifically alters the expression of a small subset of genes out of 10 000 genes examined [33]. Furthermore, Pin1 regulates the activity of some transcription factors, including human c-Jun and β-catenin and *Drosophila* Cf2 [32,33,52]. c-Jun is a component of the AP-1 transcriptional complex and is phosphorylated on two Ser63/Ser73-Pro motifs by JNKs in response to various signals [2]. This phosphorylation increases the transcriptional activity of c-Jun towards its target genes, including cyclin D1. Importantly, Pin1 binds to c-Jun on Ser63/Ser73-Pro motifs and cooperates with either oncogenic Ras or JNK to increase the transcriptional activity of c-Jun towards the cyclin D1 promoter [32]. These results indicate that Pin1 is an important c-Jun regulator.

β-Catenin is a transcriptional activator and is negatively regulated by the tumor suppressor APC [53]. The interaction between β-catenin and APC is crucial for keeping β-catenin in the cytoplasm and triggering its degradation [53–55]. Pin1 directly binds to (and possibly isomerizes) the pSer246-Pro motif next to the APC-binding site in β-catenin and prevents β-catenin from binding to APC (Fig. 3). This in turn stabilizes β-catenin and increases its translocation into the nucleus, where it activates transcription of genes, including cyclin D1 [33]. These results indicate that Pin1 regulates cyclin D1 transcription through c-Jun and β-catenin. In addition, Pin1 can directly bind to the pThr286-Pro in cyclin D1 and increase its stability and nuclear accumulation, presumably by affecting nuclear export of cyclin D1 and/or proteolysis in the cytoplasm [34]. These results indicate that Pin1 regulates cyclin D1 both at the transcriptional and posttranslational levels. Given a well-established role of cyclin D1 in the G1–S transition, Pin1 probably affects this phase of the cell cycle.

Drosophila Cf2 is a transcriptional repressor in follicle cells [52]. Activation of the epidermal growth factor (EGF) receptor activates the Ras–MAP kinase pathway, resulting in Cf2 phosphorylation and cytoplasmic degradation [52]. Pin1 binds to phosphorylated Cf2 and increases its degradation [52]. This is crucial for relieving the repression of Cf2 target gene expression during oogenesis, as shown by deletion of Pin1 in *Drosophila* [52]. Interestingly, Pin1 appears to have an opposite effect on the location and/or degradation of cyclin D1 and Cf2 [34,52], suggesting that the context of the specific pSer/Thr-Pro motifs involved is important.

Phenotypes of Pin1 knockouts

Pin1 appears to be essential for cell growth in multiple genetic backgrounds in budding yeast and in several human cancer cell lines and is also required for the DNA replication checkpoint and cell-cycle progression in *Xenopus* extracts [11,25,37,39,41,51]. Furthermore, the enzymatic activity of Pin1 is required for these functions [27,38,41]. However, deletion of *Pin1* homologs in *Drosophila* [56] and fission yeast [24] does not appear to be lethal, although the mutants do display some interesting phenotypes. For example, mutant *Drosophila* display a severe oogenic defect [52], and mutant fission yeast have a synthetic growth defect with mutations of Cdc25 or Wee1 and are also hypersensitive to inhibition of phosphatases or cyclophilins [24].

Although mice lacking Pin1 were previously reported to be normal [57], these mutant mice do display a range of severe cell-proliferative abnormalities, including decreased body size, testicular atrophy and retinal degeneration [34]. Strikingly, breast epithelial cells fail to undergo the massive proliferation associated with pregnancy [34]. Interestingly, many of these *Pin1*-deficient phenotypes are characteristics of cyclin-D1-null mice [58,59], and it is known that Pin1 can positively regulate cyclin D1 function both at the transcriptional and posttranslational levels [32–34]. However, because cyclin D1 levels appear not to be significantly affected in testis [34], and inhibition of Pin1 affects cell division in other cells [11,37–40], Pin1 might affect rapid cell division during spermatogenesis [34].

It remains to be determined why disruption of *Pin1* is lethal in some systems but not in others. In multiple genetic backgrounds of budding yeast, deletion of *Pin1* is lethal, but, in one strain, mutant cells do not die but grow slowly instead [51]. Interestingly, this strain contains elevated cyclophilin A and is hypersensitive to the inhibition of cyclophilin A [51], as is the case in fission yeast mutant lacking Pin1 [24]. Furthermore, overexpression of cyclophilin A can rescue the *Pin1* phenotype [49]. These results suggest that Pin1 and cyclophilin A might have some overlapping functions under certain conditions. Interestingly, Pin1-deficient mouse cells still contain detectable phosphorylation-specific PPIase activity (K.P. Lu, unpublished). Furthermore, there are multiple *Pin1* homologs in plants [22]. These results suggest the presence of other *Pin1*-like genes. Indeed, we have recently cloned one such gene, *Pin1-A*, in *Drosophila*. Pin1-A shares a significant similarity both in the PPIase and the WW domain to Dodo, suggesting that it is probably a phosphorylation-specific PPIase (K.P. Lu, unpublished). Finally, there might be other structurally distinct phosphorylation-specific PPIases to be identified. All these possibilities might contribute to the reason why *Pin1* is not essential in some organisms.

