The peptidyl-prolyl isomerase PIN1 relieves cyclin-dependent kinase 2 (CDK2) inhibition by the CDK inhibitor p27

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PIN1 is a peptidyl-prolyl isomerase that catalyzes the cis/trans isomerization of peptide bonds between proline and phosphorylated serine/threonine residues. By changing the conformation of its protein substrates, PIN1 increases the activities of key proteins that promote cell cycle progression and oncogenesis. Moreover, it has been shown that PIN1 stabilizes and increases the level of the cyclin-dependent kinase (CDK) inhibitor p27, which inhibits cell cycle progression by binding cyclin A– and cyclin E–CDK2. Notwithstanding the associated increase in the p27 level, PIN1 expression promotes rather than retards cell proliferation. To explain the paradoxical effects of PIN1 on p27 levels and cell cycle progression, we hypothesized that PIN1 relieves CDK2 inhibition by suppressing the CDK inhibitory activity of p27. Here, we confirmed that PIN1-expressing cells exhibit higher p27 levels but have increased CDK2 activities and higher proliferation rates in the S-phase compared with Pin1-null fibroblasts or PIN1-depleted hepatoma cells. Using co-immunoprecipitation and CDK kinase activity assays, we found that PIN1 binds the phosphorylated Thr187–Pro motif in p27 and reduces p27’s interaction with cyclin A– or cyclin E–CDK2, leading to increased CDK2 kinase activity. In conclusion, our results indicate that although PIN1 increases p27 levels, it also attenuates p27’s inhibitory activity on CDK2 and thereby contributes to increased G1–S phase transitions and cell proliferation.

PIN1 catalyzes cis/trans isomerization of the peptide bond between phosphorylated serine or threonine and proline (pSer/Thr–Pro). It specifically binds proteins containing the pSer/Thr–Pro motifs through its amino-terminal WW domain and catalyzes the cis/trans isomerization with its carboxyl-terminal prolyl isomerase (PPlase) domain (1). PIN1-catalyzed isomerization mediates conformational changes of the bound proteins, thereby modulating their cellular functions, including catalytic activity, protein–protein interaction, subcellular localization, and protein stability (1). Through this mechanism, PIN1 regulates cell cycle progression, cell proliferation, and differentiation. Over-expression of PIN1 is observed in many cancers and is implicated in oncogenesis (2, 3). Previously, we demonstrated that PIN1 is over-expressed in more than 50% of human hepatocellular carcinoma (4, 5). PIN1 promotes tumor progression through up-regulation of cyclin D1 and β-catenin, enhancing the oncogenic property of the X-protein of hepatitis B virus, and modulation of the anti-apoptotic function of survivin (6–8). In general, PIN1 expression is positively associated with increased cell proliferation.

Cyclin-dependent kinase (CDK)3 inhibitor p27 retards cell cycle progression by inhibiting cyclin A– and cyclin E–CDK kinases (9, 10), and typically functions as a tumor suppressor (11). To allow entry of cell cycle and progression through the G1–S-phase transition, the protein level of p27 is tightly and precisely regulated with the maximal protein level at quiescent (Go) and early G1 phases (11). In a normal proliferating cell, synthesis of both cyclins and CDKs are required for cell progression through the G1 phase. On the other hand, the maximal p27 protein level results in the inhibition of cyclin E–CDK2 at early G1 phase to prevent premature entry to S-phase. When the protein level of cyclin E increases, it binds and activates CDK2, which in turn phosphorylates p27 on Thr187. Phosphorylation of p27 at Thr187 allows it to be targeted by the SCFskp2 ubiquitin ligase complex for protein degradation (12–15). Decreasing the protein level of p27 restores cyclin A– and cyclin E–CDK2 activities, initiating cell cycle progression through the G1–S-phase transition.

Interestingly, p27 is also regulated by PIN1 at the post-translational level. PIN1 binds p27 and inhibits its degradation by proteasome through reducing its interaction with Cks1, a subunit of the SCFskp2 ubiquitin ligase complex, and its ubiquitination (16). As a result, PIN1 expression stabilizes and increases the p27 level. Despite its effect on increasing the p27 level, PIN1 expression also promotes cell proliferation and cell cycle progression. To address this paradoxical phenomenon, we hypothesized that PIN1 also modulates p27 qualitatively and suppresses its inhibitory effect on cyclin A– and cyclin E–CDK2, negating the effect of increased p27 level.

