Furthermore, a parallel experiment revealed that the Gag-GFP puncta formed larger aggregations in the cytoplasm upon nocodazole treatment (Fig. 4D).

To further delineate the role of microtubule integrity in HIV-1 particle formation, we next performed experiments in which we co-transfected SOCS1 and pNL4-3 with or without the microtubule-destabilizing protein stathmin. Stathmin expression efficiently blocked the effects of SOCS1 upon HIV-1 particle formation in a dose-dependent manner (Fig. 4E and F). Cell viability was not strongly affected as revealed by immunoblotting analysis of either poly (ADP-ribose) polymerase (PARP) or β-actin (Fig. 4E). Our findings together indicate therefore that microtubule integrity may be required for SOCS1 to function in Gag assembly and release.

### 3.5. SOCS1 facilitates the ubiquitination of HIV-1 Gag

Our previous study has revealed that the SOCS-box of SOCS1 is required for both HIV-1 particle production and the enhancement of Gag association with microtubules [11]. The mechanism by which SOCS1 inhibits cytokine signaling is mediated by the inhibition of kinase activity through its N-terminal kinase inhibitory region (KIR) [18]. We next examined whether SOCS1 mutants lacking the function of either KIR (SOCS1-F59D) or SOCS-box (ΔSOCS) affected virus particle production. Our ELISA results indicate that the SOCS-box deletion mutant (ΔSOCS) of SOCS1 fails to promote virus production, whereas the KIR mutant, F59D, of SOCS1 partially enhances HIV-1 particle production when co-transfected with pNL4-3 in 293T cells (Fig. 5A and B). These data again suggest that the function of SOCS1 in HIV-1 particle production is not principally due to the suppression of interferon/cytokine signaling, but is mediated by its direct interaction with the HIV-1 Gag via the function of the SOCS-box.

The SOCS-box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity [19]. Indeed, several reports have demonstrated that Gag ubiquitination is related to its membrane association and particle release, although the function of HIV-1 Gag ubiquitination remains unclear [20,21]. We thus explored the possibility that SOCS1 modulates the ubiquitination of HIV-1 Gag, leading to enhanced virus particle formation. We initially examined the specific interaction of HIV-1 Gag with the SOCS1-E3 ligase complex. Purified SOCS1 and its E3 component proteins (biotinylated-Cullin 2, elongin B/C and Rbx1) in addition to HIV-1 Gag were synthesized in a wheat germ cell-free system and then subjected to pull-down assays using streptavidin coated magnetic beads. We found that Gag was co-purified with a SOCS1-E3 complex comprising SOCS1-elongin B/C-Rbx1-Cullin2 in a similar manner to RelA, a previously reported SOCS1 binding protein (Fig. 5C, left). Significantly, in the absence of SOCS1, both elongin B and C were not co-purified with Cullin 2 probably due to the unsteady condition of the E3 complex without SOCS1, and the amount of bound Gag was also reduced (Fig. 5C, right).These result indicate that the SOCS1-E3 complex associates with HIV-1 Gag and may promote its ubiquitination.

We next addressed whether SOCS1 affects the ubiquitination of HIV-1 Gag. Immunoprecipitation analysis with cells co-transfected with Gag-FLAG and myc-tagged ubiquitin, with or without SOCS1 co-transfection, revealed that SOCS1 overexpression significantly enhances the ubiquitination of the Gag protein (Fig. 5D). In contrast, the targeted depletion of SOCS1 by siRNA significantly reduced the amount of ubiquitinated Gag (Fig. 5E). These results indicate that SOCS1 could indeed be a potent ubiquitin ligase for HIV-1 Gag.

To clarify the biological significance of Gag ubiquitination via SOCS1, we performed an experiment using a dominant-negative ubiquitin construct lacking two C-terminal glycines (residues 75–76). This mutant ubiquitin (UbΔGG) cannot become conjugated to target substrates, but can bind noncovalently to ubiquitin interacting domains [14]. By immunoprecipitation analysis we revealed that the levels of HIV-1 Gag associated with microtubules were significantly reduced in cells expressing UbΔGG, as compared with those expressing wild-type ubiquitin (Ub-WT) (Fig. 5F). This trend was further revealed by a microtubule sedimentation experiment showing that the expression of UbΔGG reduced the amount of Gag associated with microtubules when compared with the expression of Ub-WT (Fig. 5G). These results together indicate a link between ubiquitin signaling and the microtubule-mediated Gag dynamics involved with HIV-1 particle formation.

## 4. Discussion

In our current study, we report that microtubule integrity is required for SOCS1 to facilitate Gag trafficking and virus particle production. We demonstrate from our experiments that (1) SOCS1 colocalizes with HIV-1 Gag along microtubules; (2) both SOCS1 and HIV-1 Gag are co-purified with microtubules and SOCS1 can augment the association of Gag with microtubules; (3) an intact microtubule network is required for the function of SOCS1 during Gag trafficking; (4) SOCS1 facilitates Gag ubiquitination; and (5) Gag association with the microtubules is significantly reduced when a dominant-negative Ub mutant is overexpressed. These results together indicate that SOCS1 can regulate the trafficking and stability of HIV-1 Gag via the microtubule-related cellular machinery, which may be in turn enhanced by Gag ubiquitination.

SOCS1 was identified initially as a negative regulator of signaling downstream of cytokines [22–24] and has been shown to localize at both the perinuclear region and the microtubule organizing center (MTOC) in cells [16]. We show from our current data that SOCS1 also forms dotted filamentous structures in the cytoplasm emanating from the perinuclear region, including the MTOC, to the cell periphery. A recent report has also indicated that Gag colocalizes at the MTOC with HIV-1 RNA and is subsequently transported to the cell periphery [25]. These observations together indicate that SOCS1 might facilitate the trafficking of HIV-1 Gag from the MTOC toward the plasma membrane by utilizing the intrinsic transport machinery of infected host cells.

The plus-end directed transport system along the microtubules could provide a means for the targeting of virus capsid proteins to the site of virus assembly and budding in the vicinity of the plasma membrane [26]. This ante-grade transport system is utilized by several viruses, such as herpes simplex virus type 1 (HSV-1), vaccinia virus and African swine fever virus (ASFV) [26–29]. Significantly, we have demonstrated in our present study that HIV-1 can utilize the microtubule-dependent transport mechanism, which may in turn be enhanced by SOCS1. Consistent with this notion, Leblanc et al. have demonstrated previously using a monoclonal antibody raised against unprocessed Gag that intracellular Gag puncta can travel along microtubules [30]. Our current microtubule pull-down analyses also clearly indicate that SOCS1 associates with Gag on microtubules and can enhance this interaction. This in turn might accelerate the intracellular trafficking of Gag to the plasma membrane along these structures, although the topological details are still unknown. Consistent with this observation, a plus-end microtubule motor KIF4 has been shown previously to associate with HIV-1 Gag and to enhance Gag trafficking [31,32]. These results further demonstrate the relevance of microtubule network in the trafficking of the HIV-1 Gag.

The involvement of the microtubule cytoskeleton in Gag assembly and HIV-1 particle egress is somewhat controversial [3,26,33,34]. However, several reports have presented convincing data to indicate the importance of this network in HIV-1 assembly

and propagation [3,35,36]. Our current study further demonstrates that the microtubule depolymerizing reagent, nocodazole, or the expression of microtubule-destabilizing protein stathmin, significantly inhibits the enhancement of HIV-1 particle production by SOCS1, suggesting a possible role of the microtubule network in regular HIV-1 particle production.

Our previous report indicated that the targeted depletion of SOCS1 results in the prominent perinuclear accumulations of HIV-1 Gag in 293T cells [11]. Our current study shows that nocodazole treatment or stathmin expression only slightly affects Gag release in non-SOCS1 overexpressing cells. This difference might be attributable to the following two possibilities. First, SOCS1 may affect Gag at multiple points during trafficking and assembly, and a critical point could be prior to the microtubule-mediated events that can be affected by nocodazole or stathmin. Second, there are multiple pathways to the delivery of exogenously expressed Gag protein from the cytoplasm to the plasma membrane in addition to microtubule-directed transport. Furthermore, we are currently uncertain whether the Gag association with microtubules is mediated by other microtubule binding proteins in cooperation with SOCS1, or whether SOCS1 directly associates with HIV-1 Gag on the microtubules. Further careful analysis must be performed to elucidate these possibilities.

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# Pin1 Catalyzes Conformational Changes of Thr-187 in p27$^{Kip1}$ and Mediates Its Stability through a Polyubiquitination Process*[S]

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**The *cis-trans* peptidylprolyl isomerase Pin1 plays a critical role in regulating a subset of phosphoproteins by catalyzing conformational changes on the phosphorylated Ser/Thr-Pro motifs. The phosphorylation-directed ubiquitination is one of the major mechanisms to regulate the abundance of p27$^{Kip1}$. In this study, we demonstrate that Pin1 catalyzes the *cis-trans* conformational changes of p27$^{Kip1}$ and further mediates its stability through the polyubiquitination mechanism. Our results show that the phosphorylated Thr-187-Pro motif in p27$^{Kip1}$ is a key Pin1-binding site. In addition, NMR analyses show that this phosphorylated Thr-187-Pro site undergoes conformational change catalyzed by Pin1. Moreover, in Pin1 knock-out mouse embryonic fibroblasts, p27$^{Kip1}$ has a shorter lifetime and displays a higher degree of polyubiquitination than in Pin1 wild-type mouse embryonic fibroblasts, suggesting that Pin1 plays a critical role in regulating p27$^{Kip1}$ degradation. Additionally, Pin1 dramatically reduces the interaction between p27$^{Kip1}$ and Cks1, possibly via isomerizing the *cis-trans* conformation of p27$^{Kip1}$. Our study thus reveals a novel regulatory mechanism for p27$^{Kip1}$ stability and sheds new light on the biological function of Pin1 as a general regulator of protein stability.**

Cellular differentiation and cell cycle inhibition are tightly controlled via sensitive molecular mechanisms. p27$^{Kip1}$, a member of the Cip/Kip family, is an essential cell cycle inhibitor that functions largely during the $G_0/G_1$ phase where it promotes the assembly of the cyclin D1-CDK4 complex and inhibits the kinase activity of the cyclin E-CDK2 complex in the $G_1$-S phase (1–4). Several review articles have elegantly summarized and discussed the detailed cellular functions of p27$^{Kip1}$ (1–6). p27$^{Kip1}$ is also a phosphoprotein with multiple Ser/Thr phosphorylation sites, including Ser-10, Ser-178, and Thr-187, followed by a proline residue. Hence, these motifs are potential substrate sites for proline-directed kinases (5, 6). Compared with Ser-178, which has not yet been well studied, the phosphorylation of Ser-10 and Thr-187 has been well characterized to be important for the regulation of p27$^{Kip1}$ function. For instance, Ser-10 has been found to be the major phosphorylation site of p27$^{Kip1}$ (7) and to play an important role in regulating cell migration (8–10), although the regulation of Ser-10 phosphorylation is still not completely defined (11, 12).

In contrast to Ser-10 and Thr-178, Thr-187 is the best characterized phosphorylation site on p27$^{Kip1}$ and is known to regulate the complex formation of p27$^{Kip1}$-cyclin E-CDK2 (1–2). In addition, it is also widely accepted that Thr-187 plays a crucial role in determining the abundance of mature p27$^{Kip1}$ proteins. The phosphorylation of Thr-187 directs p27$^{Kip1}$ to an SCF$^{Skp2}$ ubiquitin ligase complex (consisting of Skp2-Skp1-Cks1-Cul1-Roc1), which in turn promotes the polyubiquitination and degradation of p27$^{Kip1}$ (13, 14). The crystal structure of the Skp1-Skp2-Cks1-p27$^{Kip1}$ phosphopeptide complex shows that p27$^{Kip1}$ binds both Cks1 and Skp2 and that the C terminus of Skp2 and Cks1 forms the substrate recognition core of the SCF complex (15). Furthermore, the structure of this complex has revealed that the phosphorylation of Thr-187 in p27$^{Kip1}$ is recognized by the phosphate-binding site of Cks1, indicating that Cks1 is not only a facilitator but also an indispensable component in p27$^{Kip1}$ degradation machinery (15).

Pin1 is a unique peptidyl-prolyl isomerase (PPIase)[2] that recognizes only the phosphorylated Ser/Thr motif preceding a proline residue (16). In addition, Pin1 is very prominent in isomerizing the *cis-trans* conformation of prolyl-peptidyl bonds in its substrates, resulting in either the modification of their function (*e.g.* c-Jun (17), β-catenin (18), Bax (19), and Notch1 (20)) or modulation of their stability (*e.g.* cyclin D1 (21), p53 (22, 23), and NF-κB (24)). Loss of Pin1 in mice results in several phenotypes similar to those of cyclin D1-null mice (21) and neuronal degenerative phenotypes (25–28), suggesting the conformational changes mediated by Pin1 may be crucial for the normal functioning of cells. Additionally, Pin1 also plays important roles in cancer and other cellular events, which have

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Results, and Figs. S1–S3.

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[2] The abbreviations used are: PPIase, peptidylprolyl isomerase; MEF, mice embryonic fibroblast; CIP, calf intestinal phosphatase; WT, wild type; GST, glutathione S-transferase; HEK, human embryonic kidney; ROESY, rotating frame Overhauser effect spectroscopy; SCF, SKP1-CUL1-F-box; pRb, retinoblastoma protein.



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been extensively discussed in several recent review articles (29–33).

In this study, we show that Pin1 binds to p27$^{Kip1}$, mainly through the phosphorylated Thr-187-Pro motif, and causes subsequent prolyl isomerization of this cell cycle protein. Moreover, we also find that Pin1 can protect p27$^{Kip1}$ from degradation. Importantly, we demonstrate that by catalyzing conformational changes in p27$^{Kip1}$, Pin1 hinders its association with Cks1, resulting in a reduction of polyubiquitination of p27$^{Kip1}$ and protecting its degradation by SCF$^{Skp2}$ complexes. Our results suggest that the *cis-trans* isomerization catalyzed by Pin1 represents a novel regulatory mechanism during post-phosphorylation of proteins and polyubiquitination-directed degradation pathways.