Regulation of Pin1

Subcellular localization

Although Pin1 localizes primarily in the nucleus in cultured cells [11], it is readily detected in both the nucleus and cytoplasm in many dividing cells in normal and cancerous human tissues [32–34]. In AD neurons, Pin1 is redistributed to cytoplasmic tangles [31]. Given that Pin1 is a small 18-kDa protein and does not have a defined nuclear localization signal [13], the distribution of Pin1 in the cell might be driven by its target proteins. Indeed, the interaction between the WW domain and the substrate is responsible for the subcellular localization of Pin1 [39,40].

Protein levels

Pin1 expression is tightly correlated with cell division in normal tissues, readily detectable in actively dividing cells, such as those of the testis, the bottom of crypts in intestine and basal layers of skin epidermis, but low or not detectable in most differentiated tissues, with a few exceptions such as neurons [32,34]. Similarly, expression of apple Pin1 is tightly associated with cell division during fruit development and in *in vitro* culture [22]. Interestingly, Pin1 expression is significantly upregulated in many human cancer tissues and transformed cell lines, as compared with normal controls [32,33]. It will be interesting to determine how Pin1 expression is regulated.

Protein phosphorylation

Although Pin1 levels are constant during the cell cycle in transformed cells [35,41], Pin1 phosphorylation is regulated in a cell-cycle-dependent manner [40]. Pin1 is phosphorylated mainly on two sites, one of which is Ser16 [40]. Ser16 is located at the center of the pSer/Thr-Pro-binding pocket (Fig. 2c), and its phosphorylation would prevent the WW domain from interacting with pSer/Thr-Pro motifs (Fig. 2d) [29,40]. Indeed, phosphorylation of Ser16 completely abolishes the ability of Pin1 to interact with its substrates and to carry out its essential function in yeast [40]. By contrast, a Ser16Ala Pin1 mutant binds to Pin1 substrates constitutively and acts as a dominant-negative mutant to induce mitotic block and apoptosis [40]. Given that Pin1 interacts with a large number of substrates, phosphorylation might prevent Pin1 from acting on unintended substrates and/or cause the relocation of Pin1 to different substrates during the cell cycle [40].

Pin1 pathological roles

Alzheimer's disease

Many proteins, including tau, are hyperphosphorylated on Ser/Thr-Pro motifs in the brains of AD patients [5]. There is increasing evidence that inappropriate activation of mitotic events might contribute to hyperphosphorylation and disease development [5]. However, it remains to be determined how mitotic

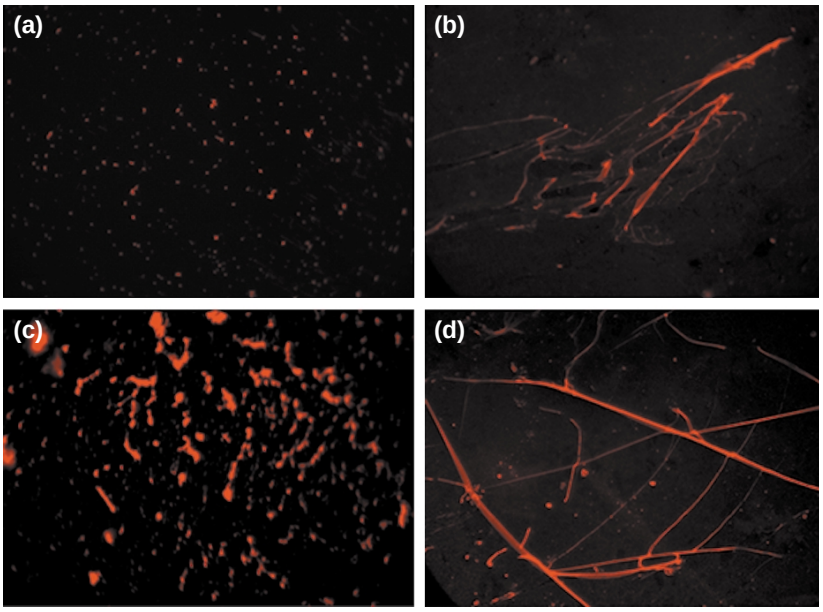

Fig. 4. Restoration of the function of phosphorylated Tau by Pin1. (a, b) Promotion of microtubule (MT) assembly by tau. The assembly of microtubules was initiated by incubating tubulin in the absence (a) or presence (b) of tau. (c) Phosphorylation of tau by Cdc2 abolishes its ability to promote MT assembly. Tau was phosphorylated by Cdc2 before the MT assembly assay. (d) Restoration of the microtubule-promoting activity of phosphorylated tau by Pin1. Pin1 was pre-incubated with Cdc2-phosphorylated tau before the MT assembly assays. Assembly of microtubules was visualized microscopically after immunostaining with antibodies against tubulin (detailed experimental methods are described in Refs [31,61]).