**Results**

**PIN1-expressing cells exhibited increased CDK2 kinase activity and higher proliferation rate**

Western immunoblot demonstrated that wild-type mouse embryonic fibroblasts (MEFs) possessed a higher p27 level as compared with Pin1-null (PIN1KO) MEFs (Fig. 1A). Restoring the expression of wild-type PIN1, but not the PIN1 W34A

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This article contains Figs. S1–S7.

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3 The abbreviations used are: CDK, cyclin-dependent kinase; MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; pRb, retinoblastoma protein.
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Figure 1. PIN1-expressing MEFs possessed higher proliferation rate and CDK2 kinase activity in S-phase. A, PIN1 enhanced p27 level. Endogenous p27 protein expression was compared between wild-type (WT) and PIN1KO MEFs. In addition, PIN1KO MEFs were transiently transfected with plasmids encoding wild-type PIN1 and the respective mutant (W34A) or the relevant control for 48 h. Western blot analysis was performed on whole cell lysates. β-Actin was used as an internal control. B, PIN1 expression enhanced cell proliferation. MTT assay was performed to compare the cell proliferation as follows: top, PIN1KO MEFs reconstituted with wild-type PIN1 or mutants (W34A or K63A), and bottom, WT MEFs transfected with siRNA against PIN1 (siPIN1) or control siRNA (siCTL). Data presented were mean ± S.D. from six independent experiments. **, p < 0.01; *, p < 0.05. C and D, PIN1 expression increased the proportion of S-phase proliferating cells. C, flow cytometric analysis of BrdU-positive cells and DNA content generated cell cycle profiles. D, BrdU-positive cells were compared as follows: wild-type (WT) versus PIN1KO MEFs, WT MEFs transfected with siRNA against PIN1 (siPIN1) versus control siRNA (siCTL), and PIN1KO MEFs transiently transfected with wild-type PIN1 versus the respective mutants (W34A or K63A). In addition, the proportions of G1 and G2–M phase cells are summarized in the supporting data (Fig. S1). *, p < 0.05. E, WT MEFs showed significantly higher CDK2 kinase activities. WT and PIN1KO MEFs were subjected to immunoprecipitation against cyclin A, cyclin E, and both cyclins A and E. CDK2 kinase activity was determined by in vitro fluorescence-based kinase assay. The kinase activity was normalized to the amount of CDK2 recovered from immunoprecipitated cyclin A and/or cyclin E (Fig. S2). **, p < 0.01; *, p < 0.05.

mutant, with a mutation in the WW domain defective for protein binding (17), in PIN1KO MEFs resulted in an increase in the p27 protein level (Fig. 1A). These findings were consistent with the previous report that PIN1 enhances protein stability of p27 (16). Despite the higher p27 level, we found that restoration of the wild-type PIN1, but not the PIN1-binding mutant W34A or the PPIase domain mutant K63A defective for isomerase activity (18, 19), in PIN1KO MEFs enhanced cell proliferation (Fig. 1B). In addition, down-regulation of PIN1 by siRNA in wild-type MEFs resulted in decreased cell proliferation (Fig. 1B). These results demonstrated that PIN1 promoted cell proliferation. To further examine whether a higher p27 protein level could delay S-phase entry and reduce the proportion of S-phase-proliferating cells, we compared the proportion of cells in S-phase in wild-type and PIN1KO MEFs with a BrdU assay in asynchronously growing cells. Interestingly, wild-type cells, which have a higher p27 expression, had a higher proportion of BrdU-positive cells as compared with the PIN1KO cells (Fig. 1, C and D). In addition, an increase in proportion of BrdU-positive cells was demonstrated in PIN1KO MEFs transfected with wild-type PIN1, but not with PIN1 mutants W34A or K63A (Fig. 1, C and D). Similarly, down-regulation of PIN1 by siRNA in wild-type MEFs resulted in a decrease in the BrdU-positive cells, suggesting that PIN1 expression promoted G1–S
phase transition (Fig. 1, C and D). As active CDK2 is essential for S-phase progression, we compared the CDK2 kinase activity between wild-type and PIN1KO MEFs. Immunoprecipitation was performed with specific antibodies against cyclin A and/or cyclin E, and the co-immunoprecipitated cyclin–CDK2 was then subjected to an in vitro fluorescence-based kinase activity assay. The kinase assay showed that wild-type MEFs displayed significantly higher cyclin A–CDK2, cyclin E–CDK2, and total CDK2 kinase activities as compared with those of PIN1KO cells (Fig. 1E). These results suggested that PIN1 expression increased CDK2 kinase activity, facilitating S-phase progression.