## EXPERIMENTAL PROCEDURES

*Constructs, Reagents, and Antibodies*—Full-length cDNAs for p27$^{Kip1}$, Cks1, and Skp2 were cloned from a HeLa cDNA library and inserted into the pXJ-40-FLAG vector and/or pXJ-40-GFP vector (a gift from Dr. B. C. Low, Department of Biological Sciences, National University of Singapore) or into p3x-FLAG-CMV vector (Sigma). Recombinant proteins of the full-length human Pin1, and its WW and PPIase domains, were expressed from the pET42b(+) vector (Novagen). All mutant constructs were generated using the QuikChange® site-directed mutagenesis kit (Stratagene). Cycloheximide, FLAG-M2 beads, and MG132 were purchased from Sigma. Okadaic acid was purchased from Santa Cruz Biotechnology. Antibodies used for Western blotting and pulldown assays were as follows: polyclonal antibody against p27$^{Kip1}$ (C-19), monoclonal antibody against p27$^{Kip1}$ (F-8), polyclonal antibodies against Ser(P)-10-p27$^{Kip1}$, Skp2 (H-435), Cks1 (FL-79), and ubiquitin (P4D1) (Santa Cruz Biotechnology); polyclonal antibody against Thr(P)-187-p27$^{Kip1}$ (Zymed Laboratories Inc.); monoclonal antibodies against $\alpha$-tubulin and FLAG (Sigma).

*Cell Culture and Transfection*—Human embryonic kidney (HEK) epithelial 293T cells and Pin1-WT (wild-type) and Pin1-KO (knock-out) mouse embryonic fibroblasts (MEFs; a gift from Dr. K. P. Lu, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin unless otherwise noted. For overexpression analysis, all constructs into MEFs were transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. HEK 293T cells were transfected using the calcium phosphate method. Following transfection, cells were harvested in mammalian lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, supplemented with protease inhibitors and phosphatase inhibitors, including 1 $\mu$M pepstatin, 1 $\mu$M leupeptin, 50 $\mu$M $\beta$-glycerophosphatase, 1 mM okadaic acid, 1 mM $Na_3VO_4$).

*GST Pulldown and Co-immunoprecipitation Assays*—For GST pulldown assay, recombinant GST-full-length Pin1, -WW, or -PPIase domain proteins were conjugated to glutathione-Sepharose 4B beads. Beads were rocked with lysates from cells overexpressing WT or mutant p27$^{Kip1}$ constructs for 3 h at 4 °C, followed by five washes with mammalian lysis buffer. The bound proteins were eluted with SDS-loading dye and resolved by 12–15% SDS-PAGE, followed by Western blotting. Calf intestinal phosphatase (CIP) treatments were performed as described previously (23). CIP, 1 unit/ml (Promega), was added to the cell lysates for 30 min at 30 °C, which were then subjected to GST pulldown. For co-immunoprecipitation, FLAG-p27$^{Kip1}$ and mutant constructs were overexpressed in HEK 293T cells. Cell lysates were then cleared by centrifugation at 13,000 rpm for 10 min, and the resulting supernatants were incubated with FLAG-M2 beads for 3–5 h at 4 °C. The bound proteins were then analyzed by Western blotting.

*Protein Stability Assay*—Pin1-WT and -KO MEF cells were transfected with FLAG-p27$^{Kip1}$ WT or mutant constructs. Within 24 h of transfection, cycloheximide (100 $\mu$g/ml) was added to the cells to block protein synthesis. Cells were then harvested at 4-h intervals, followed by Western blotting.

*In Vivo Ubiquitination Assay*—FLAG-p27$^{Kip1}$ and Myc-ubiquitin constructs were co-transfected into Pin1-WT and -KO MEF cells, respectively. At 24 h following transfection, cells were treated with 10 $\mu$M MG132 to inhibit protein degradation. After 3 h of drug treatment, cells were harvested. The supernatants of cell lysates were then incubated with FLAG-M2 beads at 4 °C for 3 h, followed by five washes with mammalian lysis buffer and Western blotting using anti-ubiquitin antibody. The membranes were subsequently stripped and re-blotted with anti-p27$^{Kip1}$ antibody to evaluate the amount of p27$^{Kip1}$ that had bound to the beads. Reciprocal experiments were carried out by co-overexpressing FLAG-ubiquitin and GFP-p27$^{Kip1}$ in Pin1-WT and KO MEF cells. After MG312 treatment, cell lysates were subjected to pulldown assays using FLAG-M2 beads. Consequently, the beads were cooked at 95 °C for 5 min. Western blotting analyses were done using either anti-p27$^{Kip1}$ or anti-ubiquitin antibody.

*Conformational Change by NMR Spectroscopy*—All NMR experiments were performed using a Bruker 800-MHz NMR spectrometer at 25 °C. All spectra were recorded for a 2.0 mM peptide concentration dissolved in a 20 mM phosphate buffer (90% $H_2O$ and 10% $D_2O$, pH 6.5) in the presence or absence of 0.03 mM Pin1. For all experiments, 256 × 512 complex points were acquired with spectral widths of 7200 × 9600 Hz in $t_1 \times t_2$ dimensions and a relaxation delay of 1 s. ROESY spectra (34) were acquired at a mixing time of 110 ms, a spin-lock field strength of 4 kHz, and 16 scans. The mixing times were 30, 50, 70, 90, and 110 ms. TOSCY experiments (35) were carried out at a mixing time of 75 ms and 8 scans.

## RESULTS

*Interaction of Pin1 and Phosphorylated p27$^{Kip1}$*—Observations from our several preliminary experiments led us to speculate that Pin1 may associate with p27$^{Kip1}$ (supplemental Fig. S1). Sequence analysis also showed that p27$^{Kip1}$ contains three potential Pin1-binding Ser/Thr-Pro motifs as follows: S10P, S178P, and T187P (Fig. 1*A*). To confirm that Pin1 could interact with p27$^{Kip1}$, we performed glutathione *S*-transferase (GST) pulldown assays using recombinant GST-Pin1, GST-Pin1-W34A (impaired WW domain-binding site), and GST-Pin1-R68A/R69A mutants (impaired PPIase domain-binding sites) or GST proteins to pull down FLAG-p27$^{Kip1}$ protein overex-

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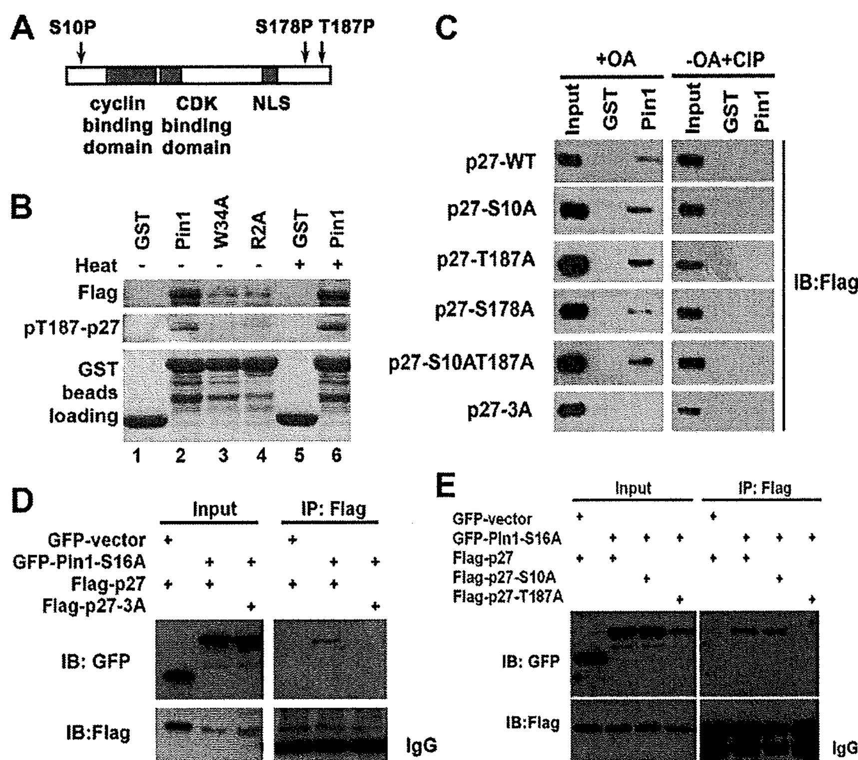


FIGURE 1. **Pin1 binds the Ser/Thr-Pro motifs of p27$^{Kip1}$ through its WW and PPIase domain.** *A*, schematic illustration of the potential Pin1-binding sites in p27$^{Kip1}$, which includes the S10P (Ser-10-Pro), S178P (Ser-178-Pro), and T187P (Thr-187-Pro) motifs. *NLS*, nuclear localization signal. *B*, immunoblotting analysis following the use of GST-Pin1, GST-Pin1-W34A (W34A mutant), and GST-Pin1-R2A (R68A/R69A mutants) to pull down FLAG-p27$^{Kip1}$. GST beads were used as a control. GST and GST-Pin1 proteins were stained as loading controls. *C*, immunoblotting (*IB*) analyses following the use of GST-Pin1 or GST beads to pull down either FLAG-p27$^{Kip1}$ or -p27$^{Kip1}$ mutants, in the absence (*left panel*) or presence (*right panel*) of CIP. *OA*, okadaic acid; *CIP*, calf intestinal phosphatase. *D*, co-immunoprecipitation (*IP*) assay using FLAG-M2 beads. GFP-Pin1-S16A was co-immunoprecipitated with FLAG-p27$^{Kip1}$, but not a FLAG-p27$^{Kip1}$-3A mutant. *E*, GFP-Pin1-S16A was co-immunoprecipitated with either the FLAG-p27$^{Kip1}$-WT or the FLAG-p27$^{Kip1}$-S10A mutant but not with the FLAG-p27$^{Kip1}$-T187A mutant.

pressed in HEK 293T cells. As shown in Fig. 1*B*, our results indicate that GST-Pin1 interacts with FLAG-p27$^{Kip1}$, but not GST control, indicating that Pin1 can bind p27$^{Kip1}$ *in vitro* (Fig. 1*B*, *1st* and *2nd lanes*). It is also known that both WW and PPIase domains of Pin1 can bind phosphorylated Ser/Thr-Pro motifs (36). Therefore, we further performed GST pulldowns using Pin1-W34A and Pin1-R68A/R69A mutants to identify the region of Pin1 that is responsible for p27$^{Kip1}$ binding. Notably, neither GST-Pin1-W34A nor GST-Pin1-R68A/R69A mutant associates strongly with p27$^{Kip1}$ protein compared with GST-Pin1-WT, suggesting that both the WW and PPIase domains are required for an efficient Pin1-p27$^{Kip1}$ interaction (Fig. 1*B*, *3rd* and *4th lanes*). To further test for a direct interaction of p27$^{Kip1}$ with Pin1, which does not rely on any bridging proteins, cell lysates with overexpressed FLAG-p27$^{Kip1}$ were subjected to heating at 95 °C for 10 min before GST pulldown because p27$^{Kip1}$ is an intrinsically heat-stable protein (13). As shown in Fig. 1*B* (*5th* and *6th lanes*), the Pin1-p27$^{Kip1}$ interaction is intact after the heat treatment, indicating that p27$^{Kip1}$ directly interacts with Pin1 *in vitro*. In addition, using an antibody specifically against phosphorylated p27$^{Kip1}$, we also found that Pin1 interacts with phosphorylated p27$^{Kip1}$ (Fig. 1*B*).

To identify the preferred Pin1-binding motif in p27$^{Kip1}$, we constructed a series of p27$^{Kip1}$ mutants by substituting Ser/Thr for Ala at its three putative Pin1-binding sites. We then performed GST pulldown and co-immunoprecipitation assays. Fig. 1*C* shows that Pin1 binds WT, all single mutants, and one double mutant of p27$^{Kip1}$. However, the triple mutation (3A) of p27$^{Kip1}$ totally abolishes this association (Fig. 1*C*, *left panel*), indicating that Ser-10, Ser-178, and Thr-187 of p27$^{Kip1}$ may all be involved in Pin1-p27$^{Kip1}$ binding *in vitro*. Given that Pin1 only binds phosphoproteins, and to confirm that the binding of Pin1 to p27$^{Kip1}$ is phosphorylation-dependent, we incubated cell lysates with CIP before performing GST pulldown assays. As shown in Fig. 1*C* (*right panel*), the interactions between Pin1 and p27$^{Kip1}$ WT/mutants are completely disrupted by CIP treatment, suggesting that the binding of Pin1 to p27$^{Kip1}$ is phosphorylation-dependent. By next co-overexpressing FLAG-p27$^{Kip1}$ and GFP-Pin1-S16A, a stronger binding derivative of Pin1 (37), the co-immunoprecipitation assays further demonstrated that Pin1 binds p27$^{Kip1}$ WT but not the triple mutant (3A) *in vivo* (Fig. 1*D*), consistent with the GST pulldown results (Fig. 1*C*). Interestingly, we observed that the p27$^{Kip1}$ S10A mutant co-immunoprecipitated significantly with Pin1 (Fig. 1*E*) but that there was almost no interaction between Pin1 and the p27$^{Kip1}$ T187A mutant. Taken together, these results suggest that Thr-187 could be the major Pin1-binding site in p27$^{Kip1}$ *in vivo* (Fig. 1*E*).

