

The Loss of PIN1 Deregulates Cyclin E and Sensitizes Mouse Embryo Fibroblasts to Genomic Instability*[§]

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During the G₀/G₁-S phase transition, the timely synthesis and degradation of key regulatory proteins is required for normal cell cycle progression. Two of these proteins, c-Myc and cyclin E, are recognized by the Cdc4 E3 ligase of the Skp1/Cul1/Rbx1 (SCF) complex. SCF^{Cdc4} binds to a similar phosphodegron sequence in c-Myc and cyclin E proteins resulting in ubiquitylation and degradation of both proteins via the 26 S proteasome. Since the prolyl isomerase Pin1 binds the c-Myc phosphodegron and participates in regulation of c-Myc turnover, we hypothesized that Pin1 would bind to and regulate cyclin E turnover in a similar manner. Here we show that Pin1 regulates the turnover of cyclin E in mouse embryo fibroblasts. Pin1 binds to the cyclin E-Cdk2 complex in a manner that depends on Ser³⁸⁴ of cyclin E, which is phosphorylated by Cdk2. The absence of Pin1 results in an increased steady-state level of cyclin E and stalling of the cells in the G₁/S phase of the cell cycle. The cellular changes that result from the loss of Pin1 predispose Pin1 null mouse embryo fibroblasts to undergo more rapid genomic instability when immortalized by conditional inactivation of p53 and sensitizes these cells to more aggressive Ras-dependent transformation and tumorigenesis.

Mitogenic stimuli initiate a sequence of events that result in the entry of quiescent cells into S phase (1). Critical for this important transition is the ordered synthesis and degradation of transcription factors such as c-Jun and c-Myc (Myc) and cyclins such as cyclin D and cyclin E (1). Deregulation of the turnover of these proteins, such that they remain active at inappropriate times during cell cycle progression, is frequently found in human cancer. Thus, an understanding of the molecular mechanisms that regulate protein turnover is crucial to provide insight into the oncogenic process. Three proteins important for the progression of cells into S phase, c-Jun, c-Myc, and cyclin E, are ubiquitylated by a common member of the Skp1/Cul1/Rbx1 (SCF)³ group of ubiquitin enzymes in which the F-box component, which serves as the ubiquitin E3 ligase, is Cdc4 (SEL-10, Fbw7, Ago) (2). SCF^{Cdc4} binds to a component of each protein that has been termed the “phosphodegron” (3) to promote the ubiquitylation and degradation of these proteins via the 26 S proteasome. c-Jun and Myc have an additional binding protein in common, the peptidyl prolyl *cis/trans*-isomerase Pin1,

which binds and isomerizes prolyl bonds in the context of phospho-Ser/Thr-Pro motifs (4, 5).

Previously, we described the mechanism by which Pin1 promotes Myc degradation (5). The Cdc4 phosphodegron of Myc is present in a domain termed Myc box 1 (MB1), containing the sequence LPpTP-PLpSP (where pT represents phosphothreonine and pS represents phosphoserine), in which the two phosphorylation events occur sequentially and are catalyzed by ERK (Ser⁶²) and GSK3β (Thr⁵⁸), respectively (6, 7). Pin1 binds to the doubly phosphorylated motif in a manner requiring phospho-Thr⁵⁸ and promotes a conformational change that presents phospho-Ser⁶² as a substrate for the protein phosphatase PP2A (5). Ubiquitylated Myc is only phosphorylated on Thr⁵⁸ (5), suggesting that either dephosphorylation of Ser⁶² occurs before Cdc4 can bind to Myc or before ubiquitylation can occur. Regardless of the precise mechanism involved, inhibition of PP2A or the absence of Pin1 results in a stabilization of Myc (5). Myc can also be stabilized by mutating Thr⁵⁸ to Ala, and this mutation renders Myc oncogenic in a primary human cell transformation assay (5). Since residues in the Myc phosphodegron, including Thr⁵⁸ and Pro⁵⁷, are frequently mutated in lymphomas (8–11), deregulation of Myc due to mutations of this region of the protein can play a role in human cancer (9).