**PIN1 did not regulate subcellular localization of p27**

In addition to the quantity of p27, subcellular localization of p27 will also affect its inhibition on CDK2 activity (20, 21). p27 resides in the cell nucleus, and translocates to the cytoplasm when it is phosphorylated at Ser10 during the G0/G1 phase of the cell cycle (22, 23). Only nuclear p27 efficiently inhibits CDK2 kinase activity, whereas cytoplasmic p27 regulates cytoskeleton dynamics (21). To examine if PIN1 may also alter the subcellular localization of p27, rendering p27 ineffective in inhibiting CDK2, we investigated the subcellular localization of PIN1 and p27 in wild-type and PIN1KO MEFs. Immunostaining and fluorescence microscopy revealed that both wild-type and PIN1KO cells showed a similar nuclear staining pattern of p27 (Fig. S3), indicating that PIN1 expression did not affect the subcellular localization of p27.

**PIN1 did not modulate cyclin A and cyclin E levels during S-phase progression**

Cyclin A and cyclin E bind and activate CDK2, and their levels control CDK2 kinase activity (24, 25). Cell cycle progression from S to G2–M phase of wild-type and PIN1KO cells were investigated. Cell cycle profiles showed that most of the wild-type and PIN1KO cells were still retarded at the S-phase from 1 to 4 h after release from the double thymidine block (Fig. S4A). To determine whether the protein levels of cyclin A and cyclin E change during S-phase progression, Western blotting was performed at different time points after release from the double thymidine block. Our results showed that comparable levels of CDK2 were found during S-phase progression between wild-type and PIN1KO cells (Fig. S4B). Also, the higher level of p27 in wild-type MEFs was maintained during S-phase progression. More importantly, both wild-type and PIN1KO MEFs had comparable cyclin E protein levels, and PIN1KO cells had an even higher cyclin A level than wild-type MEFs. These results suggested that the lower CDK2 kinase activity observed in PIN1KO MEFs was not due to reduction in cyclin A and cyclin E levels.

**PIN1 reduced p27 binding to cyclin and its inhibitory effect on CDK2 activity**

Through PIN1-catalyzed isomerization, PIN1 has been shown to induce a conformational change of p27 to reduce its interaction with Cks1 (16). This led us to hypothesize that PIN1 might also inhibit the interaction between p27 and cyclin A–and cyclin E–CDK2, resulting in higher CDK2 activities. To examine this hypothesis, co-immunoprecipitation experiments were carried out with cell lysates from wild-type and PIN1KO cells. Western blot analysis demonstrated that less cyclin A and cyclin E was co-immunoprecipitated with p27 from wild-type cells as compared with PIN1KO cells (Fig. 2A). In the reciprocal experiments, the reduced p27 level was detected in the cyclin A or cyclin E immunoprecipitates from wild-type cells as compared with PIN1KO cells (Fig. 2, B and C). Likewise, wild-type MEFs with PIN1 knocked-down showed more cyclin A and cyclin E in the p27 immunoprecipitates as compared with control siRNA-transfected cells (Fig. 2D). These results indicated that PIN1 reduced the protein binding between p27 and cyclin A or cyclin E. To further confirm the negative regulatory effect of PIN1 on the CDK2 inhibitory function of p27, we performed CDK2 kinase activity assay in vitro with recombinant cyclin A–CDK2 and p27 immunoprecipitates. Our results showed that p27 immunoprecipitates obtained from wild-type MEFs inhibited the CDK2 activity to a lesser extent as compared with that of PIN1KO MEFs (Fig. 3A). In addition, p27 immunoprecipitates obtained from PIN1KO MEFs with wild-type PIN1 cDNA transfection had higher CDK2 activity than those with control or PIN1 mutant transfections (Fig. 3B). Similarly, p27 immunoprecipitates obtained from wild-type MEFs with PIN1 knocked down exhibited lower CDK2 activity as compared with that of the control (Fig. 3B). All these data suggested that PIN1 negatively regulated the CDK2 kinase inhibitory function of p27 by decreasing the interaction between p27 and cyclin A–and cyclin E–CDK2.