A single Ala residue substitution at the Ser/Thr-Pro motifs of p27$^{Kip1}$ did not totally eliminate the interaction between Pin1 and p27$^{Kip1}$ in GST pulldown assays (Fig. 1*C*). Hence, to further elucidate the binding property of the Pin1-p27$^{Kip1}$ interaction, we developed phosphopeptide chips for binding analysis. To this end, three N-terminal biotinylated phosphopeptides derived from Ser/Thr-Pro motifs in p27$^{Kip1}$ were synthesized (Fig. 2*B*). Subsequently, we immobilized these three biotinylated-p27$^{Kip1}$ peptides on glass slides coated with avidin to generate peptide chips, as described previously (38, 39). To determine the affinity of Pin1 to these peptides, different concentrations of Cy3-labeled Pin1 were titrated on chips. The fluorescence reading for each spot was then extracted from the unbound background intensities and fitted to binding curves for individual Pin1-peptide interaction (Fig. 2*A*). A smaller $K_d$ value indicates a stronger binding. As shown in Fig. 2*B*, Pin1 binds most strongly to the phosphorylated Thr-187 peptide,

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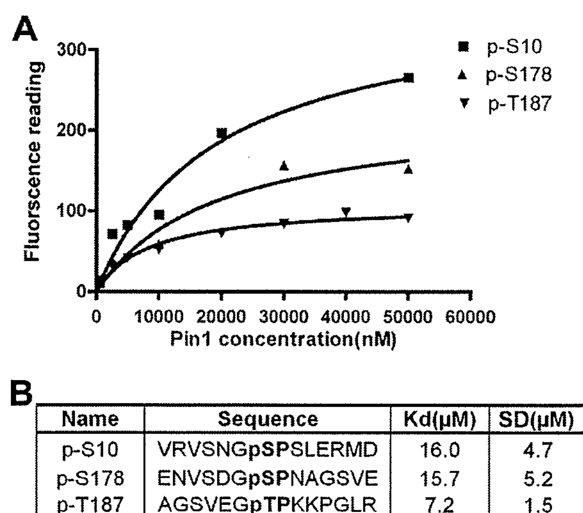


| Name | Sequence | Kd(μM) | SD(μM) |
|---|---|---|---|
| p-S10 | VRVSNG**pSP**SLERMD | 16.0 | 4.7 |
| p-S178 | ENVSDG**pSP**NAGSVE | 15.7 | 5.2 |
| p-T187 | AGSVEG**pTP**KKPGLR | 7.2 | 1.5 |

FIGURE 2. **Phosphorylated Thr-187 is the major Pin1-binding site on p27$^{Kip1}$.** *A*, fitted binding curves for Pin1 recombinant protein bound to three phosphorylated peptides derived from p27$^{Kip1}$. *B*, phosphorylated peptide sequences and $K_d$ and S.D. values calculated from the binding curve shown in *A*, indicating the affinity of Pin1 for phosphorylated peptides derived from p27$^{Kip1}$.

with a dissociation constant ($K_d$) of ~7.2 μM. In contrast, Pin1 has a weaker affinity for phosphorylated Ser-10 or Ser-178 peptides with $K_d$ values of ~16 μM. Therefore, taken together both *in vitro* and *in vivo* binding assays confirm that the phosphorylated Thr-187 residue on p27$^{Kip1}$ is the major Pin1-binding site.

*Pin1 Accelerates Conformational Changes in p27$^{Kip1}$*—Pin1 is a peptidylprolyl isomerase and accelerates conformational changes between the *cis* and *trans* forms of phosphorylated polypeptides. In addition to its binding of cognate substrates, the best way to demonstrate the function of Pin1 is to measure these catalytically driven conformational changes. To this end, we analyzed the isomerase activity of Pin1 against nonphosphorylated (Fig. 3*A*) and phosphorylated Thr-187-Pro peptides (GSVEG**p**T**P**KKPGA, where boldface indicates positions 6 and 8, respectively), in the absence of (Fig. 3B) or presence of Pin1 (Fig. 3C). Because of the slow exchange between *cis* and *trans* conformations of proline, several residues in both peptides displayed two distinct sets of $^1$H signals in the ROESY and total correlation spectroscopy spectra (Fig. 3, *B* and *C*). The *cis* and *trans* populations of both peptides were ~10 and 90%, respectively, as estimated from the one-dimensional $^1$H spectrum. In a normal situation, the exchange between the *cis* and *trans* conformations was so slow on an NMR time scale; therefore, no cross-peaks between the two conformations were observed by NMR (<0.1 $s^{-1}$, see Fig. 3*B*). In contrast, in the presence of Pin1, the proline isomerization rate of the phosphorylated peptide is greatly enhanced by Pin1. Cross-peaks from conformational exchange were also identified (Fig. 3*B*). Both cross-peaks and diagonal peaks of Thr-6 and Lys-8 amide protons from the phosphorylated Thr-187 peptides were also found (Fig. 3*B*). On the other hand, the nonphosphorylated peptide displayed no exchange peaks, even in the presence of Pin1 (Fig. 3*A*). Hence, Pin1 only accelerates the isomerization of the phosphorylated Thr-187 peptide in p27$^{Kip1}$ (Fig. 3*C*) but not the nonphosphorylated control (Fig. 3*A*).

Taking the intensities of cross-peaks and diagonal peaks of Thr-6 and Lys-8 amide protons of phosphopeptide, we further calculated the isomerization rates from the *cis* to *trans* ($k_{ct}^{cat}$) and from the *trans* to *cis* ($k_{tc}^{cat}$) conformations of Thr-6 (Fig. 3, *D* and *E*). The values of $k_{ct}^{cat}$- and $k_{tc}^{cat}$-obtained intensities for Thr-6 were 1.08 and 0.12 $s^{-1}$, respectively. Similarly, the values of $k_{ct}^{cat}$- and $k_{tc}^{cat}$-obtained intensities for Thr-8 were 1.05 and 0.15 $s^{-1}$, respectively (data not shown). The enhanced *cis-trans* conformational exchange rate by more than 10-fold suggests that the isomerization rates catalyzed by Pin1 are significantly faster than those without Pin1 (Fig. 3*F*).

*Pin1 Protects p27$^{Kip1}$ from Degradation*—Phosphorylation on Thr-187 was known to be very important for p27$^{Kip1}$ degradation (6, 40). Our current results show that Pin1 catalyzes p27$^{Kip1}$ through its phosphorylated Thr-187-Pro motif (Fig. 3). We next speculated whether Pin1 is involved in regulating p27$^{Kip1}$ stability. To test this possibility, we measured endogenous p27$^{Kip1}$ levels and found them to be dramatically lower, by about 50%, in Pin1-KO MEFs compared with Pin1-WT MEFs (Fig. 4, *A* and *B*); as previously reported, in our Pin1-KO MEFs, the cyclin D1 levels are markedly decreased (21), and cyclin E is significantly increased (41) (Fig. 4*A*). Consistently, we also find in our present experiments that a knockdown of Pin1 using small interfering RNA causes a decrease of the p27$^{Kip1}$ levels in HEK 293T cells (supplemental Fig. S2). This observation indicates that Pin1 is able to stabilize p27$^{Kip1}$ protein. Subsequently, we compared the half-life of endogenous p27$^{Kip1}$ in both Pin1-WT and -KO MEFs exposed to cycloheximide. As shown in Fig. 4*C*, the endogenous p27$^{Kip1}$ levels in Pin1-KO MEFs are significantly less stable than those in Pin1-WT MEFs. After 12 h of cycloheximide inhibition, endogenous p27$^{Kip1}$ is markedly reduced by up to ~76% in the absence of Pin1. In contrast, only a 50% reduction of p27$^{Kip1}$ is evident in Pin1-WT MEFs (Fig. 4, *C* and *D*). To demonstrate that the destabilization of p27$^{Kip1}$ is a direct effect of the absence of Pin1, we investigated the turnover rate of p27$^{Kip1}$ by re-introducing GFP-Pin1 into Pin1-KO MEFs. As shown in Fig. 4, *C* and *D*, the stability of p27$^{Kip1}$ is markedly enhanced by re-overexpression of GFP-Pin1, but not GFP alone, in Pin1-KO MEFs, suggesting that Pin1 plays a critical role in modulating p27$^{Kip1}$ stability.

To clearly determine whether Pin1 specifically regulates p27$^{Kip1}$ stability through the phosphorylated Thr-187 site, we investigated this stability in the presence or absence of Pin1 using the p27$^{Kip1}$ double mutants, S10A/S178A (potential Thr(P)-187), S10A/T187A (potential Ser(P)-178), and S178A/T187A (potential Ser(P)-10), instead of single mutants of p27$^{Kip1}$. We found that the stability of the two mutants containing T187A and the triple mutant (3A) showed no significant difference in the presence or absence of Pin1 (Fig. 4*E*). Furthermore, even though Pin1 may potentially interact with Ser(P)-10 (Fig. 1), the S178A/T187A mutant (potential Ser(P)-10) has no significant difference in turnover rate between Pin1-WT (73% remaining) and -KO MEFs (76% remaining), suggesting that Pin1 has little or no effect on p27$^{Kip1}$ degradation through the Ser-10 site. Most importantly, the double mutant S10A/S178A (potential Thr(P)-187) displays a shorter half-life in the absence of Pin1, degrading 66% of its total level (Fig. 4*E*), underscoring the significance of Thr-187 of p27$^{Kip1}$ for Pin1 function.

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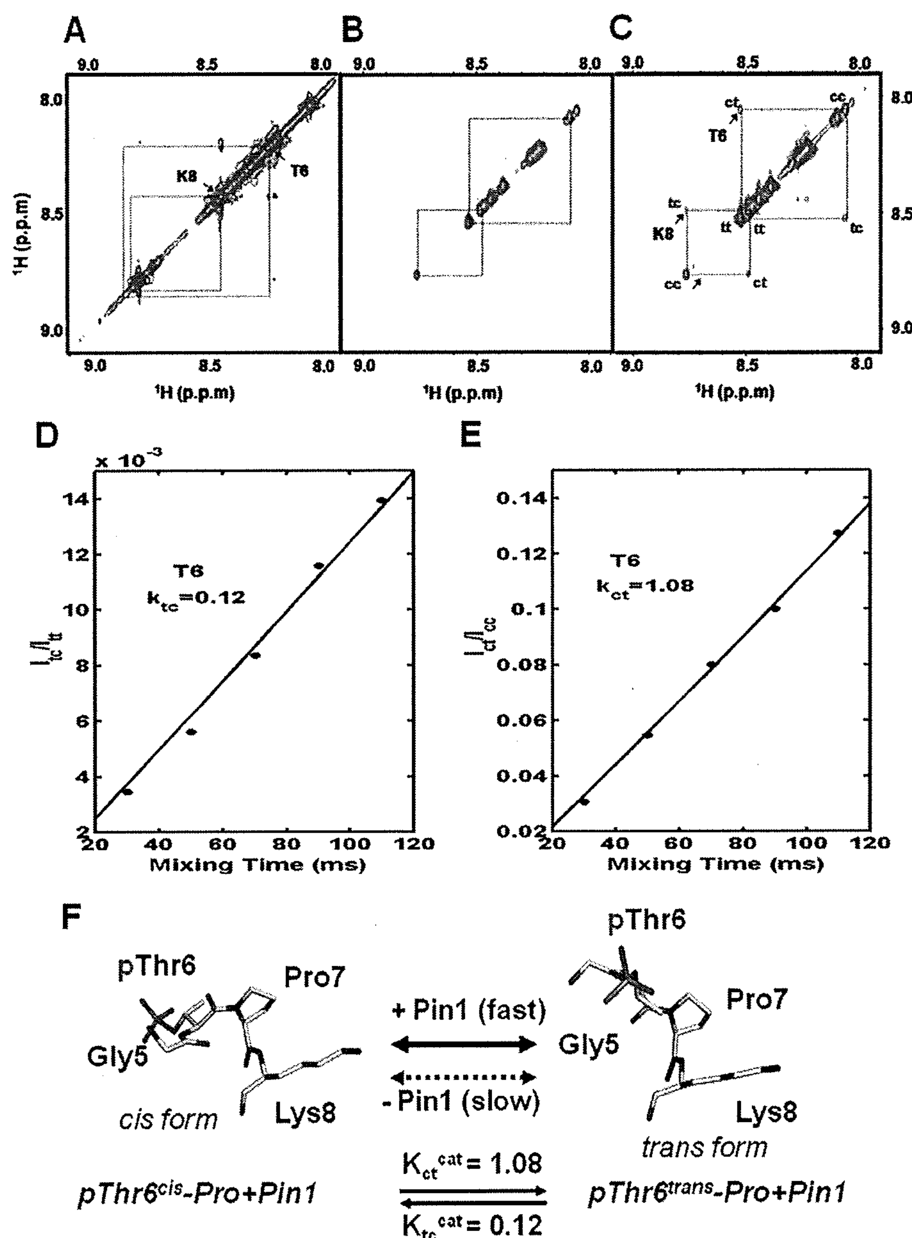


FIGURE 3. **Pin1 catalyzes conformational changes in phosphorylated $p27^{Kip1}$.** *A*, selected region of a two-dimensional ROESY spectrum of a nonphosphorylated Thr-187 peptide (AGSVEGTPKKPGLR) at a concentration of 2.4 mM and in the presence of 0.03 mM Pin1 (mixing time 110 ms) is shown. There is no cross-peak found (as indicated by *arrows*). *B* and *C*, selected regions of a two-dimensional ROESY spectrum of a phosphorylated Thr-187 peptide (GSVEG**pT**P**K**KPGA, where boldface indicates positions 6 and 8, respectively) at a concentration of 2.0 mM are shown in the absence (*B*) or in the presence (*C*) of 0.03 mM Pin1 (mixing time 110 ms). Negative and positive peaks are indicated by *arrows*. Diagonal peaks from *cis* and *trans* conformers are indicated by *cc* and *tt*, respectively. Exchange peaks resulting from Pin1-catalyzed isomerization are labeled *ct* and *tc*. Note that rotating frame nuclear Overhauser enhancement and exchange cross-peaks are indicated by *arrows*. Diagonal peaks of Thr (*T6*) and Lys (*K8*) amide protons from the phosphorylated Thr-187 peptides are identified by *arrows*. *D* and *E*, ratios of cross-peak and diagonal peak intensities for the *cis* and *trans* conformations of Thr-6 on rotating frame nuclear Overhauser enhancement mixing times and its isomerization rates are indicated; *D*, from the *cis* to *trans* ($k_{ct}^{cat}$) conformation; *E*, from the *trans* to *cis* ($k_{tc}^{cat}$) conformation. *F*, illustration of the conformational change of phosphorylated Thr-187 peptides by Pin1. The peptide models were generated using PyMOL. In the absence of Pin1, the isomerization rates between the *cis* and *trans* conformations are very slow; however, in the presence of Pin1, the isomerization rates are greatly enhanced by Pin1. The rates of Thr-6 are presented.