events occur in the neuron and how they lead to neurodegeneration. Interestingly, Pin1 is depleted in AD brain owing to its tight interactions with the tangles [31]. Importantly, Pin1 can restore the biological function of phosphorylated tau directly or indirectly [27,31]. Pin1 can bind to phosphorylated tau (ptau) and fully restore the ability of tau to bind to microtubules and promote microtubule assembly directly (Fig. 4) [31] or indirectly through facilitating ptau dephosphorylation [27]. Pin1 can also facilitate the dephosphorylation of other MPM-2 antigens, such as CTD that is known to be hyperphosphorylated in AD [5,27,48]. In addition, Pin1 can inhibit Cdc25C [27,35,43], and its depletion can induce mitotic arrest and apoptosis [11,27,39,41]. These results suggest that Pin1 might be needed to control the function of phosphoproteins in the event that they become phosphorylated. However, Pin1 dysfunction or sequestration might lead to hyperphosphorylation and accelerate the formation of tangles and/or cause neuronal cell death [27,31]. A crucial test for this hypothesis will be to determine whether disruption of Pin1 function causes any AD phenotypes in animals.

Cancer

In contrast to AD, where Pin1 depletion is associated with cell death, Pin1 is overexpressed in many human cancers such as malignancies of the breast and prostate tissues [32,33]. Interestingly, although Pin1 appears to be predominantly phosphorylated in normal breast tissues, overexpressed Pin1 in breast cancer tissues is mainly nonphosphorylated [32]. Since phosphorylation inactivates Pin1 [40], Pin1 activity becomes hyperactive in tumor tissues. The following three sets of experiments have substantiated the significance of Pin1 overexpression in breast cancer.

- First, Pin1 overexpression correlates with cyclin D1 levels in human breast cancer tissues, and Pin1 can cooperate with either activated Ras or JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter [32].
- Second, Pin1 prevents β-catenin from binding to APC and increases accumulation of β-catenin in the nucleus, activating genes such as cyclin D1 [33]. Indeed, Pin1 overexpression strongly correlates with β-catenin levels and with the nuclear accumulation of β-catenin in human breast cancer tissues [33]. Thus, Pin1 overexpression might contribute to upregulation of β-catenin in tumors, where APC or β-catenin mutations are not common.
- Third, Pin1 can also directly increase the stability of cyclin D1, and the phenotypes of *Pin1* knockouts resemble cyclin-D1-null phenotypes [34].

These results together provide strong evidence for a role for Pin1 in regulating cyclin D1. Cyclin D1 overexpression occurs in many different tumors and contributes to cell transformation, whereas deletion of cyclin D1 blocks the development of breast cancer in mice in response to oncogenic Ras or Neu [58–60]. Given the crucial roles of Ras signaling, β-catenin and cyclin D1 in oncogenesis, Pin1 overexpression might promote tumor growth. Since inhibition of Pin1 induces apoptosis in tumor cells [11,39,40], Pin1 inhibitors might offer a novel anticancer therapy.

Phosphorylation-specific prolyl isomerization as a timing mechanism

Why would the cell employ phosphorylation-specific prolyl isomerization as an additional regulatory mechanism after proteins have been phosphorylated? The answer might lie in the unique structure and the crucial regulatory role of the pSer/Thr-Pro motifs. Based on analysis of ~1% of total Ser/Thr-Pro bonds available in the protein structure database, the propensity of *cis* bond formation is in the range of 7–25% [27]. Because phosphorylation on Ser/Thr-Pro motifs in peptides does not greatly affect the ratio of *cis* and *trans* prolyl bonds [20], the probability of pSer/Thr-Pro bonds in the *cis* conformation is estimated to be similar in proteins [27]. Therefore, even though kinases and phosphatases are *trans*-specific [18,19], both *cis* and *trans* pSer/Thr-Pro bonds are probably present in phosphoproteins [27]. Given that phosphorylation reduces the isomerization rate of the pSer/Thr-Pro bonds [12,20], Pin1-catalyzed prolyl isomerization would be needed to accelerate either *trans* to *cis* or *cis* to *trans* isomerization, depending on specific target sites (Fig. 5). Such conversion might induce dramatic changes in the conformation of proteins. Importantly, such