**Suppression of PIN1 enhanced p27 binding to cyclin and its inhibitory effect on CDK2 activity in hepatoma PLC/PRF/5 cells**

To determine whether PIN1 inhibits CDK2 inhibitory activity of p27 in cancer cells, knocking down PIN1 expression in hepatoma PLC/PRF/5 cells was performed. Our results demonstrated that down-regulation of PIN1 by shPIN1 in PLC/PRF/5 cells reduced cell proliferation, the proportion of BrdU-positive cells, and cyclin A– and cyclin E–CDK2 kinase activities (Fig. 4, A–C). In addition, PLC/PRF/5 cells with PIN1 knocked down showed higher levels of cyclin A and cyclin E in the p27 immunoprecipitates as compared with those of control shRNA-transfected cells (Fig. 4D). Moreover, p27 immunoprecipitates obtained from PIN1-depleted PLC/PRF/5 cells inhibited CDK2 activity to a greater extent as compared with that of the control (Fig. 4E). These results indicated the negative regulatory effect of PIN1 on CDK2-inhibitory activity of p27 in hepatoma cells.

**PIN1 bound p27 at Thr187–Pro to decrease its binding to cyclin and its inhibitory effect on CDK2 activity**

PIN1-induced stabilization of p27 requires binding of PIN1 to the phosphorylated Thr187–Pro motif of p27 (16). To study if PIN1-induced reduction of CDK2 inhibitory activity of p27 was also mediated through the same binding site, the expression plasmid encoding the p27 (T187A) mutant was generated. Wild-type and PIN1KO cells were transfected with expression plasmids encoding HA-tagged wild-type p27 or p27 (T187A) mutant. Immunoprecipitation with anti-HA antibody was performed to selectively isolate the exogenous HA-tagged p27 and p27 (T187A) mutant from the transfected cells. Our results...
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showed that in the wild-type cells, higher levels of cyclin A and cyclin E were co-immunoprecipitated with the p27 (T187A) mutant than with wild-type p27. However, no significant difference was found in the levels of co-immunoprecipitated cyclin A and cyclin E between wild-type and mutant p27 immunoprecipitates from the PIN1KO cells (Fig. 5A). These results indicated that PIN1 did not reduce the binding of p27 (T187A) mutant to cyclin A and cyclin E. We then further investigated if the CDK2-inhibitory activity of the p27 (T187A) mutant could be modulated by PIN1. As for the wild-type MEFs, immunoprecipitated p27 (T187A) mutant decreased the CDK2 activity to a greater extent as compared with immunoprecipitated wild-type p27. However, no significant difference was found in the CDK2-inhibitory activities between wild-type and mutant forms of p27 in the PIN1KO cells (Fig. 5B). More importantly, wild-type p27 immunoprecipitated from PIN1KO MEFs decreased CDK2 activity to a lesser extent after incubation with purified recombinant wild-type PIN1, but not with PIN1 W34A mutant (Fig. 5C and Fig. S5). However, recombinant wild-type PIN1 did not alter the CDK2-inhibitory activity of immunoprecipitated p27 (T187A) mutant. These results indicated that PIN1 reduced the CDK2-inhibitory activity of wild-type p27 but not p27 (T187A) mutant. To investigate if PIN1 regulates CDK2 activity through modulation of p27 inhibitory function, we have determined the CDK2 activity in the wild-type and PIN1KO MEFs with over-expression of wild-type or mutant (T187A) forms of p27. Our results showed that in wild-type MEFs, wild-type p27 decreased the cyclin A- and cyclin