*Pin1 Plays a Role in the Ubiquitination of $p27^{Kip1}$*—To further explore the molecular mechanisms underlying Pin1 regulation of $p27^{Kip1}$ stability, we evaluated whether Pin1 is in fact involved in the $p27^{Kip1}$ ubiquitination machinery. To this end, we performed *in vivo* ubiquitination assays in which following the treatment of MG132, FLAG-$p27^{Kip1}$ and Myc-ubiquitin proteins were co-immunoprecipitated from Pin1-WT and -KO MEF cell lysates, respectively. Western blotting analyses revealed that $p27^{Kip1}$ is strongly polyubiquitinated in Pin1-KO MEFs but only modestly so in Pin1-WT MEFs (Fig. 5*A*). To rule out the possibility that the polyubiquitination bands detected were not because of a contamination by the polyubiquitinated $p27^{Kip1}$-associated proteins, we performed a reciprocal pulldown experiment by co-expressing the FLAG-ubiquitin and GFP-$p27^{Kip1}$ in Pin1-WT and KO MEF cells. Consistent with previous results shown in Fig. 5*A*, loss of Pin1 significantly enhanced the polyubiquitination of $p27^{Kip1}$ (Fig. 5*B*). These results are in a good agreement with our previous findings that in the presence of Pin1, $p27^{Kip1}$ protein accumulates at a higher level (Fig. 4*A* and supplemental Fig. S2), whereas in the absence of Pin1, the half-life of $p27^{Kip1}$ is much shorter because of a more rapid degradation rate (Fig. 4*C*).

The crystallographic structure of Skp2-Cks1-$p27^{Kip1}$ complex shows that Cks1 directly binds to the phosphorylated Thr-187 site in $p27^{Kip1}$ and promotes its degradation (15). On the other hand, we find in our present study that Pin1 enhances $p27^{Kip1}$ stability also through the phosphorylated Thr-187. Given that these two proteins bind to the same site on $p27^{Kip1}$ but exert an opposite effect, we hypothesized that Pin1 might compete with Cks1 for binding to $p27^{Kip1}$. To test this hypothesis, a gradient concentration of recombinant human Pin1 proteins was added to co-immunoprecipitation lysates in which FLAG-$p27^{Kip1}$-T187D (a mutant that mimics the phosphorylated-Thr-187 of $p27^{Kip1}$) and HA-Cks1 were co-overexpressed. As shown in Fig. 5*C*, a lower level of Cks1 remains bound to $p27^{Kip1}$ in the presence of a higher concentration of Pin1, suggesting that Pin1 competes with Cks1 for binding to $p27^{Kip1}$. Moreover, we fur-

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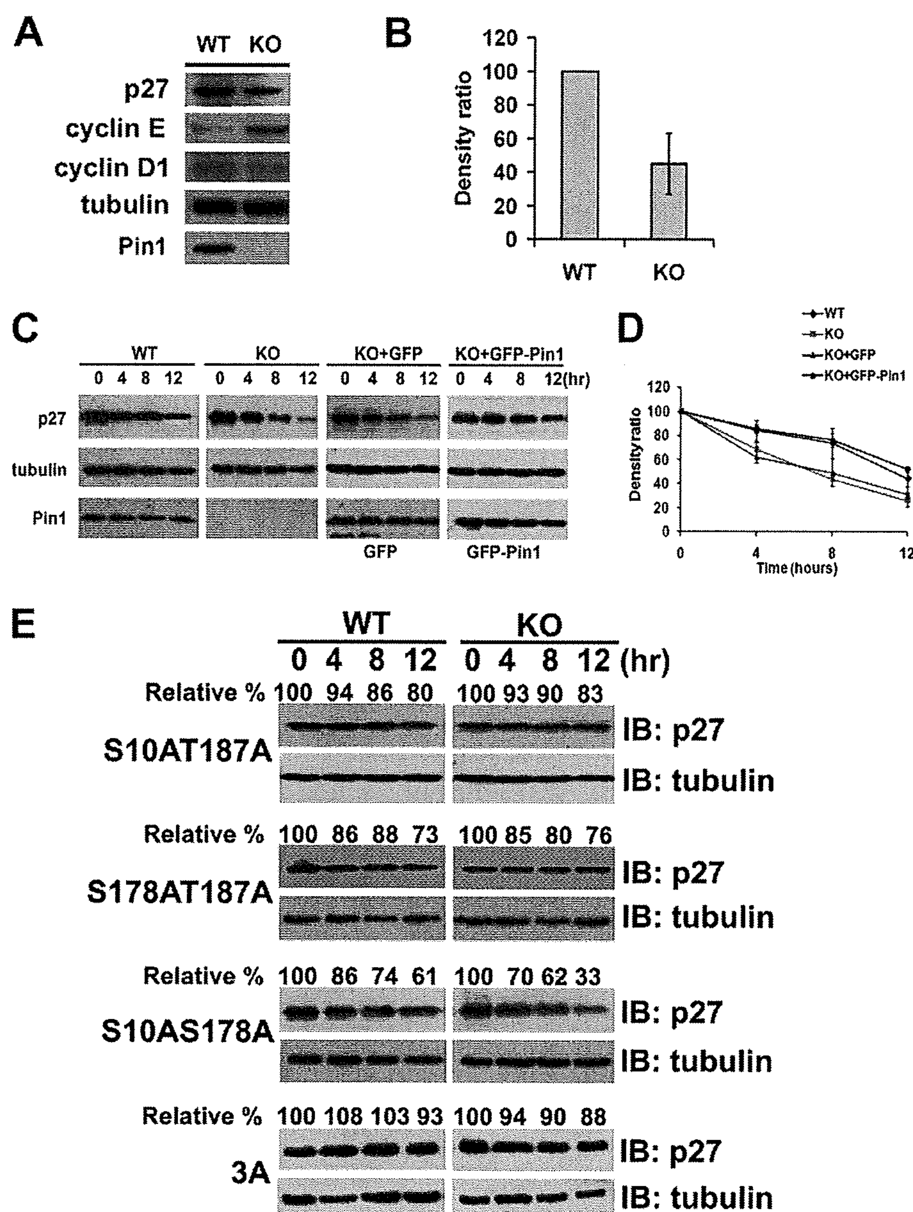


FIGURE 4. **Pin1 regulates p27$^{Kip1}$ stability.** *A*, immunoblotting analysis of the endogenous p27$^{Kip1}$, cyclin E, and cyclin D1 levels in Pin1-WT and -KO MEFs. *B*, quantification of endogenous p27$^{Kip1}$ levels shown in *A*, normalized to tubulin levels. *C*, protein stability assay of endogenous p27$^{Kip1}$ in both Pin1-WT and -KO MEFs. Cells were starved for 36 h before their arrest at $G_0$ phase. The cells were then treated with cycloheximide, harvested at 4-h intervals (*left panel*), and analyzed by immunoblotting. To confirm the function of Pin1 in regulating p27$^{Kip1}$ stability, GFP-Pin1 and GFP vectors were re-introduced into Pin1-KO MEF cells, respectively, followed by a protein stability assay. *D*, densitometric analysis of the degradation assays from *C*, normalized to tubulin levels. *E*, protein stability assay of exogenous FLAG-p27$^{Kip1}$ or its mutants in either Pin1-WT or -KO MEFs. Cells were treated with cycloheximide after 24 h of transfection and then harvested at 4-h intervals, followed by immunoblotting (*IB*) analyses. Tubulin was used as a loading control. *KO*, knock-out.

ther confirmed this observation *in vivo* by co-overexpressing FLAG-p27$^{Kip1}$-T187D and HA-Cks1 in Pin1-WT and -KO MEFs. Our co-immunoprecipitation results show that a markedly higher level of Cks1 is precipitated by p27$^{Kip1}$ in the Pin1-null background compared with the WT background (Fig. 5*D*), suggesting that the interaction of p27$^{Kip1}$ with Cks1 is significantly impaired in the presence of Pin1. Collectively, however, our results illustrate that Pin1 stabilizes p27$^{Kip1}$, possibly due to its inhibition of the association of Cks1 with p27$^{Kip1}$.

## DISCUSSION

We report herein that Pin1 plays a protective role during the turnover of p27$^{Kip1}$ in a phosphorylation-dependent manner. We describe a novel molecular mechanism by which Pin1 alters the conformation of phosphorylated p27$^{Kip1}$, thereby suppressing the association of Cks1 and p27$^{Kip1}$ and preventing p27$^{Kip1}$ degradation via the SCF complex (Fig. 6). Our study thus sheds new light on the biological function of Pin1 as an important regulator of specific protein abundance and also uncovers evidence of a cross-talk between post-phosphorylation regulation and the ubiquitination-mediated degradation of proteins.

Pin1 provides a novel mechanism for p27$^{Kip1}$ ubiquitin-proteasome degradation. It has been extensively shown that the degradation of p27$^{Kip1}$ is regulated by its subcellular compartmentalization (11, 42), phosphorylation status (43–44), and the availability of components in the degradation complex (13–15). In this study, we show that the ubiquitination status of p27$^{Kip1}$ is noticeably increased in the absence of Pin1, indicating that the role of Pin1 is significant in regulating this process. Our NMR studies clearly show that the phosphorylated p27$^{Kip1}$ undergoes a conformational change mediated by Pin1, suggesting that the post-phosphorylation modification may be crucial for preventing the recognition of p27$^{Kip1}$ by its degradation machinery. Cks1 is an important factor in p27$^{Kip1}$ degradation (13–15), and we further find that Pin1 disrupts the binding of Cks1 to phosphorylated p27$^{Kip1}$. Therefore, one of the possible mechanisms could be that the conformational changes of p27$^{Kip1}$ catalyzed by Pin1 is unfavorable for the binding of Cks1, resulting in a reduced interaction of p27$^{Kip1}$ with the SCF complex and an enhancement of p27$^{Kip1}$ stability (Figs. 5 and 6). From the structure of Skp1-Skp2-Cks1-p27$^{Kip1}$ complex, we further found that Cks1 indeed interacts with a *trans* conformation of the Thr-187-Pro motif in p27$^{Kip1}$ (15), suggesting

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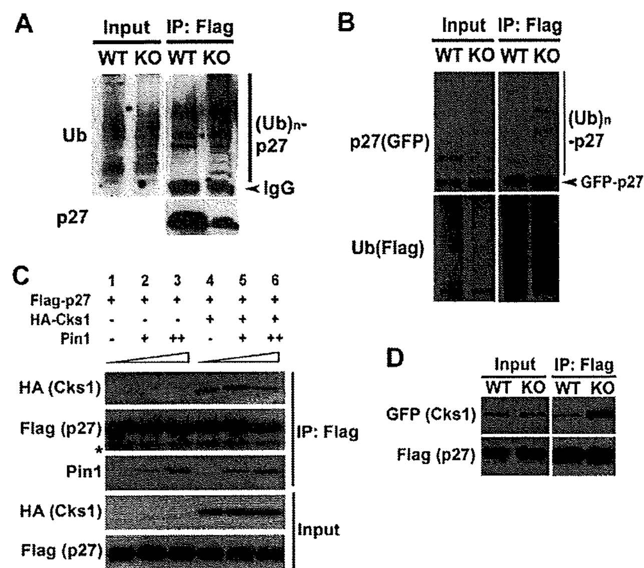


FIGURE 5. **Pin1 is involved in p27$^{Kip1}$ polyubiquitination.** *A*, *in vivo* ubiquitination (*Ub*) assay of FLAG-p27$^{Kip1}$ in Pin1-WT and -KO MEFs. At 24 h following transfection, cells were treated with 10 μM MG132 to inhibit protein degradation. After 3 h of MG132 treatment, cells were harvested, and lysates were then incubated with FLAG-M2. Western blots were carried out using anti-FLAG or anti-ubiquitin antibody. *IP*, immunoprecipitation; *KO*, knock-out. *B*, reciprocal experiments were carried out as in *A*, after FLAG beads pulldown, and the beads were cooked at 95 °C for 5 min, and Western blots were done by blotting with either anti-p27$^{Kip1}$ or anti-ubiquitin antibody. *C*, effects of Pin1 on co-immunoprecipitation of HA-Cks1 and FLAG-p27$^{Kip1}$. Immunoblotting analysis of HA-Cks1 bound to FLAG-p27$^{Kip1}$ in the presence of different concentrations of recombinant Pin1 protein are shown. *D*, co-immunoprecipitation of HA-Cks1 and FLAG-p27$^{Kip1}$ in the presence or absence of Pin1. Immunoblotting analysis of HA-Cks1 bound to FLAG-p27$^{Kip1}$ in Pin1-WT and -KO MEFs, respectively, is shown.

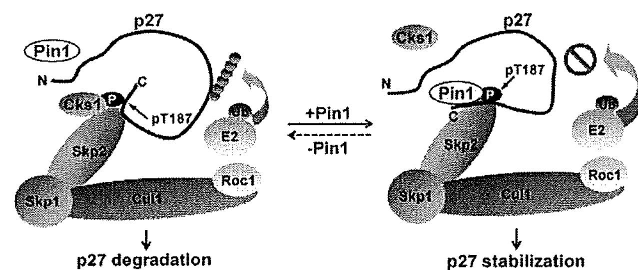


FIGURE 6. **A putative mechanism for Pin1 in the regulation of p27$^{Kip1}$.** Cks1 binds to phosphorylated p27$^{Kip1}$ and promotes its degradation through ubiquitination (*Ub*). However, to regulate p27$^{Kip1}$ stability at specific time points, Pin1 binds phosphorylated p27$^{Kip1}$ and catalyzes its conformational change, which hinders the association of p27$^{Kip1}$ with Cks1. This process in turn inhibits the polyubiquitination and degradation of p27$^{Kip1}$.

that Cks1 may have a structural preference to its target. In the presence of Pin1, the *cis-trans* isomerization rate of Thr-187-Pro motif is increased by more than 10-fold. This result suggests that the conformational change catalyzed by Pin1 is a critical regulatory mechanism in mediating the interaction of Cks1 and p27$^{Kip1}$. Another possible mechanism could be that the Pin1 enhances the dephosphorylation of p27$^{Kip1}$ by its downstream phosphatase, resulting in a reduction of the binding to Cks1 and rescuing p27$^{Kip1}$ stability. It has been reported that Pin1 can regulate the conformation and dephosphorylation of its substrates (25, 29). Pin1 facilitates the dephosphorylation of Cdc25 and Tau by PP2A, a conformation-specific proline-directed phosphatase that effectively dephosphorylates only the *trans*-Ser/Thr-Pro motifs (29). It would be interesting to further investigate whether Pin1 can regulate the dephosphorylation of p27$^{Kip1}$ in the future. The third possible mechanism is that Pin1 competes with Cks1 or other components of the SCF complex for binding to phosphorylated p27$^{Kip1}$ (Figs. 5 and 6). Indeed, we also find that GST-Pin1 can pulldown Skp2 *in vitro* (data not shown), indicating that Pin1 may become incorporated into the p27$^{Kip1}$ degradation complex and is likely to have a regulatory role. Our current study provides the first experimental evidence of a novel "post-phosphorylation" regulation mechanism for the polyubiquitination of p27$^{Kip1}$. Therefore, overall the post-phosphorylation mechanisms mediated by prolyl isomerases may be a crucial determinant of the abundance of some proteins such as β-catenin (18), cyclin D1 (21), and NF-κB (24) *in vivo*.