The phosphodegron of cyclin E, LpTPXpSG, is remarkably similar to that of Myc (12). In the case of cyclin E, the phosphorylation events are catalyzed by Cdk2 (Ser³⁸⁴) and GSK3β (Thr³⁸⁰) (11), respectively, and x-ray structural studies have shown that phospho-Thr³⁸⁰ plays a crucial role in the binding of cyclin E by Cdc4 (3, 12). As is the case for Myc, deregulation of cyclin E can result in cell cycle defects (13–17) that predispose cells to oncogenesis (18–23). Such defects include aberrant DNA replication and the loss of genomic integrity. Indeed, cyclin E deregulation is associated with many human cancers including breast (18, 20, 24–26), ovarian (19), and bladder cancer (27), yet the precise mechanism responsible for ensuring the timely cell cycle-dependent turnover of cyclin E is incompletely understood. For these reasons and because of the similarities in the phosphodegron motif and degradation process between Myc and cyclin E, we investigated whether cyclin E was also a Pin1-binding protein. Here we show that Pin1 binds cyclin E and modulates cyclin E levels. In the absence of Pin1, cyclin E is deregulated in a way that leads to its stabilization, which, in combination with other protein alterations in Pin1 null cells, leads to cell cycle defects. We also demonstrate a correlation between the cell cycle defects that occur in mouse embryo fibroblasts (MEFs) null for Pin1 and increased rate in the progression of genomic instability when these cells are immortalized by inactivating p53 function. Finally, we show that the cell cycle defects resulting from the loss of Pin1 sensitize immortalized Pin1 null cells to more extensive and aggressive transformation and tumorigenesis induced by the Ras oncogene.

MATERIALS AND METHODS

Cells and Cell Culture—HEK 293 cells were acquired from the American Type Culture Collection. 293 cells were grown in Dulbecco's mod-

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³ The abbreviations used are: SCF, Skp1/Cul1/Rbx1; MEF, mouse embryo fibroblast; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BrdUrd, bromodeoxyuridine; WT, wild type; MMTV, murine mammary tumor virus.

Loss of Pin1 Deregulates Cyclin E

ified Eagle's medium, containing 10% fetal bovine serum. Phoenix cells (293 derivative) were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. Pin1 null mice, originally generated by Fujimori *et al.* (28), were obtained from Hoffmann-LaRoche. The *pin1* gene deletion was transferred into an isogenic C57BL6 background using marker-assisted speed congenic breeding by Jackson Laboratory. *pin1*^{+/+} and *pin1*^{-/-} embryonic fibroblasts were isolated from the isogenic C57BL6 strain as previously described (29). MEFs were grown in Dulbecco's modified Eagle's medium containing sodium pyruvate and 10% heat-inactivated fetal bovine serum.

293 cells were transfected using Lipofectamine or Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen) serum-reduced media per the manufacturer's suggestion. Cells were plated 1 day prior to transfection at a density of $\sim 1 \times 10^6$ cells/100-mm plate. Briefly, 2.5 μ g of DNA was mixed with 15–30 μ l of Lipofectamine 2000 or Lipofectamine, respectively, in 0.5 ml of Opti-MEM and incubated for 15 min at room temperature. The mixture was added to the cells for 12–18 h prior to treatment with drug or lysis.

The following plasmids were used for transfections. pCS2-Myc-cyclin E, cyclin E^{T62A}, cyclin E^{T62A/T380A}, cyclin E^{S372A}, and cyclin E^{S384A} were generous gifts from J. Wade Harper (Harvard Medical School, Boston, MA). pBabe-Puro-Ras was kindly provided by Chris Counter (Duke University, Durham, NC), and pBabe-Hygro-p53_{DD} was from William Hahn (Dana Farber Research Institute, Boston, MA). pWZL-Blast-Myc was generated as previously described (5).

Protein Binding Assays—GST pull-down assays were performed as previously described (5). Briefly, GST and GST-Pin1 fusion proteins with either wild type *Xenopus* Pin1, an xPin1 WW domain mutant with substitutions at W11A and W34A, or a xPin1 catalysis-deficient mutant with a substitution at C109A was prepared as described (30). The GST and GST-Pin1 recombinant proteins were maintained on GSH-agarose beads and stored at 4 °C. Cells were lysed in buffer containing PBS, 10 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100 supplemented with Pefabloc (Roche Applied Science) and NaF. Lysates were clarified by centrifugation prior to binding assays. Binding was performed at 4 °C for 1 h using 10 μ g of GST proteins incubated with 500 μ g of total lysate protein. Beads were washed four times in 1 ml of lysis buffer per wash. Subsequently, proteins were solubilized in 2 \times SDS sample buffer and separated by SDS-PAGE. Bound proteins were analyzed by Western blotting.