Figure 2. PIN1 reduced binding between p27 and cyclin A or cyclin E. A–C, binding between p27 and cyclin A or cyclin E was reduced in wild-type (WT) MEFs as compared with that of PIN1KO cells. Lysates from WT and PIN1KO MEFs were immunoprecipitated (IP) with nonspecific IgG, (A) anti-p27, (B) anti-cyclin A, or (C) anti-cyclin E antibodies and immunoblotted with the indicated antibodies. The amounts of co-immunoprecipitated cyclin A, cyclin E, and p27 were normalized with the amount of indicated immunoprecipitated proteins and expressed as intensity ratio against WT MEFs for PIN1KO MEFs. *, \( p < 0.05 \). D, knocking down PIN1 expression in WT MEFs increased the binding between p27 and cyclin A or cyclin E. Lysates from WT MEFs transfected with siRNA against PIN1 (siPIN1) or control siRNA (siCTL) were immunoprecipitated with anti-p27 antibody and immunoblotted with the indicated antibodies. The amounts of co-immunoprecipitated cyclin A and cyclin E were normalized with the amount of immunoprecipitated p27 and expressed as intensity ratio against siCTL for siPIN1. *, \( p < 0.05 \).
E-CDK2 activities to a lesser extent as compared with the p27 (T187A) mutant (Fig. 5D). On the other hand, PIN1KO MEFs with wild-type p27 over-expression did not possess higher CDK2 activities as compared with p27 (T187A) mutant over-expression. These results indicated that PIN1 increased CDK2 kinase activity through modulation of the inhibitory function of
wild-type p27, but not the p27 (T187A) mutant, in the transfected cells. To investigate if the effect of PIN1 on cell proliferation is mediated solely through p27, PIN1 expression was knocked-down in MEFs with or without depleted p27 by co-transfecting specific siRNAs against PIN1 and p27. Suppression of PIN1 expression resulted in decreased cell proliferation and proportion of BrdU-positive cells in both wild-type and p27-depleted MEFs (Fig. 6, A and B). However, the negative effect of PIN1 was significantly more pronounced in the wild-type than the p27-depleted cells, suggesting that PIN1 promotes cell proliferation via both p27-dependent and -independent mechanisms.

Discussion

Through cis/trans isomerization, PIN1 has been shown to regulate various biological functions of its interacting proteins by altering their subcellular localization, protein stability, and protein–protein interaction. In this study, PIN1 was found...
to reduce the binding affinity of p27 with CDK2–cyclin A and –cyclin E, thereby enhancing CDK2 kinase activity in PIN1-expressing cells. Based on the previous report that PIN1 induced conformational changes of p27 to reduce its interaction with Cks1 (16), we speculated that the same conformational changes of p27 might also regulate its binding affinity with other interacting proteins such as cyclin A or cyclin E.

In addition to the quantity and subcellular localization of p27, post-translational modification of p27 has been found to

Figure 5. PIN1 bound p27 at Thr187–Pro to decrease its binding to cyclin and CDK2 inhibitory activity. A, PIN1 did not reduce the binding of PIN1-binding defective p27 (T187A) mutant with cyclin A or cyclin E. Lysates from wild-type (WT) and PIN1KO MEFs transiently transfected with pHAT187A mutant plasmid were immunoprecipitated with anti-HA antibody and immunoblotted with the indicated antibodies. The amounts of co-immunoprecipitated cyclin A and cyclin E were normalized with the amount of immunoprecipitated HA-tagged p27 proteins. Results were presented as intensity ratio against HA-tagged pHAT187A mutant. Protein levels of cyclin A and cyclin E in the transfected cells are provided in the supporting data (Fig. S6). *, p < 0.05. B, PIN1 did not decrease the CDK2-inhibitory activity of the PIN1-binding defective p27 (T187A) mutant. Lysates from WT and PIN1KO MEFs transiently transfected with pHAT187A mutant plasmid were immunoprecipitated with anti-HA antibody. Immunoprecipitated HA-tagged p27 proteins were incubated with recombinant cyclin A–CDK2, and CDK2 kinase activity was then determined. The kinase activity was normalized to the amounts of immunoprecipitated HA-tagged p27 protein and presented as percentage relative to the recombinant cyclin A–CDK2 kinase alone. *, p < 0.05. C, recombinant PIN1 decreased the CDK2-inhibitory activity of wild-type p27 but not PIN1-binding defective p27 (T187A) mutant. After incubation with recombinant wild-type PIN1 or its W34A mutant, immunoprecipitated HA-tagged p27 proteins (wild-type or T187A mutant) from PIN1KO MEFs were incubated with recombinant cyclin A–CDK2, and CDK2 kinase activity was then determined. The kinase activity was normalized to the amounts of immunoprecipitated HA-tagged p27 protein and presented as percentage relative to the recombinant cyclin A–CDK2 kinase alone. *, p < 0.05. D, WT MEFs with wild-type p27 over-expression possessed higher CDK2 kinase activity as compared with the PIN1-binding defective p27 (T187A) mutant over-expression. Lysates from WT and PIN1KO MEFs transiently transfected with pHAT187A mutant plasmid were immunoprecipitated with anti-cyclin A or cyclin E antibodies. Cyclin A– and cyclin E–CDK2 kinase activities were determined by in vitro fluorescence-based kinase assay and expressed as percentage relative to untransfected WT MEFs (parental). *, p < 0.05.
affect its inhibitory effect on CDK2 activity. Phosphorylation of p27 at Ser10 and Thr198 lead to its dissociation from CDK2, resulting in activation of CDK2 kinase activity (26, 27). In this study, we demonstrated that PIN1 did not reduce the binding affinity and CDK2-inhibitory activity of p27 (T187A) mutant, suggesting that the PIN1-binding site (Thr187–Pro) in p27 is critical for PIN1-mediated modulation of its binding affinity with cyclin A and cyclin E, and its inhibitory effect on CDK2 kinase activity. In addition, the suppression of CDK2-inhibitory activity of p27 by purified recombinant PIN1 further supported our hypothesis that PIN1 alone is able to inhibit p27 inhibitory activity on CDK2. Thus, our findings have provided evidence for a novel regulatory mechanism of p27 function mediated through PIN1-catalyzed isomerization. Nonetheless, PIN1 may