There are three Ser/Thr-Pro motifs on p27$^{Kip1}$, Ser-10, Ser-178, and Thr-187, which are potential Pin1-binding sites. Among these, Thr-187 is the most well characterized site for p27$^{Kip1}$ degradation, as revealed in a number of previous studies (13, 40, 45). Using peptide chips and co-immunoprecipitation assays, our current results further indicate that phosphorylated Thr-187 of p27$^{Kip1}$ is the most favored Pin1-binding site. At the same time, we also find that this interaction is fully abolished only when all three of these Ser/Thr-Pro sites are mutated, indicating that all Ser/Thr-Pro motifs, at least partially, might be involved in the Pin1-p27$^{Kip1}$ interaction. Although Ser-10 is reported to be the major phosphorylated site in p27$^{Kip1}$ (6, 7), our stability assay shows that the stability of S178A/T187A mutant has no significant difference in Pin1-WT and -KO MEF cells (Fig. 4*E*), suggesting that the interaction of Pin1 with phosphorylated Ser-10 may not have a significant effect on p27$^{Kip1}$ stability. On the other hand, because Pin1 can associate both sites, it would also be interesting to test whether Pin1 can induce conformational changes on Ser(P)-10 or Ser(P)-178 peptides in the future. In addition, FOXO4 is known to regulate the p27$^{Kip1}$ level through transcriptional regulation. Recently, it was reported that the overexpression of Pin1 inhibits FOXO4 transcriptional activity resulting in an impairment of p27$^{Kip1}$ expression (46). To confirm this, we also tested the turnover rate of p27$^{Kip1}$ in HEK 293T cells with or without ectopically overexpressed Pin1 (supplemental Fig. S2), but we observed no ectopic effects of Pin1 on p27$^{Kip1}$ stability. However, because of a high endogenous level of Pin1 in HEK 293T cells, additional ectopic expression may not have significant effects, similar to the results shown previously (46). On the other hand, we show that the loss of Pin1 in cells significantly enhances the polyubiquitination levels of p27$^{Kip1}$ resulting in reduced stability (Fig. 4, *A* and *C*). Furthermore, we also demonstrated that there is little or no detectable FOXO4 in our MEFs (supplemental Fig. S3), suggesting the regulatory function of Pin1 on p27$^{Kip1}$ degradation is not likely to be mediated through FOXO4 in our study.

Isomerization of the phosphorylated Ser/Thr-Pro motifs by Pin1 is a key mechanism underlying a post-phosphorylation regulation in many proteins. In general, Pin1 catalyzes the conformational changes of its substrates and thereby alters their properties, *e.g.* transcriptional activity, protein-protein interac-

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tion, and subcellular localization. The *cis-trans* isomerization of proline is thus likely to be a key regulatory switch in signal transduction. Given the cumulative evidence to date, we also propose that by switching the *cis-trans* conformations of the proline residue, Pin1 plays a pivotal role in the protein degradation machinery. For instance, Pin1 stabilizes cyclin D1 (21), NF-κB (24), β-catenin (18), p53 (22, 23), p73 (47), and Tax (48). On the other hand, Pin1 negatively regulates protein stability, including that of c-Myc (49), SRC-3 (50), IRF3 (51), cyclin E (41), Daxx (52), and SMRT (53). Additionally, many of these substrates undergo ubiquitination-mediated proteosomal degradation. The turnover of Pin1 substrates is generally through the *cis-trans* conformational changes. In this study, we report that Pin1 stabilizes p27$^{Kip1}$ by a direct involvement in its degradation machinery, which adds weight to a general role for Pin1 in regulating protein stability. Hence, the post-phosphorylation isomerization by Pin1 may act as a molecular switch that determines the fate of its substrates. However, the reason why Pin1 can act as both a stabilizer and a de-stabilizer is unclear at present. Future studies focusing on the general function of Pin1 in ubiquitination-mediated protein degradation may help to elucidate this issue.

The molecular mechanisms underlying the regulation of p27$^{Kip1}$ by Pin1 are likely to be highly complex given the roles that both proteins play in different phases of the cell cycle. As an example of this, the overexpression of Pin1 in mammalian cells leads to a $G_2$ arrest, whereas its inhibition causes mitotic arrest (16). Moreover, Pin1 regulates the turnover of c-Myc and cyclin E (49, 41), both of which play critical roles in the $G_1$/S phase transition, and the cyclin E protein has been shown to be further destabilized by Pin1 in MEFs (41). On the other hand, we have also reported that Pin1 can regulate cyclin D1 through both transcriptional and translational mechanisms (17–18, 21). Pin1 directly stabilizes cyclin D1 and regulates its localization; in the absence of Pin1, the cyclin D1 protein levels are markedly reduced (21). Furthermore, the cell cycle reentry of Pin1-KO MEFs is retarded in response to serum starvation (55). Taken together, these results suggest that Pin1 plays a pivotal role in the $G_0$/$G_1$-S transition. You *et al.* (56) have reported in their previous study that in response to IGF-1 treatment, Pin1-KO MEFs display a delayed entry into S phase. Conversely, IGF-1 was found to stimulate Pin1 expression, resulting in an increased expression of cyclin D1 and the phosphorylation of pRb, thus further promoting the $G_0$/$G_1$-S transition (56). The transcription factor E2F is known to be regulated by pRb, the hyperphosphorylation of which releases E2F1 thereby activating the downstream essential genes for the $G_1$/S phases of the cell cycle. In addition, E2F1 cannot only bind to the p27$^{Kip1}$ promoter (57), but also the Pin1 promoter (58) and activates the expression of these two proteins. On the other hand, the Cdk inhibitor p27$^{Kip1}$ can prevent pRb phosphorylation by inhibiting the activities of cyclin D1/Cdk4 and cyclin E/Cdk2 (59, 60). Interestingly, the phosphorylation of Thr-187 on p27$^{Kip1}$ by cyclin E/Cdk2 and the subsequent recognition by the ubiquitin ligase Skp2 SCF proteasome complex are the predominant mechanisms that regulate the protein abundance of p27$^{Kip1}$. In this study, we show that the phosphorylation-dependent ubiquitination of p27$^{Kip1}$ is highly controlled by Pin1, further highlighting the complexity of the cell cycle regulatory processes in which Pin1 and p27$^{Kip1}$ function.

Interestingly and unexpectedly, Pin1 knock-out mice are viable and undergo relatively normal development despite several age-dependent and cell-proliferative abnormalities (21). Accordingly, Pin1-KO MEFs are slightly slower growing than their wild-type counterparts but otherwise show no significant differences. Conversely, overexpression of Pin1 not only confers transforming properties on epithelial cells but also enhances the transformed phenotypes of Neu/Ras activated mammary epithelial cells, indicating an important role of Pin1 in tumor formation (58). On the other hand, it is also surprising that the inactivation of the Thr-187-dependent p27$^{Kip1}$ turnover pathway has no severe impact on cell cycle regulation, as revealed by studies of p27$^{Kip1}$T187A knock-in mice. In addition, these mice are viable and display only modest cell-proliferative alterations (61). More interestingly, the p27$^{Kip1}$Thr-187A can also be down-regulated in activated K-*ras*-induced lung tumors, and the p27$^{Kip1}$Thr-187A mice have a same tumor-dependent death rate as the p27$^{Kip1}$ wild-type mice, implying that an alternative degradation pathway other than Skp2-dependent mechanism plays a significant role in regulating p27$^{Kip1}$ stability (54). Taken together, these observations suggest, at least in part, that there are negative feedback mechanisms in different phases of the cell cycle that control p27$^{Kip1}$ degradation and Pin1 isomerase activity. Given our current data showing that Pin1 also interacts with and stabilizes p27$^{Kip1}$, further studies of the temporal and spatial regulation of phosphorylated p27$^{Kip1}$ mediated by Pin1 may provide a better understanding of cell cycle control. Also, it would be interesting to cross Pin1 knock-out mice with cyclin D1, cyclin E, or p27$^{Kip1}$ transgenic/knock-out mice to dissect each of their roles in this complicated cell cycle progression.

In conclusion, our current study elucidates a novel molecular mechanism by which phosphorylated p27$^{Kip1}$ is further regulated by the peptidylprolyl isomerase Pin1. This may underscore the significance of prolyl isomerization in the post-phosphorylation regulation and polyubiquitination-directed degradation of proteins in the cell.

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Research Focus

# Pinning down HER2–ER crosstalk in SMRT regulation

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**SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) is a transcriptional co-repressor that mediates the repressive function of nuclear hormone receptors such as the estrogen receptor (ER). Decreased SMRT levels correlate with acquired tamoxifen resistance in breast cancer, and SMRT restoration might resensitize breast cancer cells to tamoxifen. A new study demonstrates that SMRT protein stability is regulated by phosphorylation-dependent Pin1-catalyzed prolyl-isomerization. Pin1 functions downstream of HER2, positioning it as an important modulator of the crosstalk between ER and growth factor signaling.**

## Breast cancer and the estrogen receptor

Breast cancer is one of the most common malignancies in women and the second most common cause of female cancer-related deaths [1]. However, deaths due to breast cancer have decreased in recent years owing to the development of targeted therapies, including hormone therapy, in addition to conventional chemotherapy and surgical interventions [1]. The majority of breast cancers in post-menopausal women express the estrogen receptor (ER), and after surgery they can be treated with hormonal therapy alone, in the absence of more toxic chemotherapy, resulting in a relatively favorable prognosis [2]. However, a significant fraction of these hormone-sensitive breast cancer patients will experience disease progression that is attributable to the resistance to endocrine agents, such as tamoxifen, thus resulting in mortality [3]. Therefore, it is desirable to develop new strategies to treat hormone refractory breast cancer; this goal will probably be facilitated by elucidating the underlying molecular mechanisms of hormone resistance. A new study by Stanya and colleagues [4] delineates an important hint in solving the riddle of endocrine resistance. They find that Cdk2 (cyclin dependent kinase 2)-mediated phosphorylation of SMRT (silencing mediator for retinoid and thyroid receptors), an ER co-repressor, creates binding sites for protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 (Pin1), which in turn induces conformational changes to promote SMRT degradation (Figure 1a). Moreover, this event crucially mediates human epidermal growth factor receptor 2 (HER2)-dependent SMRT protein degradation and resultant endocrine resistance. These findings shed new light on the molecular mechanism of SMRT regulation and warrant further investigation of the role and therapeutic potential of Pin1 in the treatment of endocrine-resistant breast cancers.

## ER regulation

ER is a member of the nuclear hormone receptor family, which has important roles in cell proliferation, differentiation and oncogenesis [5]. In response to the hormone estrogen, ER can recruit steroid receptor coactivator-3 (SRC-3; also called amplified in breast cancer-1, AIB1) to enhance estrogen-dependent transcriptional gene activation. In the absence of 17β-estradiol (E2), ER can interact with co-repressors such as SMRT and N-CoR (nuclear receptor co-repressor) to repress target gene expression [6]. Although both SMRT and N-CoR mediate the most repressive function of unliganded ER by recruiting histone deacetylase 3 (HDAC3), only SMRT inhibition is sufficient to de-sensitize cells to tamoxifen-mediated inhibition of ER-induced gene expression [7].

## Crosstalk between HER2 and ER

HER2 (also known as ErbB2, ERBB2 and Neu) is a member of the epidermal growth factor receptor family that has a notable role in breast cancer pathogenesis; it is the target of the anti-breast-cancer drug trastuzumab [8]. Both preclinical and clinical evidence implicates HER2 overexpression in the development of endocrine resistance, especially to tamoxifen [9]. The receptor crosstalk between the ER and growth factor receptors affects ER transcriptional activity. For example, HER2-dependent mitogen-activated protein kinase (MAPK) activation triggers both ER and SRC-3 phosphorylation thereby increasing their transcriptional activity [10,11]. MAPK can also phosphorylate SMRT thereby reducing its binding affinity for transcription factors and enhancing its nuclear export [12]. However, it is unknown whether growth factor signaling modulates co-repressor stability. Several previous reports indicate that estrogen markedly downregulates N-CoR protein levels in ER-positive breast cancer cells without affecting SMRT levels [13]. Therefore, it will be of great importance to elucidate the regulatory mechanism(s) underlying SMRT stability in relation to tamoxifen resistance and growth-factor signaling in breast cancer.