For immunoprecipitations, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 1% Triton X-100 supplemented with Pefabloc (Roche Applied Science) and NaF. Lysates were clarified by centrifugation. 500 μ g of total lysate protein was used per sample. Prior to binding, the lysate samples were precleared with Protein A/G-Sepharose for 10 min at 4 °C. Binding was performed using 2 μ g (per sample) of anti-9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for Myc-tagged protein. For experiments using *Xenopus* interphase extracts, extracts were prepared as previously described (30). Binding was performed by preincubating anti-Pin1 to Protein A beads (Amersham Biosciences) in the presence of bovine serum albumin. The beads were washed three times in PBS prior to incubation with 400 μ g of total extract protein. Beads were washed four times in a modified radio-immune precipitation buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. Bound proteins were separated and analyzed as described above.

Western Blotting—The following antibodies were used for the detection of proteins by Western blotting: anti-Cdk2 M2 (Santa Cruz Biotechnology), anti-cyclin E M20 (Santa Cruz Biotechnology), anti-cyclin

E HE12 (Upstate Biotechnology), anti-Myc 9E10 (Santa Cruz Biotechnology), anti-Pin1 (30), anti-p27 C-19 (Santa Cruz Biotechnology), anti-p53 FL-393 (Santa Cruz Biotechnology), anti-phosphocyclin E Thr³⁸⁰ (Santa Cruz Biotechnology), and anti- β -actin (Sigma). Equal amounts of protein for each sample were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore Corp.). Membranes were blocked in TBS-0.2% Tween 20 containing 5% milk for 1 h prior to incubation with primary antibody. Primary antibodies were diluted 1:1000 in the blocking solution with the exception of anti-Pin1, which was diluted 1:10,000 unless otherwise stated. Membranes were washed a minimum of three times in Tris-buffered saline, 0.2% Tween 20 for 15 min each wash. Horseradish peroxidase-conjugated secondary antibodies were diluted into the blocking buffer and incubated for 2 h at room temperature. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies using the ECL reagent (Amersham Biosciences) per the manufacturer's instructions.

Retroviral Transduction—The *pin1*^{+/+} and *pin1*^{-/-} cells containing p53_{DD} and Ras were generated by amphotrophic retroviral transduction and polyclonal selection using 10 μ g/ml hygromycin B (Calbiochem) or 0.5 μ g/ml puromycin (Calbiochem). Viral medium was obtained by Lipofectamine transfection of the Phoenix packaging cell line with 10 μ g of DNA. Cells were allowed to recover for 24 h, after which time the medium was collected and filtered through a 0.45- μ m filter (16) to remove any cellular debris. Filtered viral medium was supplemented with 4 μ g/ml Polybrene (Sigma) and added to primary MEFs at passage 3 for 24 h. Cells were allowed to recover in complete medium for 24 h at 37 °C prior to drug selection.

Cellular Transformation—The ability of cells to undergo anchorage independent growth was tested as previously described (32). 5×10^4 cells were plated in 0.3% Nobel agar, Dulbecco's modified Eagle's medium and heat-inactivated fetal bovine serum on 35-mm plates containing a 2-mm grid. Plates were incubated at 37 °C for 21 days and assessed for colony growth in soft agar. Cells were fed one time weekly with 1 ml of agar/medium mixture. Colonies were counted by light microscopy. The mean \pm S.D. represents two independent experiments, each performed in triplicate.

Tumor Formation in Immunodeficient (SCID) Mice—SCID mice were obtained from Charles River and housed in the Comprehensive Cancer Center Isolation Facility under a 12-h light, 12-h dark cycle. Food and water were provided *ad libitum*, and all care was given in compliance with National Institutes of Health and institutional guidelines on the use of laboratory and experimental animals. Prior to injection, cells were free of micoplasma, drugs, and antibiotics. Five mice were injected per cell type with a single subcutaneous injection of 1×10^7 cells/animal. Data are represented as number of mice that developed tumors over total number of animals.