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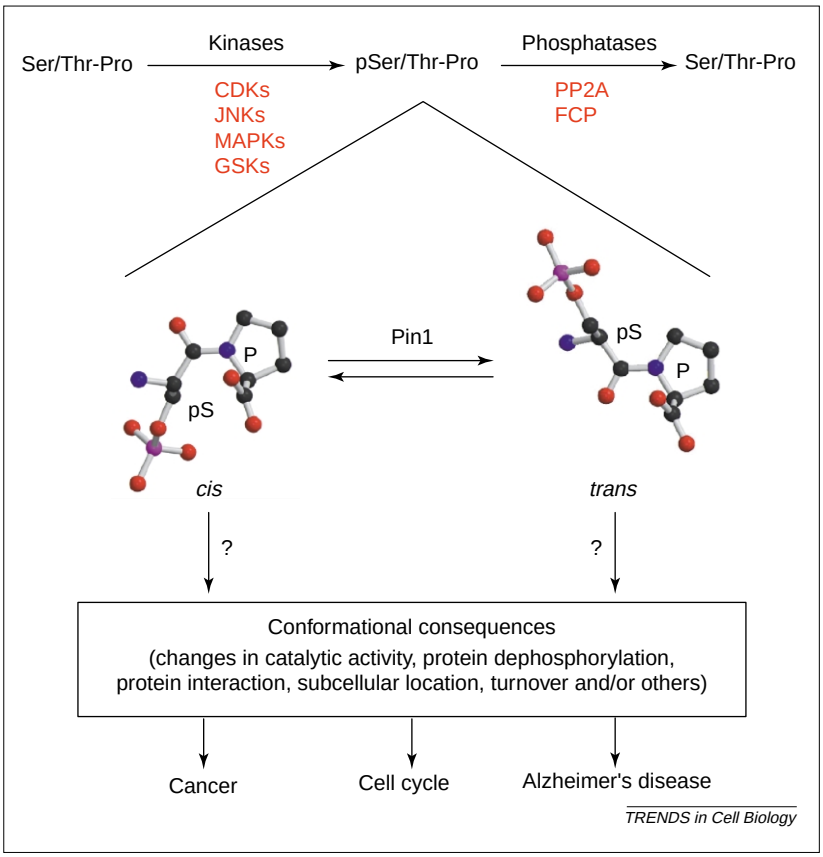

Fig. 5. A model for phosphorylation-specific prolyl isomerization as a timing mechanism. Phosphorylation of proteins on certain regulatory Ser/Thr-Pro motifs produces the substrate for Pin1, which alters the conformation of proteins by catalyzing either the *trans* to *cis* or the *cis* to *trans* isomerization of pSer/Thr-Pro, depending on specific target sites. The results so far support the notion that it is the conformational changes following phosphorylation, rather than the initial phosphorylation, that regulate the function of proteins. The functional changes include catalytic activity, dephosphorylation, protein interaction, subcellular location and/or turnover, although it remains to be determined which isomer is biologically active. Given that some of these functional changes lead to the same consequences, phosphorylation-specific prolyl isomerization probably functions as a timing mechanism, which would allow the cell to turn the function/activity of phosphorylated proteins on or off with high efficiency and precise timing. Listed are the Pro-directed kinases and phosphatases that have been shown to phosphorylate and dephosphorylate, respectively, Pin1 target sites. Abbreviations: CDK, cyclin-dependent protein kinase; FCP, RNA polymerase II C-terminal domain phosphatase; JNK, Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; GSK, glycogen synthase kinase; PP2A, protein phosphatase 2A.

conformational changes can regulate catalytic activity, dephosphorylation, protein interaction, subcellular location and/or turnover of Pin1 substrates. These results support the notion that it is the conformational changes following phosphorylation, rather than the initial phosphorylation, that have profound effects on protein function (Fig. 5). Interestingly, some of these functional changes lead to the same consequences at least for Cdc25 and tau [27,31,35]. Therefore, phosphorylation-specific prolyl isomerization probably functions as a timing mechanism that would allow the cell to turn the function of phosphorylated proteins on or off with high efficiency and precise timing.

Concluding remarks

Recent studies indicate that phosphorylation-dependent prolyl isomerization is a post-phosphorylation signaling mechanism that might play an important role in diverse cellular processes such as the cell cycle and transcription. Furthermore, this mechanism might provide novel insights into the pathogenesis of some human diseases such as AD and cancer. A major challenge for the future will be to determine how Pin1 actually induces conformational changes in phosphoproteins at the molecular level, whether the substrates undergo a conformation change that correlates with their activity, and whether there are other Pin1-type PPIases or other structurally distinct phosphorylation-specific PPIases, which could explain why Pin1 is essential in some systems but not in others. Another major challenge will be to determine the role of Pin1 in the development and treatment of human disease such as cancer and AD.

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