Figure 6. PIN1 promoted cell proliferation through both p27-dependent and -independent mechanisms. A, suppressed PIN1 expression with siRNA against PIN1 (siPIN1) resulted in decreased cell proliferation in MEFs co-transfected with control siRNA (siCTL) or siRNA against p27 (sip27). Cell proliferation was analyzed by MTT assay. Data presented were mean ± S.D. from six independent experiments. **, p < 0.01; ns, no significance. B, suppressed PIN1 expression with siRNA against PIN1 (siPIN1) resulted in a decreased proportion of S-phase proliferating cells in MEFs co-transfected with control siRNA (siCTL) or siRNA against p27 (sip27). Flow cytometric analysis of BrdU-positive cells and DNA content-generated cell cycle profiles. The proportions of G1 and G2–M phase cells are shown in the supporting data (Fig. S7). Data presented were mean ± S.D. from four independent experiments. **, p < 0.01; ns, no significance.
also promote G1–S phase progression through its effect on other proteins including cyclin D1 and cyclin E (28, 29), in addition, the negative regulatory effect of PIN1 on CDK2-inhibitory activity of p27 might facilitate S-phase progression.

To coordinate progression of the G1–S phase transition, expression and phosphorylation of cell cycle-regulated proteins are tightly regulated. In normal proliferating cells, the highest protein level of p27 was found at G0 and early G1 phases (30). However, during G1–S phase transition, activation of cyclin E- and cyclin A-CDK2 kinases phosphorylates p27, that in turn undergoes SCF<sub>skp2</sub>-mediated ubiquitination and proteasomal degradation (12). In general, the degradation of p27 by SCF<sub>skp2</sub> ubiquitin is widely considered to contribute to G1–S phase progression through reduction of the p27 inhibitory effect on CDK2 kinases. In this study, we showed that PIN1 further impairs CDK2 inhibitory activity of p27, revealing another mechanism of regulating G1–S phase transition by PIN1. Recently, PIN1 has been found to promote hyperphosphorylation of retinoblastoma protein (pRb) upon S-phase DNA damage (31). Hyperphosphorylation of pRb inhibits its binding with E2F transcription factors and, subsequently activate the transcription of genes that are involved in G1–S phase transition. Overexpression of p27 has also been shown, however, to induce dephosphorylation and degradation of pRb (32). Nonetheless, the interplay between PIN1, p27, and hyperphosphorylated pRb in the coordination of G1–S phase progression remains to be defined.

In conclusion, we have demonstrated that the binding of PIN1 to p27 at Thr<sup>187</sup>–Pro impaired the inhibitory effect of p27 on CDK2, leading to an increase in CDK2 kinase activity. Our findings therefore partly explain the paradoxical phenomenon of the higher level of p27 but enhanced cellular proliferation due to PIN1 expression.