Real Time PCR—Quantitation of cellular mRNA levels was determined by real time PCR using the SyberGreen Supermix (Bio-Rad) reagent per the manufacturer's suggestions. Briefly primers to the NH₂-terminal region of cyclin E were created to contain the following sequences: 5'-ACG GAC CAC AGC AAC ATG AA-3' and 5'-AAA CAC GGC CAC ATT TGC CT-3' (IDT). Total cellular RNA was isolated by harvesting confluent 100-mm plates of *pin1*^{+/+} and *pin1*^{-/-} MEFs using the TRIzol (Invitrogen) reagent. Cells were harvested in 1 ml of TRIzol and cleaned with chloroform. Resulting material was purified by ethanol precipitation. First strand cDNA was generated using Superscript III reverse transcriptase (Invitrogen). Reaction mixtures were denatured at 65 °C for 6 min followed by cDNA synthesis at 50 °C for 50 min, and then the reactions were terminated by incubation at 85 °C for 5 min. Resultant cDNA was mixed with designated primers

and SyberGreen dye. The mixture was analyzed by real time PCR (Bio-Rad) under the following conditions: step 1, 95 °C for 3 min; step 2, 60.8 °C for 0–15 min; step 3, 72 °C for 0–15 min; repeat steps 2 and 3 for 45 cycles; step 5, 72 °C for 2 min; step 6, 45 °C for 0–10 min. Samples were analyzed two times in duplicate per trial.

Cell Proliferation— 2.5×10^5 cells were seeded into 60-mm plates in duplicate. Every 24 h, cells were trypsinized and counted by Coulter counter. Data are represented as the average of the two samples for both cell types at each time point. These data are representative of five individual experiments.

BrdUrd Incorporation— 1×10^5 cells were seeded onto coverslips in 6-well plates and incubated overnight or until 70–80% confluent. Cells were pulsed with BrdUrd for 30 min at a final concentration of 10 μ M. Following incubation with BrdUrd, cells were fixed in cold MeOH for 5 min at -20 °C and then rehydrated in PBS. 1 ml of 2 M HCl/Triton X-100 was added to each well, followed by incubation at room temperature for 30 min. Cells were washed once for 5 min in 1 ml of 0.1 M sodium tetraborate (pH 8.5) and twice for 5 min in PBS followed by blocking in 1% bovine serum albumin/PBS for 30 min. To perform staining, anti-BrdUrd, diluted 1:100 in 0.1% bovine serum albumin/PBS, was added to each coverslip and incubated at 4 °C overnight. Coverslips were washed three times in PBS for 5 min and then incubated with anti-mouse fluorescein isothiocyanate diluted 1:200 in 0.1% bovine serum albumin/PBS and incubated at room temperature for 60 min followed by three washes in PBS. Coverslips were mounted on slides with fluor antioxidant containing 1:5000 4',6-diamidino-2-phenylindole and analyzed by fluorescence microscopy (Zeiss-Axiscop). Each of three individual experiments were performed in triplicate, and ≥ 300 cells were counted per replicate.

Flow Cytometry—MEFs were grown until ~ 70 –80% confluent in 60-mm plates. Cells were harvested by washing once in Hanks' balanced salt solution followed by trypsinization. Cells were pelleted by centrifugation at $500 \times g$ for 10 min and washed once in PBS, followed by fixation with cold 70% ethanol. At this time, cells were either stored at -20 °C until used or kept on ice for 2 h prior to staining. DNA staining was performed by resuspending cells in PBS containing propidium iodide (5 μ g/ml) and RNase A (50 units) and then analyzed by fluorescence-activated cell sorting (Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource, Durham, NC). Three separate experiments were performed with each cell type analyzed in triplicate in each experiment.

RESULTS

Pin1 Binds to the Cyclin E-Cdk2 Complex and Influences the Stability of Cyclin E Protein—Cyclin E has been reported to undergo phosphorylation on several Ser and Thr residues in cells (12, 26, 33–36). At least four of these sites are implicated in its degradation (12, 26, 33–36) (Fig. 1A). Three sites, Thr⁶², Thr³⁸⁰, and Ser³⁸⁴ (shown in *black*), influence the Cdc4-dependent turnover of cyclin E, whereas Ser³⁷² (shown in *gray*) may be involved in cyclin E degradation but by an ill defined pathway (12, 26, 33). To determine whether endogenous Pin1 and cyclin E interact, we used *Xenopus* interphase extracts and immunoprecipitated Pin1 using our previously described anti-*Xenopus* Pin1 antibody (29). As shown in Fig. 1B, endogenous cyclin E and Pin1 do interact in this assay. Next, we transfected 293 cells with wild type (WT), Myc-tagged cyclin E and subjected the extracts to GST pull-down assays using GST alone, as a negative control, or GST-Pin1. As shown in the *first two lanes* of Fig. 1C, GST-Pin1 also binds Myc-tagged cyclin E. We next evaluated the importance of the three Cdc4-specific phosphorylation sites, Thr⁶², Thr³⁸⁰, and Ser³⁸⁴, in Pin1 binding by transfecting