## The role of Pin1 in HER2–ER crosstalk

The peptidyl-prolyl isomerase Pin1 is an important regulator in HER2-mediated growth signaling and ER-mediated transcription in breast cancer [14]. Unlike other prolyl isomerases, Pin1 binds to discrete phosphorylated Ser/Thr-Pro motifs in a specific group of proteins and catalyzes their *cis–trans* isomerization to regulate protein

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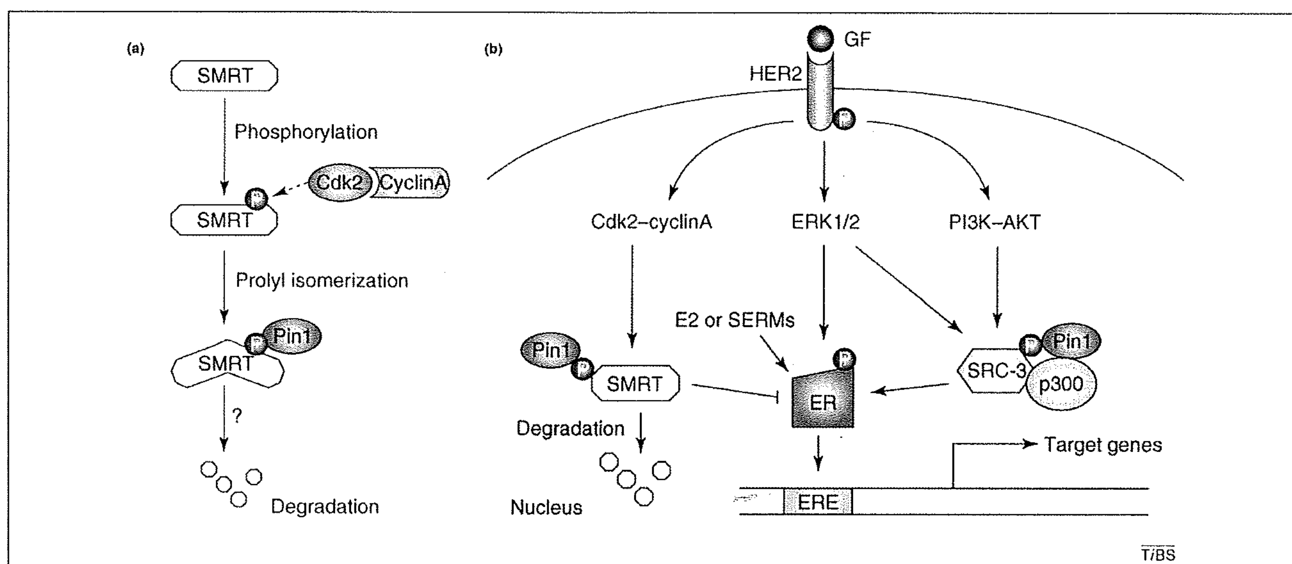


**Figure 1.** Pin1 functions as a crucial catalyst in the crosstalk between HER2 and ER. **(a)** A novel post-phosphorylation regulatory mechanism for SMRT stability. The phosphorylation of SMRT by the proline-directed kinase Cdk2 (shown with its binding partner, cyclinA) generates binding modules for the prolyl isomerase Pin1. Subsequent prolyl isomerization by Pin1 induces conformational changes and thereby enhances SMRT degradation. **(b)** Pin1 orchestrates signaling cascades from HER2 to ER. Growth-factor-mediated signaling via HER2 activates several downstream kinases, including MAPK (ERK1/2), phosphoinositide 3-kinase (PI3K)–Akt and Cdk2–cyclinA. These kinases, in turn, phosphorylate both the co-repressor SMRT and coactivator SRC-3 (shown in a complex with p300), in addition to ER, creating Pin1-binding sites. Subsequent Pin1-mediated prolyl isomerization activates ER and renders it resistant to SERMs (selective estrogen receptor modulators). These data indicate that Pin1 has a central role in the crosstalk between growth factor signaling and ER during ER-mediated transcription and tamoxifen resistance. GF, growth factor; ERE, estrogen response element.

conformation as a mode of post-phosphorylation regulation. Such Pin1-catalyzed prolyl isomerization can regulate a wide spectrum of phosphorylation-dependent activities, including protein stability [14]. Importantly, Pin1 is highly overexpressed in several human cancers including breast cancer, and its expression levels parallel the malignant properties of many tumors [14,15]. Pin1 is transcriptionally upregulated by the E2F transcription factors in response to growth factors and other stimulating conditions such as HER2 or Ras activation in breast cancer [16]. Moreover, Pin1 inhibition or deletion efficiently suppresses the ability of oncogenic HER2 or Ras to transform normal mammary epithelial cells or to induce breast cancer *in vivo* [16,17]. Thus, Pin1 is essential for growth-factor-induced breast cancer development.

A previous report indicated that Pin1 can regulate SRC-3 activity and turnover [18]. Pin1 binds to phosphorylated SRC-3 and enhances SRC-3-mediated recruitment of cAMP-response-element binding protein (CREB) binding protein (CBP; similar to p300) to the promoters of ER-dependent genes to activate transcription. In addition, Pin1 enhances SRC-3 degradation, an event that seems to promote SRC-3 turnover and sustained activation, rather than its functional attenuation, although a detailed mechanism remains elusive [18]. It remains unclear whether Pin1 can regulate transcriptional co-repressors during and after oncogenesis or whether Pin1 participates in hormone therapy resistance in conjunction with aberrant growth-factor signaling in breast cancers.

## Newly identified: Pin1-dependent SMRT regulation

Stanya and colleagues [4] now provide the first evidence that Pin1-catalyzed, phosphorylation-dependent prolyl isomerization promotes SMRT protein degradation, thereby reducing SMRT-mediated ER transcriptional repression. The authors first identified Pin1 as a SMRT interacting partner in a yeast two-hybrid screen, and they confirmed that this interaction relies upon the Pin1 Trp-Trp domain binding phosphorylated SMRT.

One of the functional consequences of the Pin1–SMRT association is to promote SMRT degradation. As Pin1 overexpression compromises SMRT repressor activity, Stanya and colleagues [4] hypothesized that Pin1 might modulate SMRT-mediated repression by affecting its protein stability, as has been shown for other Pin1 targets [14,19]. Indeed, Pin1 overexpression promoted SMRT protein degradation in a dose-dependent manner, whereas Pin1 knockdown or knockout significantly increased SMRT stability. Moreover, the effects of Pin1 knockdown on SMRT stability could be rescued by ectopic Pin1 expression, but not expression of its catalytically inactive mutant [4]. The authors thus concluded that Pin1 regulates SMRT protein half-life.

The authors then mapped the Pin1-binding sites in SMRT via mutational analysis. They also identified multiple Ser and Thr residues in SMRT that are phosphorylated *in vivo*, including two consensus Cdk target sites: Ser1241 and Ser1469. Indeed, Pin1 can interact with SMRT on these two sites *in vitro*. Furthermore, a triple mutant at 3 potential Cdk consensus sites (Ser1241Ala; Thr1445Ala; Ser1469Ala) is neither phosphorylated by Cdk2 nor pulled down by Pin1. Importantly, the SMRT triple mutant is also more stable than wild-type SMRT. In addition, Cdk2 overexpression can reduce exogenous SMRT protein levels, which can be rescued by Pin1 knockdown [4]. Furthermore, siRNA-mediated Cdk2 knockdown

**Box 1. Tamoxifen resistance in breast cancer**

Tamoxifen is currently the most widely prescribed orally active selective ER modulator for the treatment of breast cancer [21]. Although tamoxifen is an ER antagonist in breast tissue, it has variable cell-type-specific partial agonist or antagonist activities. The antagonist activity enables the drug to block ER-mediated transcription and cancer cell growth in ER-positive breast cancer cells. However, tamoxifen resistance might occur when its agonistic activity overcomes its antagonistic effect [21]. This variability could be related, in part, to the cellular milieu of ER coactivators and co-repressors [10]. For example, increased levels of coactivators, such as SRC-3, enhance the estrogen agonist properties of tamoxifen, whereas decreased levels of co-repressors, such as SMRT and N-CoR, correlate with acquired tamoxifen resistance [9,10]. Therefore, tamoxifen resistance might depend on the relative abundance or activity between co-repressors and coactivators that are often determined by HER2-iniated upstream growth-factor signaling [22].

upregulates endogenous SMRT levels. These data indicate that SMRT phosphorylation by Cdk2 is crucial for Pin1-mediated SMRT degradation.

HER2 signaling can increase Cdk activity and upregulate Pin1 expression in breast cancers [19,20]. Therefore, Stanya and colleagues [4] next explored how ER activity might be regulated by functional crosstalk between HER2 signaling and the Cdk2- and Pin1-dependent SMRT-degradation pathway. A HER2 agonist, for example heregulin, decreases SMRT protein levels, whereas a HER2 inhibitor, for example AG825, increases both SMRT half-life and protein levels. Moreover, Pin1 or Cdk2 knockdown blocks heregulin from decreasing SMRT stability [4]. These data point to HER2 functioning upstream of Cdk2 and Pin1 as a potential regulator of SMRT protein stability.

Finally, to delineate the possible role of the SMRT degradation pathway in tamoxifen resistance (Box 1), the authors investigated the effect of SMRT, Pin1 or Cdk2 knockdown on ER activity after tamoxifen treatment of ER-positive breast cancer cells. They found that tamoxifen treatment represses the expression of two different ER target genes; this effect is compromised by SMRT knockdown. By contrast, Pin1 or Cdk2 knockdown enhances the repression of both ER target genes. Moreover, SMRT knockdown increases cell proliferation, whereas Cdk2 inhibition produces a slight decrease in cell proliferation. Interestingly, Pin1 inhibition does not significantly reduce cell growth despite suppressing ER activity. It thus seems that complex crosstalk between Pin1 or Cdk2 and other proteins involved in cell growth might explain the discrepancy between ER-mediated transcription and cell proliferation on increased SMRT stability.

This new study [4] highlights the potential role for Pin1 as a target for preventing improper ER activation; moreover, it could provide important insight toward understanding the nature of tamoxifen resistance in breast cancer. What remains unclear, however, is the mechanism underlying Pin1-dependent SMRT degradation. The identification of factors involved in the Pin1-mediated SMRT-degradation pathway might uncover vital information regarding the enigmatic cell-type-specific function of Pin1 in SMRT regulation. Furthermore, relevant factors could be potential new therapeutic targets for treating hormone-resistant breast cancers. Because Pin1 can bind to many cellular proteins, it becomes important to ask whether Pin1 fosters ER-mediated transcription and hormone resistance only by decreasing SMRT stability. Indeed, because Pin1 can also increase ER function by acting on SRC-3, it could act together with HER2 signaling as a molecular determinant of the balance between ER co-repression and coactivation (Figure 1b). However, it remains unclear why Pin1-mediated degradation of both the coactivator and co-repressor triggers similar ER activation and how these events are coordinated. Further careful analysis of the interplay between Pin1 and relevant signaling pathways will be necessary to fully delineate the function of Pin1 and its target proteins in the regulation of ER and endocrine resistance in breast cancer.

## Concluding remarks

Pin1 regulates both the co-repressor SMRT and the coactivator SRC-3 as a downstream effector of HER2 signaling [4,14,16–18]. Growth-factor-receptor signaling is often increased in endocrine-resistant breast tumors, and it contributes to coactivator upregulation and co-repressor downregulation, thus activating proliferation and/or survival pathways and hormone resistance [9,10]. Interestingly, Pin1 inhibitors (if identified) potentially could be administered to re-sensitize tumors to endocrine therapies. Furthermore, such Pin1 inhibitors could be used along with hormonal therapies to block ER proliferation and/or survival activity and the agonistic effects of selective estrogen receptor modulators such as tamoxifen, which both are often deregulated by aberrant growth factor receptor signaling.

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# Pin1 Promotes Transforming Growth Factor-β-induced Migration and Invasion*

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**Transforming growth factor-β (TGF-β) regulates a wide variety of biological activities. It induces potent growth-inhibitory responses in normal cells but promotes migration and invasion of cancer cells. Smads mediate the TGF-β responses. TGF-β binding to the cell surface receptors leads to the phosphorylation of Smad2/3 in their C terminus as well as in the proline-rich linker region. The serine/threonine phosphorylation sites in the linker region are followed by the proline residue. Pin1, a peptidyl-prolyl cis/trans isomerase, recognizes phosphorylated serine/threonine-proline motifs. Here we show that Smad2/3 interacts with Pin1 in a TGF-β-dependent manner. We further show that the phosphorylated threonine 179-proline motif in the Smad3 linker region is the major binding site for Pin1. Although epidermal growth factor also induces phosphorylation of threonine 179 and other residues in the Smad3 linker region the same as TGF-β, Pin1 is unable to bind to the epidermal growth factor-stimulated Smad3. Further analysis suggests that phosphorylation of Smad3 in the C terminus is necessary for the interaction with Pin1. Depletion of Pin1 by small hairpin RNA does not significantly affect TGF-β-induced growth-inhibitory responses and a number of TGF-β/Smad target genes analyzed. In contrast, knockdown of Pin1 in human PC3 prostate cancer cells strongly inhibited TGF-β-mediated migration and invasion. Accordingly, TGF-β induction of *N*-cadherin, which plays an important role in migration and invasion, is markedly reduced when Pin1 is depleted in PC3 cells. Because Pin1 is overexpressed in many cancers, our findings highlight the importance of Pin1 in TGF-β-induced migration and invasion of cancer cells.**

Transforming growth factor-β (TGF-β)[3] is a multifunctional cytokine that controls various fundamental biological activities such as cell proliferation, differentiation, migration, adhesion, and apoptosis (1). Disruption of the TGF-β signaling pathways is associated with a number of human diseases, especially cancer (2–8). TGF-β has two opposite roles in cancer. It is a potent tumor suppressor during the early stages of tumorigenesis through its growth-inhibitory effects and apoptosis-promoting function (2–8). TGF-β promotes cancer progression and metastasis at later stages (2–8).

TGF-β signal transduction is mediated by two types of cell surface serine/threonine kinase receptors (TβRI and TβRII) and downstream effectors, the Smad family proteins (9–14). TGF-β binding induces the formation and activation of a receptor complex containing TβRI and TβRII. The activated TβRI directly phosphorylates Smad2 and Smad3 at the SSXS motif in their C-tails. The phosphorylated Smad2 and Smad3 then form a complex with Smad4 and together accumulate in the nucleus to regulate transcription of a wide variety of target genes, leading to distinct biological effects in a cell context-dependent manner (9–14).

In addition to the C-tail phosphorylation sites for the receptor kinase, Smad2 and Smad3 contain multiple serine/threonine phosphorylation sites in the proline-rich linker region that connects the N- and C-terminal domains. Among them, several phosphorylation sites are followed by the proline residue and can be phosphorylated by proline-directed kinases, such as the mitogen-activated protein kinase superfamily members that include ERK, c-Jun N-terminal kinase, and p38, the cyclin-dependent kinase family members, and glycogen synthase kinase-3 (11, 15–38).

We and others have recently shown that three sites Thr-179, Ser-204, and Ser-208 in the Smad3 linker region are phosphorylated in response to TGF-β (30, 36–38) and that the cy-

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[3] The abbreviations used are: TGF-β, transforming growth factor-β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; EGF, epidermal growth factor; ERK, extracellular-signal regulated kinase; Smurf2, Smad ubiquitination regulatory factor 2; Pin1, protein interacting with NIMA (never in mitosis A); shRNA, small hairpin RNA; GST, glutathione *S*-transferase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; FBS, fetal bovine serum; EPSM, Erk/proline-directed kinase site mutant.