**Experimental procedures**

**Cell culture and transfection**

Wild-type and Pin1-null (PIN1KO) MEFs (a kind gift from Kun-Ping Lu, Harvard Medical School) were maintained in cell culture medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and penicillin/streptomycin) supplemented with nonessential amino acids at 37 °C in a humidified incubator with a 5% CO<sub>2</sub>-enriched atmosphere. For cell synchronization experiments, cells were synchronized at the beginning of S-phase by using a double thymidine block. Briefly, asynchronously growing cells were incubated with 2 mM thymidine (Sigma) for 18 h. Cells were released from the first block by washing with phosphate-buffered saline (PBS) and replacing with fresh culture medium for 9 h and then incubated with 2 mM thymidine for another 17 h. After incubation, cells were released by washing with PBS and replaced with fresh culture medium and then harvested at various time points. The efficiency of cell synchronization was determined by cell cycle analysis using flow cytometry. Transient transfections with various plasmids were performed using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA). siRNA targeting PIN1 (sense, 5’-GACGGCCAGATTCTCCC-TTAA-3’) and negative control siRNA (sense, 5’-AATTCTC-GAACGTGTACGT-3’) were transfected into cells with Oligofectamine transfection reagent (Thermo Fisher Scientific). All siRNAs were obtained from Qiagen (Valencia, CA). Stable clones of human hepatoma PLC/PRF/5 cells with PIN1 depletion (shPIN1) or control shRNA (shCTRL) were generated as described previously (6).

**Plasmids and site-directed mutagenesis**

Plasmids pcDNA3.1-PIN1, pcDNA3.1-PIN1 mutants (W34A and K63A), pEGFP-PIN1, and pEGFP-PIN1 W34A mutant were generated as described previously (6, 8). Plasmid expressing HA-tagged p27 was generated by PCR amplification of the HA-tagged full-length mouse p27 cDNA using the following primers (with restriction sites underlined and HA tag coding sequence shown in bold): 5’-AAAGGATCCAAATGTACCCATACGATGTCTCAGGTCGAATTCCGAGGTCGGAAGAAGAAATGTCATAAAACCAGGTGAAGTTCTCAAAC-3’ (sense); 5’-TTTGGATTCTTTTACGTCTGGGCCTGCA-3’ (antisense).

PCR products were digested with BamHI and EcoRI and cloned into pcDNA3.1+ vector (Thermo Fisher Scientific) to generate the pHA-p27 plasmid. PIN1-binding defective p27 (T187A) mutant was generated with site-directed mutagenesis (QuickChange Lightning; Agilent Technologies, Santa Clara, CA). The mutagenic primers were as follows (with locations of mismatches underlined): PIN1 W34A, 5’-GGGCTTCTTGG-GGGCGCCTGCCAGAGT-3’ (sense) and 5’-ACTGTGGAG-CAGGGCCCGAAGACGCC-3’ (antisense). All DNA constructs were verified by DNA sequencing.

**MTT cell proliferation assay**

Transfected cells were plated in 96-well culture plates prior to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. After 24 and 48 h, cells were incubated with 1 mg/ml of MTT solution (Sigma) at 37 °C for 4 h. 10% SDS (w/v) solution was then added and incubated with the cells overnight. Absorbance at 550 nm with a reference wavelength of 690 nm was measured by the CLARIOstar microplate reader.

**BrdU assay and cell cycle analysis**

Asynchronously growing cells were pulse-labeled with BrdU (5-bromo-2’-deoxyuridine) at a final concentration of 1 μM for 30 min. After BrdU incorporation into the cells, cells were washed with PBS and incubated with culture medium in the absence of BrdU for 24 h. Harvested cells were then fixed with ice-cold 70% ethanol overnight, treated with 2 N HCl, 0.5% Triton X-100 for DNA denaturation and 0.1 M borate buffer for neutralization of residual acid, and stained with FITC-conjugated anti-BrdU antibody (eBioscience, San Diego, CA) and propidium iodide. For cell cycle analysis, cells were harvested at different time points after release from double thymidine block. Cells were fixed with ice-cold 70% ethanol overnight, washed with PBS, and then stained with propidium iodide/RNase A staining solution. All the stained cells were analyzed by Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA) and BrdU-positive cells and DNA content were quantified using FlowJo 8.7 software.
Expression and purification of recombinant PIN1 protein