Myc-tagged cyclin E cDNA constructs that were mutated in one or more of the phosphorylation sites illustrated in Fig. 1A. Surprisingly, mutation of Thr⁶², Thr³⁸⁰, or the combination of Thr⁶² and Thr³⁸⁰ does not affect Pin1 binding (Fig. 1C, *lanes 3–8*). On the other hand, mutation of Ser³⁸⁴ nearly abolishes Pin1 binding (Fig. 1C, *lanes 9 and 10*). Since Ser³⁸⁴ is phosphorylated by the Cdk2 component of the cyclin E-Cdk2 heterodimer (12), this result suggested that Pin1 might bind to the heterodimer in a manner that requires an active Cdk2 protein to catalyze the phosphorylation of Ser³⁸⁴ on cyclin E. This hypothesis is supported by the data in Fig. 1, *D–F*. First, when Pin1 is associated with WT endogenous or Myc-tagged cyclin E, immunoblot analysis reveals the presence of both cyclin E and Cdk2 (Fig. 1, *D and E, lanes 1 and 2, and F, lanes 1 and 2, respectively*). Second, preincubation of the cells containing Myc-cyclin E with the selective Cdk2 inhibitor roscovitine prior to lysis and the addition of GST-Pin1 markedly attenuated the binding of both cyclin E and Cdk2 (Fig. 1E, *lanes 3 and 4*). This result was repeated three times with similar results, and quantitation of these results can be found in supplemental Fig. 1. Although roscovitine is an inhibitor of Cdk2 activity, it may also affect Cdk2 and/or cyclin E protein levels (37). Thus, we evaluated Cdk2 and cyclin E levels by immunoblotting extracts derived from cells that had been treated with either vehicle (Me₂SO) or 30 μ M roscovitine. As shown in Fig. 1E (*right panel*), treatment with 30 μ M roscovitine resulted in minimal changes in cyclin E or Cdk2 levels. Collectively, these results suggest that the efficient binding of Pin1 to cyclin E depends on Cdk2 activity and an intact Ser³⁸⁴ residue, which is the site on cyclin E that is phosphorylated by Cdk2.

To confirm the observation that phosphorylation of Ser³⁸⁴ is important for Pin1 binding to cyclin E, we expressed the Myc-tagged cyclin E constructs in 293 cells and examined Pin1 binding by immunoprecipitation using antibody to the Myc tag. We then probed the immunoprecipitates with antibodies to Cdk2, Pin1, or Myc (to detect the immunoprecipitated cyclin E proteins). In addition to WT, the T62A/T380A double mutant, and the S384A mutant cyclin E proteins, we also expressed a S372A mutant, since this site has also been implicated in cyclin E protein turnover (12) and is a potential Ser(P)-Pro binding site for Pin1. As shown in the *left panel* of Fig. 1F, Pin1 was present in the immunoprecipitates of WT, T62A/T380A and S372A cyclin E but was considerably less abundant in the immunoprecipitate containing the S384A mutant protein. This was true, although a similar amount of Cdk2 (as shown by Western blot) and of each cyclin E protein (based on Coomassie staining) was immunoprecipitated (Fig. 1F, *left*). In addition, each of the whole cell extracts contained similar amounts of Cdk2, Pin1, and the appropriate cyclin E protein (Fig. 1F, *right*). Collectively, these results show that Ser³⁸⁴ phosphorylation is important for the association of Pin1 with cyclin E-Cdk2 but not for the assembly of the cyclin E-Cdk2 complex.