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clin-dependent kinase family members and glycogen synthase kinase-3 are responsible for the phosphorylation (36–38). The Thr-179 and Ser-208 are phosphorylated by the cyclin-dependent kinase family members in response to TGF-β (36), whereas the Ser-204 is phosphorylated by glycogen synthase kinase-3 in response to TGF-β (36, 37). We have further shown that the C-tail phosphorylation is necessary for the linker phosphorylation in response to TGF-β (36). When the C-tail phosphorylation sites in Smad3 are mutated, the linker sites are not phosphorylated in response to TGF-β (36).

Interestingly, these three same sites, Thr-179, Ser-204, and Ser-208, in the Smad3 linker region are also phosphorylated by ERK in response to EGF treatment and Ser-208 is the best ERK phosphorylation site in Smad3 (29). The ERK phosphorylation does not require the C-tail phosphorylation. When the C-tail phosphorylation sites in Smad3 are mutated, ERK still phosphorylates these three sites in response to EGF treatment (36).

The Smad2 linker region is also phosphorylated in response to TGF-β or EGF (11, 15–17, 19–25, 30, 31, 36). However, due to the lack of specific phosphopeptide antibodies against each of the putative phosphorylation sites and the lack of mapping of the phosphorylation sites by other methods, the exact sites in the Smad2 linker region that are phosphorylated in response to TGF-β or EGF remain to be determined.

Pin1 is a peptidyl-prolyl cis/trans isomerase that recognizes the phosphorylated serine/threonine-proline motifs in certain proteins and catalyzes prolyl cis/trans isomerization (39–41). The prolyl isomerization induces conformational changes, leading to distinct effects in different target proteins, such as increased stability, increased turnover, alteration of the sensitivity to phosphatases, alteration in subcellular localizations, and altered enzymatic activities, and enabling protein-protein interactions (39–41). Since its discovery, a number of Pin1 targets have been identified, indicating the importance of Pin1 in the cellular physiology (39–41). The various studies also suggest that the effects of Pin1 are cell context-dependent (39–41). Pin1 is overexpressed in many cancers, such as in prostate, breast, lung, colon, and hepatocellular carcinoma (39–51). In prostate cancer, overexpression of PIN1 is correlated with a higher probability of tumor recurrence and a shorter period to tumor recurrence after radical prostatectomy (52). In addition to cancer, Pin1 is also linked with other diseases, such as Alzheimer disease and asthma (39, 41).

Because the Thr-179, Ser-204, and Ser-208 phosphorylation sites in Smad3 are followed by the proline residue, they constitute putative Pin1 binding sites. It is possible that certain or all of these sites serve as the Pin1-binding site(s) when they are phosphorylated. Recently, Nakano *et al.* (53) reported that Pin1 can associate with Smad2 and Smad3 to enhance their interaction with Smurf2 (Smad ubiquitination regulatory factor 2), a HECT domain E3 ubiquitin ligase, resulting in enhanced Smad ubiquitination and reduced Smad2/3 levels. We show in this report that Pin1 binds to Smad2/3 in a TGF-β-dependent manner and that the phosphorylated Thr-179-proline is the major binding site for Pin1 in Smad3 in response to TGF-β. We further show that knockdown of Pin1 does not have a significant effect on TGF-β-induced growth-inhibitory response, as analyzed in the human HaCaT keratinocytes. On the contrary, knockdown of Pin1 in human PC3 prostate cancer cells significantly inhibited TGF-β-induced cell migration and invasion. Our study uncovered an important role of Pin1 in TGF-β-mediated cancer cell migration and invasion.

## EXPERIMENTAL PROCEDURES

*Constructs, Antibodies, and Chemical Inhibitors*—Mammalian expression plasmids for Smad3, its phosphorylation mutants, and TβRI were described previously (26, 36). Plasmids for GST-Pin1, pSUPER-puro-shRNA against Pin1 or the scrambled control were also described previously (54, 55). The Smad3-specific peptide antibody and the Smad2-specific peptide antibody were from Invitrogen. The Smad2/3 antibody that was raised against the full-length Smad3 and recognizes both Smad2 and Smad3, the Pin1 polyclonal antibody, the SIP1 polyclonal antibody, and the actin antibody was purchased from Santa Cruz Biotechnology, Inc. The N-cadherin antibody was purchased from Upstate Biotechnology. The Slug monoclonal antibody (clone 2B6) was obtained from the Millipore Corp. The Snail polyclonal antibody was from ABGENT. The E-cadherin monoclonal antibody (clone 67A4) was from Chemicon International. Smad3 phospho-specific antibodies were described previously (26, 36). The Smad3 (C-tail) phosphopeptide antibody was generously provided by Dr. Edward B. Leof (Mayo Clinic Cancer Center, Rochester, MN). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce or Chemicon International. ECL Western blot reagents were purchased from Roche Applied Science or Millipore Corp. PiB, diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[lmn][3,8]phenan throline-2,7-diacetate, a Pin1 inhibitor, was purchased from Merck.

*Cell Culture, Transfection, and Retroviral Infection*—Human PC3 prostate cancer cell line was cultured in RPMI1640 with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Amphotropic Phoenix cells (Phoenix A) were grown in Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin/streptomycin. Human HaCaT keratinocytes and human HEK293T cells were cultured in minimum essential medium, 10% FBS, 1% penicillin/streptomycin. L17, a TβRI-deficient cell line derived from the Mv1Lu mink lung epithelial cell line, was maintained in minimum essential medium without histidine but plus histidinol, 10% FBS, 1% penicillin/streptomycin. The L17 cells and the 293T cells were transfected by DEAE-dextran and Lipofectamine Plus reagent (Invitrogen), respectively, as previously described (26, 36). For shRNA retrovirus production, Phoenix A cells were transfected with pSUPER-puro-shRNA targeting Pin1 or a scrambled control by Lipofectamine Plus reagent. HaCaT and PC3 cells were infected several times with the retrovirus targeting Pin1 or the scrambled control. The infected cells were selected with 5 μg/ml puromycin.

*GST Pulldown Assay and Coimmunoprecipitation Assay*—GST and GST-Pin1 were expressed in bacteria strain BL21 (DE3). GSH-Sepharose beads containing GST or GST-Pin1 were prepared as described previously (26). For analysis of Smad interaction with GST-Pin1, HaCaT cells were serum-starved before the addition of EGF or TGF-β. Cells were then treated with either 50 ng/ml EGF for 15 min or 300 pM TGF-β for 1 h for maximal induction of Smad3 phosphorylation in the linker sites. Cells were lysed with the lysis buffer (10 mM Tris-

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Cl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1× complete protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 μg/ml RNase A). Cell lysates (300 μg) were incubated with 10 μl of either GST or GST-Pin1 beads at 4 °C for 2 h. The beads were washed with the cell lysis buffer 5 times, and the bound proteins on the beads were eluted by SDS sample buffer. For analysis of Smad3 and its mutants for interaction with GST-Pin1, L17 cells were cotransfected with expression plasmids for wild type or a mutant Smad3 together with TβRI, and the cells were treated with or without TGF-β for 1 h. Alternatively, the HEK293T cells were transfected with the wild type Smad3 or a mutant Smad3 and treated with TGF-β for 1 h. The cell lysates were subjected to GST pulldown assays as described above.

For coimmunoprecipitation assays to detect Smad2/3 and Pin1 interaction at endogenous levels, HaCaT cells were treated with or without TGF-β for 1 h, and the cell lysates (800 μg) were precleared by incubation with 20 μl of protein A/G beads at 4 °C for 0.5 h. The precleared lysates were then incubated with 2.5 μg of either control IgG or the Smad2/3 antibody at 4 °C overnight. Protein A/G-beads were then added and incubated for 2 h. The beads were washed 5 times with the lysis buffer and eluted with SDS sample buffer. Proteins eluted from the beads were subjected to Western blot analysis using the polyclonal Pin1 antibody by fresh ECL reagents (Roche Applied Science).

*Thymidine Incorporation Assay*—[$^3$H]Thymidine incorporation assay was performed as described previously (26). In brief, $2 \times 10^5$ shRNA-transduced HaCaT cells were seeded on 6-well plates in triplicate for 24 h and then treated with or without TGF-β at various concentrations for 24 h. 5 μCi of [$^3$H]thymidine was added to each well during the last 4 h of the incubation. Cells were then washed 3 times with phosphate-buffered saline, fixed with 95% methanol, and extracted with 0.2 N NaOH. The extracts were counted for radioactivity. The results represent the average and S.D. of three independent experiments.

*RNA Preparation and Northern Blot Analysis*—shRNA-transduced HaCaT cells were treated with or without 300 pM TGF-β for 8 h. Poly(A)$^+$ RNA was prepared by the FAST track kit (Invitrogen). Northern blot was performed as previously described (56). Briefly, poly(A)$^+$ RNA was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a random primed $^{32}$P-labeled probe. The following probes were used: Pin1, p15, p21, Smad7, JunB, PAI-1, Bcl-2, Bub-1, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

*Cell Migration and Invasion Assays*—Cell migration and invasion assays were performed essentially as previously described (57, 58). shRNA-transduced PC3 cells were serum-starved (0.2% FBS) overnight and then treated with or without 500 pM TGF-β for 48 h. The cells were trypsinized and resuspended in RPM1640, 0.1% bovine serum albumin at $3.3 \times 10^5$ cells/ml for migration assay and at $6.6 \times 10^5$ cells/ml for invasion assay. 300 μl of the suspension was applied onto a Millicell Hanging Cell Culture Insert (8 μm pore size, Millipore) in 24-well plates with the media containing 10% FBS at the bottom. For the invasion assay, the insert was precoated with Matrigel (BD Biosciences, 1:4 diluted with RPM1640, 0.1% bovine serum albumin). TGF-β (500 pM) was included in the medium for those cells that had been treated with TGF-β. The cells were incubated at 37 °C for 15 h for migration assay or 24 h for invasion assay. Medium was removed from the insert, and the upper side of the membranes was wiped with a cotton swab to remove un-migrated cells. Migrated cells at the bottom of the insert were stained with 0.1% crystal violet, 20% ethanol, and 1% formaldehyde. The number of migrated cells was counted for quantification. The results represent the average and S.D. of four independent experiments.

## RESULTS

*Pin1 Binds to Smad2/3 Specifically in a TGF-β-dependent Manner*—We previously showed that phosphorylation of three serine/threonine residues (Thr-179, Ser-204, and Ser-208) in the proline-rich linker region of Smad3 is induced by both EGF and TGF-β (29, 36). These phosphorylation sites are followed by the proline residue, thus constituting putative Pin1 binding sites. We therefore examined the possibility of whether Smad3 binds to Pin1 in response to EGF or TGF-β. We treated human HaCaT keratinocytes with either EGF or TGF-β. The cell lysates were then subjected to GST pulldown assays with GST-Pin1 beads or the control GST beads followed by immunoblot with an antibody against Smad3. As shown in Fig. 1*A*, Pin1 binds specifically to the TGF-β-stimulated Smad3. To confirm this result, we analyzed the precipitates by immunoblot with phospho-specific antibodies against Thr-179, Ser-204, and Ser-208 in Smad3. Although the phosphorylation of Thr-179, Ser-204, and Ser-208 in Smad3 was induced to a similar extent by EGF and TGF-β (Fig. 1*A*), the phosphorylated Thr-179, Ser-204, and Ser-208 were detected in GST-Pin1 beads only in response to TGF-β treatment (Fig. 1*A*). Very little of Thr(P)-179, Ser(P)-204, or Ser(P)-208 was detected in the GST-Pin1 beads in response to EGF treatment (Fig. 1*A*).

Because the linker region of Smad2 is also phosphorylated in response to EGF or TGF-β (11, 15–17, 19–25, 30, 31, 36), we analyzed whether Pin1 binds to Smad2 after EGF or TGF-β treatment. HaCaT cells were treated with EGF or TGF-β. The cell lysates were incubated with GST-Pin1 beads or the GST control beads. The precipitates were then analyzed by immunoblots with an antibody against Smad2. As shown in Fig. 1*B*, Pin1 binds to Smad2 in response to TGF-β but not in response to EGF.

To provide further evidence that Smad2/3 binds to Pin1 in response to TGF-β, we analyzed whether Smad2/3 interacts with Pin1 after TGF-β treatment at endogenous levels. HaCaT cells were treated with or without TGF-β. Cell lysates were then immunoprecipitated with a Smad2/3 antibody that was raised against the full-length Smad3 and recognizes both Smad3 and Smad2. The immunoprecipitates were then analyzed by immunoblot with a Pin1-specific antibody. As shown in Fig. 1*C*, Pin1 binds to Smad2/3 in response to TGF-β at endogenous levels.