Recombinant GST-PIN1 and its W34A mutant fusion proteins were expressed in *Escherichia coli* BL21 cells as described previously (6, 8). Cells were lysed by sonication in lysis buffer (50 mmol/liter of Tris-HCl, pH 7.5, and 100 mmol/liter of NaCl) containing protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and 1 mmol/liter of phenylmethylsulfonyl fluoride. Supernatants containing GST fusion proteins were collected and added to 1 ml of glutathione-immobilized columns (Thermo Fisher Scientific). Subsequently, the column was washed 5 times with wash solution, and bound proteins were eluted with 10 mmol/liter of glutathione elution buffer. To cleave PIN1 protein from the GST moiety, eluted GST-PIN1 fusion proteins were subjected to thrombin (Sigma) digestion at room temperature for 16 h. Cleaved PIN1 protein was then desalted with microcon centrifugal filters (Millipore, Billerica, MA), and the successful cleaving of recombinant PIN1 from GST fusion protein was confirmed by SDS-PAGE.

Immunoprecipitation and Western blot analysis

Immunoprecipitation experiments were performed using Pierce Direct IP kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, nonimmune serum or the indicated antibodies were immobilized onto agarose resin. Cells were lysed by sonication in lysis buffer with the addition of protease inhibitor mixture (Roche Diagnostics), 1 mmol/liter of phenylmethylsulfonyl fluoride, 0.3 mmol/liter of aprotinin, 1 mmol/liter of leupeptin, 1 mmol/liter of pepstatin, 20 mmol/liter of sodium fluoride, 1 mmol/liter of sodium orthovanadate, and 1 mmol/liter of disodium glycero phosphate. Protein concentration of the cell lysate was determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of proteins from the cell lysates were incubated with immobilized antibody with constant rotation overnight at 4 °C. Subsequently, the immunoprecipitated proteins were washed, eluted with low pH elution buffer, and analyzed by Western blot. For Western blot analysis, antibodies against the following proteins were used: β-actin (Sigma), CDK2, cyclin A, cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA), GFP (Clontech Laboratories, Mountain View, CA), HA tag (Sigma), p27 (BD Transduction Laboratories, San Jose, CA), and PIN1 (Calbiochem; EMD Millipore Chemicals, Billerica, MA). For detection of bound antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo Fisher Scientific) were used. Chemiluminescent signals of Western blots were developed by Western HRP substrates (Millipore, Billerica, MA), and secondary antibodies (Thermo Fisher Scientific) were used.

For CDK2 kinase activity assay, co-immunoprecipitation of cyclins A/E–CDK2 and p27 was performed using Pierce Direct IP kit. To preserve the kinase activity of CDK2 or inhibitory activity of p27, immunoprecipitated proteins were eluted with a neutral pH, high-salt elution buffer. Eluted proteins were desalted with microcon centrifugal filters (Millipore, Billerica, MA) and then incubated with histone H1 substrate and ATP for 30 min at 30 °C for kinase reaction. After incubation, the kinase activity was determined by the ADP-Glo™ Kinase assay (Promega). Briefly, the kinase reaction mixture was incubated with ADP-Glo reagent and Kinase Detection Reagent. The luminescence was then measured by luminometer (Berthold Technologies). For determination of p27 inhibitory activity, recombinant cyclin A–CDK2 kinase (Promega, Madison, WI) was used as a positive control in the experiment.

Immunofluorescence microscopy

Cells plated on glass slides were fixed with paraformaldehyde solution (4% (w/v) in PBS) and permeabilized with Triton X-100 solution (0.5% (v/v) in PBS). Cells were rinsed with PBS and incubated with primary antibody overnight at 4 °C. After washing with PBS, cells were incubated with fluorochrome-conjugated secondary antibody at room temperature for 1 h in the dark and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. After washing with PBS, slides were mounted using ProLong® Gold antifade reagent (Life Technologies®) and visualized with a Carl Zeiss LSM 710 confocal microscope (Germany). Fluorescent images were collected and analyzed with ZEN software (Carl Zeiss, Germany).

Statistical analysis

If not stated otherwise, data were expressed as mean ± S.D. for at least three independent experiments. Statistical analysis was performed by Student’s *t* test using GraphPad Prism version 6 for Mac OS, GraphPad Software, La Jolla, CA. *p* value < 0.05 was considered statistically significant.

Author contributions—C. W. C. designed and performed the experiments, wrote, and approved the manuscript; K. W. L. and Y. M. N. performed the experiments and approved the manuscript; Y. L. K. conceived the project and approved the manuscript; E. T. conceived the project, designed the experiments, and wrote and approved the manuscript.

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