Since the phosphorylation of Thr³⁸⁰ and Ser³⁸⁴ on cyclin E are important for regulating the Cdc4-mediated turnover of cyclin E protein (12, 26, 33), we next asked whether, in the absence of Pin1, cyclin E levels were elevated. We subjected equal amounts of extracts from MEFs, either WT or null for Pin1, to Western blotting and found that steady-state levels of cyclin E were up-regulated 2-fold in the absence of Pin1 (Fig. 2A and supplemental Fig. 2). Interestingly, this up-regulation of cyclin E in Pin1 null cells was accompanied by a considerable decrease in the level of phosphorylated Thr³⁸⁰. Since phosphorylation of Thr³⁸⁰ promotes the degradation of cyclin E and this process is impaired in the absence of Pin1, these results are compatible with a role for Pin1 in regulating the turnover of cyclin E. Because the cyclin E gene is activated during the progression from G₀/G₁ to S phase, we also questioned whether the changes in cyclin E protein levels in the absence of Pin1

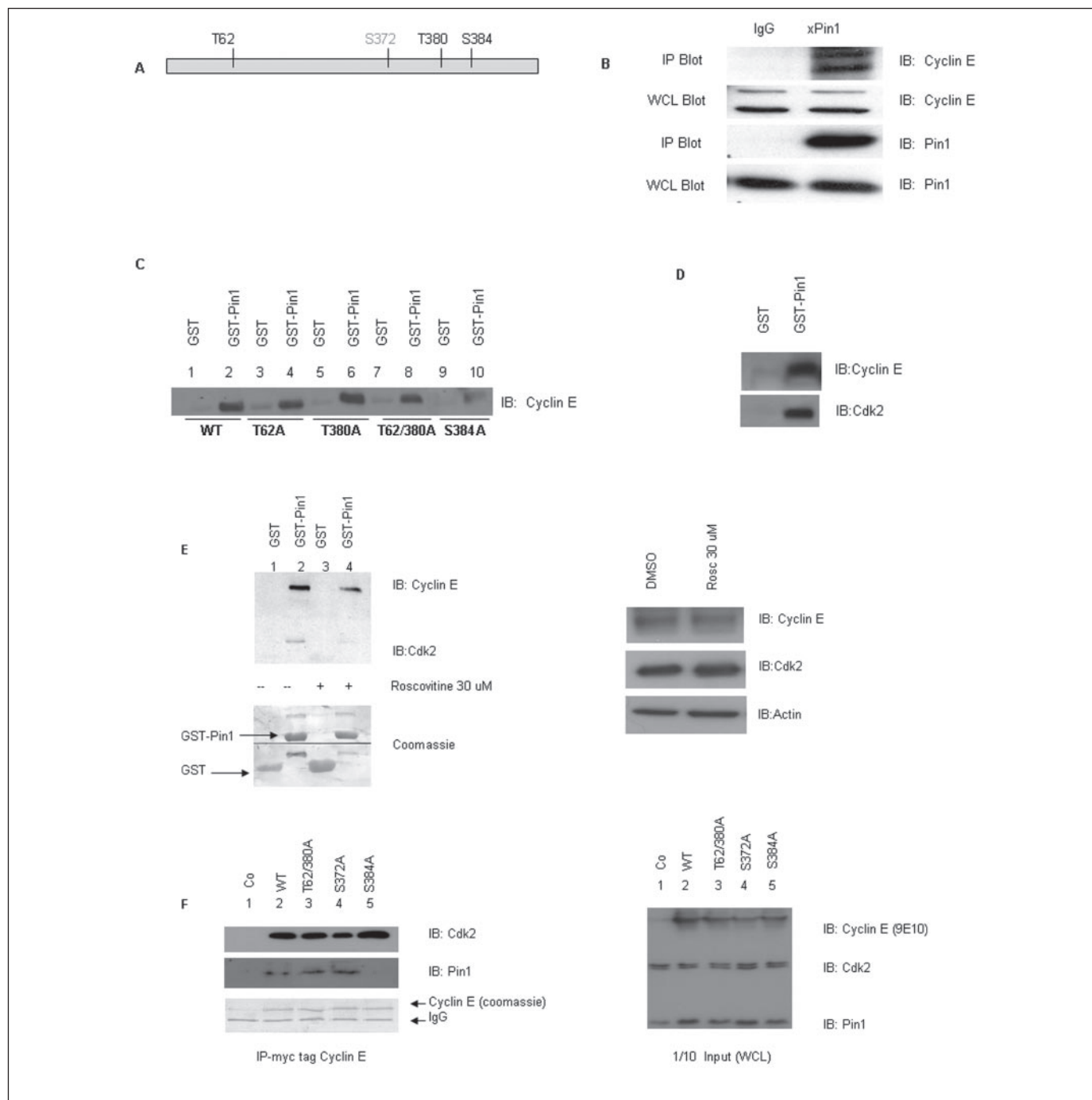


FIGURE 1. Cyclin E binds to Pin1. A, phosphorylation sites on cyclin E protein that are involved in cyclin E turnover. Thr⁶², Thr³⁸⁰, and Ser³⁸⁴ have been described to influence cyclin E turnover promoted by Cdc4. Ser³⁷² is also involved in cyclin E turnover by an unknown mechanism. B, endogenous Pin1 interacts with endogenous cyclin E. *Xenopus* Pin1 was immunoprecipitated, using anti-xPin1 antibody or rabbit IgG as a negative control, from *Xenopus* interphase extracts. Cyclin E was found only with the xPin1 co-immunoprecipitate. C, mutation of Ser³⁸⁴ impairs binding of cyclin E to Pin1. 293T cells were transfected with either Myc-tagged wild type cyclin E, cyclin E^{T62A}, cyclin E^{T380A}, cyclin E^{T62A/T380A}, or cyclin E^{S384A} prior to lysis. Lysates were subjected to GST pull-down with either GST beads as a negative control or GST-Pin1. Bound wild type and mutant cyclin E proteins were detected by Western blot, using anti-9E10 antibody for the Myc tag. Wild type cyclin E, cyclin E^{T62A}, cyclin E^{T380A}, and cyclin E^{T62A/T380A} protein associated with GST-Pin1, whereas cyclin E^{S384A} binding to Pin1 was attenuated. D, endogenous cyclin E-Cdk2 associates with Pin1. Nontransfected 293T cells were lysed and subjected