*Pin1 Binding Requires Smad3 Phosphorylation in the C-tail and at the Thr-179 Site in the Linker Region*—As described above, although EGF and TGF-β induces the phosphorylation of the same sites in the Smad3 linker region, Pin1 binds only to TGF-β-induced Smad3. We reasoned that there should be

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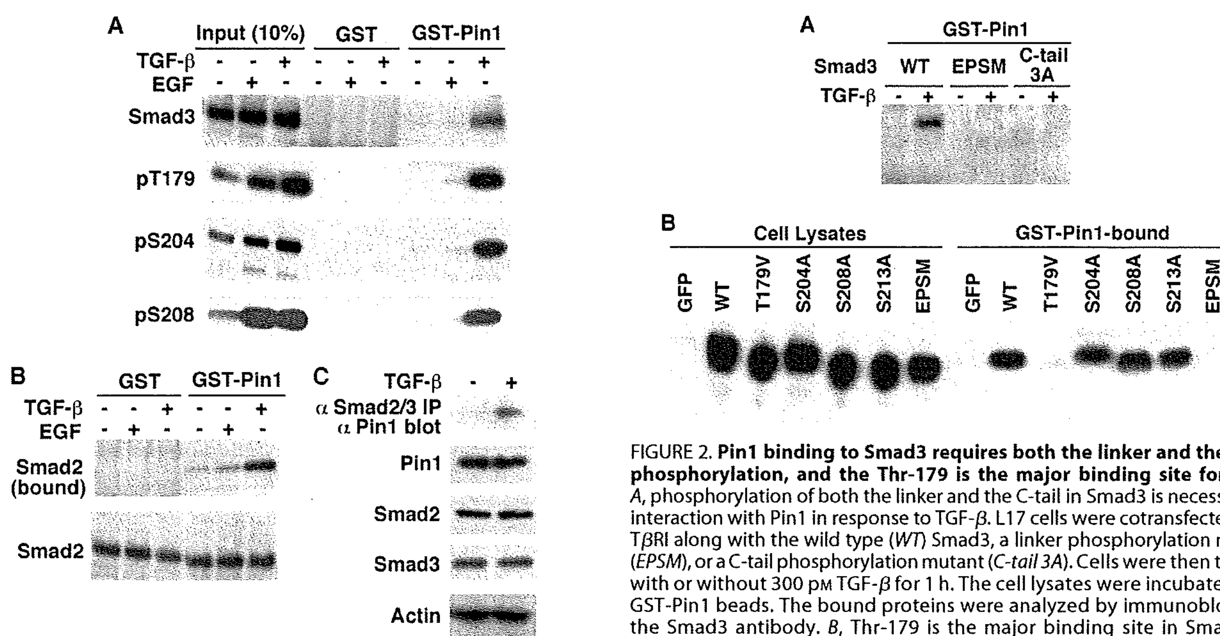


FIGURE 1. **Pin1 binds to Smad2/3 in a TGF-β-dependent manner.** *A*, TGF-β, but not EGF, induces Pin1-Smad3 interaction. HaCaT cells were treated with 300 pM TGF-β for 1 h or with 50 ng/ml EGF for 15 min for maximal induction of Smad3 phosphorylation in the linker region. The cell lysates were incubated with either GST or GST-Pin1 beads. The bound proteins were analyzed by immunoblot with an antibody against Smad3. The bound proteins were also analyzed by immunoblot with specific phosphopeptide antibodies against the phosphorylated Thr-179, Ser-204, and Ser-208 in the Smad3 linker region. *B*, TGF-β, but not EGF, induces Pin1-Smad2 interaction. HaCaT cells were treated with TGF-β or EGF as described in *A*. The cell lysates were incubated with GST or GST-Pin1 beads. The bound proteins were analyzed by immunoblot with an antibody against Smad2. The Smad2 levels in the cell lysates were also analyzed as a control. *C*, TGF-β induces Pin1-Smad2/3 interactions at endogenous protein levels. HaCaT cells were treated with or without TGF-β for 1 h. The cell lysates were immunoprecipitated with an antibody that was raised against the full-length Smad3 and recognizes both Smad3 and Smad2. The precipitates were analyzed by immunoblot with an antibody against Pin1. The expression levels of Pin1, Smad2, Smad3, and actin in the cell lysates were also analyzed as controls.

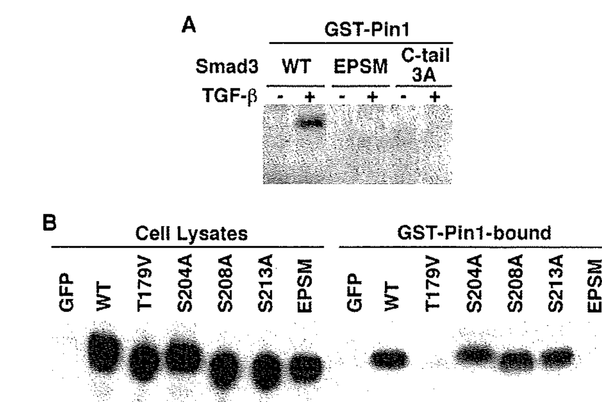


FIGURE 2. **Pin1 binding to Smad3 requires both the linker and the C-tail phosphorylation, and the Thr-179 is the major binding site for Pin1.** *A*, phosphorylation of both the linker and the C-tail in Smad3 is necessary for interaction with Pin1 in response to TGF-β. L17 cells were cotransfected with TβRI along with the wild type (*WT*) Smad3, a linker phosphorylation mutant (*EPSM*), or a C-tail phosphorylation mutant (*C-tail 3A*). Cells were then treated with or without 300 pM TGF-β for 1 h. The cell lysates were incubated with GST-Pin1 beads. The bound proteins were analyzed by immunoblot with the Smad3 antibody. *B*, Thr-179 is the major binding site in Smad3 for interaction with Pin1 in response to TGF-β. HEK293T cells were transfected with the wild type Smad3, a linker phosphorylation mutant Smad3, or a control plasmid that encodes GFP. Cells were treated with 300 pM TGF-β for 1 h. The cell lysates were incubated with the GST-Pin1 beads. The bound proteins were analyzed by immunoblot with the Smad3 antibody. The expression levels of the various Smad3 proteins were also analyzed as a control.

other components in Smad3 that contributes to this specificity. In addition to the linker sites, TGF-β induces phosphorylation of the C-tail of Smad3. Because this phosphorylation was absent in EGF-stimulated Smad3, we tested whether mutation of the C-tail phosphorylation sites had any effect on TGF-β-dependent Smad3-Pin1 interaction. We transfected the L17 cells with TβRI along with the wild type Smad3, its linker region Erk/proline-directed kinase site mutant (EPSM) that contained mutations at the four putative Pin1 sites, or the C-tail phosphorylation site mutant (C-tail 3A). As shown in Fig. 2*A*, upon TGF-β treatment, Smad3 formed a complex with Pin1. Consistent with the idea that the Pin1-binding site(s) is located in the linker region, the EPSM mutant was unable to bind to Pin1 (Fig. 2*A*). Interestingly, the C-tail 3A mutant also abolished the Pin1 binding (Fig. 2*A*), although it still contains the intact Pin1-binding site(s) in the linker region. Our previous study has shown that when the Smad3 C-tail is mutated, the TGF-β-induced linker phosphorylation is abolished but has no effect on EGF-induced linker phosphorylation (36), suggesting that the C-tail phosphorylation is necessary for the linker phosphorylation in response to TGF-β. Thus, abrogation of Pin1 binding by the C-tail 3A mutant suggests that the C-tail phosphorylation by the receptor kinase is necessary for Smad3 binding to Pin1. Although EGF and TGF-β induce phosphorylation at the same linker sites, the structure of Smad3 at the C termini is different under the two conditions. Pin1 binding may require a discrete C termini structure, which results from phosphorylation of the SS*X*S motif (10). Taken together, our result suggests that Pin1 binding requires the phosphorylation of both the C-tail and the linker.

In the Smad3 linker region there are four putative Pin1 binding sites. These include the Thr-179, Ser-204, and Ser-208. In addition, it includes the Ser-213 site, which is phosphorylated by cyclin-dependent kinase at the basal state (26). We mutated these four sites individually. GST pulldown assays with these mutants revealed that Thr-179 → Val mutation (T179V) dramatically reduced Pin1 binding in response to TGF-β. In contrast, mutation of any of the other three sites had little effects (Fig. 2*B*). As a control, the EPSM mutant was unable to bind to the Pin1 (Fig. 2*B*). Thus, we conclude that Thr-179 is the major Pin1-binding site in Smad3.

*Depletion of Pin1 Does Not Have a Significant Effect on the Smad2/3 Levels, on TGF-β-induced Smad3 Linker Phosphorylation, or on TGF-β-induced Smad2/3 Nuclear Accumulation*—To determine the consequence of Pin1 binding in TGF-β signaling, we generated stable cell lines in HaCaT cells that knock down the expression of Pin1 or a scrambled control. As shown in Fig. 3*A*, Pin1 is very effectively depleted by shRNA against Pin1. The depletion of Pin1 has little effect on Smad2 or Smad3 levels in HaCaT cells (Fig. 3*A*).

Because Pin1 binding to Smad3 may regulate TGF-β signaling by affecting the phosphorylation levels at Thr-179 and potentially also at the Ser-204 and Ser-208 sites, we analyzed

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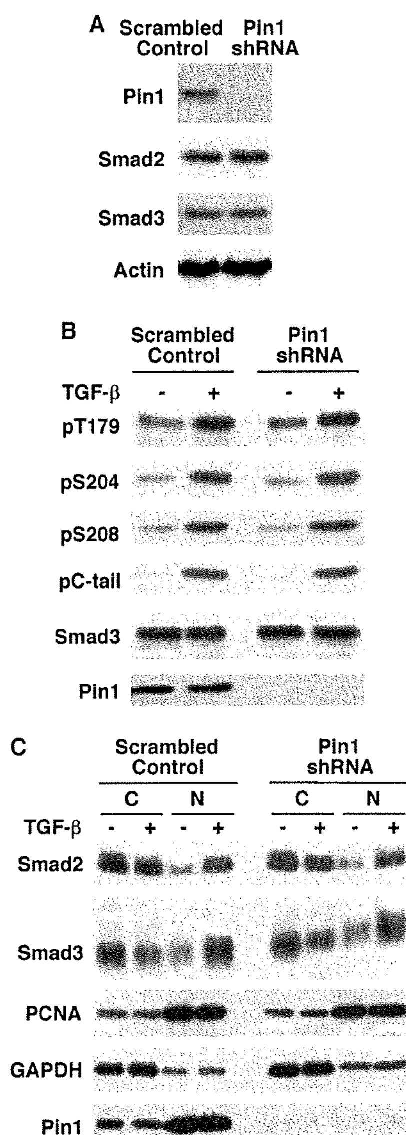


FIGURE 3. **Knockdown of Pin1 does not have a significant effect on Smad2/3 levels, on TGF-β-induced linker and C-tail phosphorylation of Smad3, or on TGF-β-induced Smad2/3 nuclear accumulation.** *A*, depletion of Pin1 has little effect on Smad2/3 levels in HaCaT cells. Stable HaCaT cell lines with an shRNA targeting Pin1 or the scrambled control were generated. The cell lysates were analyzed by immunoblot for Pin1, Smad2, and Smad3 levels. Actin levels were also analyzed as a control. *B*, depletion of Pin1 does not have a significant effect on TGF-β-induced linker and C-tail phosphorylation of Smad3 in HaCaT cells. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. The cell lysates were analyzed by immunoblot with phosphopeptide antibodies against the phosphorylated Thr-179, Ser-204, Ser-208, and the C-tail. Smad3 and Pin1 expression levels were also analyzed as controls. *C*, depletion of Pin1 does not affect TGF-β-induced Smad2/3 nuclear accumulation in HaCaT cells. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. Cells were then harvested and fractionated into the cytoplasmic (*C*) and nuclear (*N*) fractions. The same amount of proteins from the cytoplasmic fraction and nuclear fraction was analyzed for Smad2, Smad3, and Pin1 levels by immunoblot. GAPDH and proliferating cell nuclear antigen (*PCNA*) serve as cytoplasmic and nuclear markers, respectively.

whether Pin1 knockdown affected the phosphorylation levels of Thr-179, Ser-204, or Ser-208 in response to TGF-β. As shown in Fig. 3*B*, depletion of Pin1 did not have a significant effect on the TGF-β-induced phosphorylation levels at these three sites or a significant effect on the Smad3 C-tail phosphorylation in response to TGF-β (Fig. 3*B*).

We also analyzed whether depletion of Pin1 affects TGF-β-induced nuclear accumulation of Smad2 and Smad3. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. Cells were then harvested and fractionated into the cytoplasmic and nuclear fractions. As shown in Fig. 3*C*, knockdown of Pin1 does not affect Smad2 or Smad3 nuclear accumulation in response to TGF-β. Pin1 is predominantly localized in the nucleus as previously reported (59), and its subcellular localization was not affected by TGF-β treatment (Fig. 3*C*). The GAPDH serves as a marker for cytoplasmic localization, whereas the proliferating cell nuclear antigen serves as a marker for nuclear localization.

*Depletion of Pin1 Does Not Have a Significant Effect on TGF-β-induced Growth-inhibitory Effects*—One of the major functions of the TGF-β/Smad pathway is to induce growth inhibition. We therefore analyzed whether Pin1 knockdown affected TGF-β/Smad-mediated growth inhibition in HaCaT cells, which are strongly inhibited by TGF-β. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β at various doses and then subjected to [$^3$H]thymidine incorporation assay. As shown in Fig. 4*A*, the scrambled control HaCaT cells are highly sensitive to TGF-β, and the Pin1 knockdown HaCaT cells are overall similar to the scrambled control in terms of TGF-β sensitivity. At 5 pM TGF-β, the Pin1 knockdown cells are a little more sensitive to TGF-β than the control cells (Fig. 4*A*). At a higher concentration of TGF-β, the control HaCaT cells are slightly more sensitive to TGF-β (Fig. 4*A*). Although we don't understand why there is a subtle difference in TGF-β sensitivity at different concentrations between the control cells and the Pin1 knockdown cells, the results clearly indicate that knockdown of Pin1 does not have a significant effect on TGF-β-induced growth inhibition.

Previous studies have shown that TGF-β/Smad-mediated growth inhibition resulted from changes in the expression of cell cycle-related genes, such as induction of the expression of the cyclin-dependent kinase inhibitors p15 and p21 (2, 14, 60–65). We therefore analyzed whether knockdown of Pin1 affected several TGF-β/Smad target genes. The control HaCaT cells and the Pin1 knockdown HaCaT cells were treated with or without TGF-β. Poly(A)$^+$ RNA were isolated from the cells and then subjected to Northern blot analysis. As a control, the Northern blot confirmed that Pin1 expression was essentially abolished in the knockdown cells (Fig. 4*B*). Consistent with the [$^3$H]thymidine incorporation assay above, TGF-β induction of p15 and p21 was similar between the control cells and the Pin1 knockdown cells (Fig. 4, *B* and *C*). We also analyzed several other TGF-β/Smad target genes, including Smad7, JunB, and PAI-1 (2, 14, 56, 66–69). As shown in Fig. 4, *B* and *C*, TGF-β induction of Smad7, JunB, and PAI-1 was overall similar between the control cells and the Pin1 knockdown cells. For JunB, the basal levels and TGF-β-induced levels are modestly higher in Pin1 knockdown cells than in control cells. For PAI-1, the basal levels and TGF-β-induced levels are slightly higher in the control cells than in the Pin1 knockdown cells. We also analyzed the expression of Bcl2, which is reduced in response to

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