to GST pull-down using GST alone or GST-Pin1. Both endogenous cyclin E and endogenous Cdk2 were found to be associated with GST-Pin1 but not with GST alone. E, Pin1 binds to the cyclin E-Cdk2 complex, and inhibition of Cdk2 with roscovitine impairs cyclin E-Cdk2 complex association with Pin1. Left, 293T cells were transfected with wild type cyclin E and treated with either Me₂SO or 30 μM roscovitine. 500 μg of total protein from lysates were used to perform GST pull-down as described in A. Binding of cyclin E-Cdk2 to Pin1 was decreased in the presence of roscovitine. Right, 293T cells were transfected with wild type cyclin E prior to treatment with Me₂SO vehicle or 30 μM roscovitine. Whole cell lysates containing 35 μg of total protein were subjected to Western blotting using the anti-9E10 antibody to detect cyclin E. As determined by densitometry, the ratio of cyclin E to actin in the Me₂SO treatment is 1.1, whereas the ratio of cyclin E to actin under roscovitine treatment is 1.3. Similarly, the ratio of Cdk2 to actin in the Me₂SO-treated lane is 2, whereas the ratio of Cdk2 to actin under roscovitine treatment is 2.1. No significant changes in the amounts of cyclin E and Cdk2 proteins were observed with 30 μM roscovitine treatment relative to Me₂SO treatment. F, Cdk2 remains associated with cyclin E^{S384A} when Pin1 binding is impaired. Left, 293T cells were mock-transfected (lane 1) or transfected with Myc-tagged wild type cyclin E, cyclin E^{T62A/T380A}, cyclin E^{T372A}, or cyclin E^{S384A} (lanes 2–5) prior to immunoprecipitation using the Myc tag with the anti-9E10 antibody. Bound proteins were resolved using anti-Cdk2 antibody and anti-Pin1 antibody. Cdk2 was bound to cyclin E and to each cyclin E mutant protein, whereas Pin1 binding was decreased with cyclin E^{S384A} immunoprecipitate, confirming the result seen in B. Right, 25 μg of whole cell lysate for samples containing mock-transfected cells or cells transfected with Myc-tagged wild type cyclin E, cyclin E^{T62A/T380A}, cyclin E^{T372A}, or cyclin E^{S384A} were subjected to Western blotting using the anti-Myc (9E10). Cyclin E was expressed in the appropriate lanes (lanes 2–5 but not lane 1) at comparable levels between samples. IB, immunoblotting; IP, immunoprecipitation.

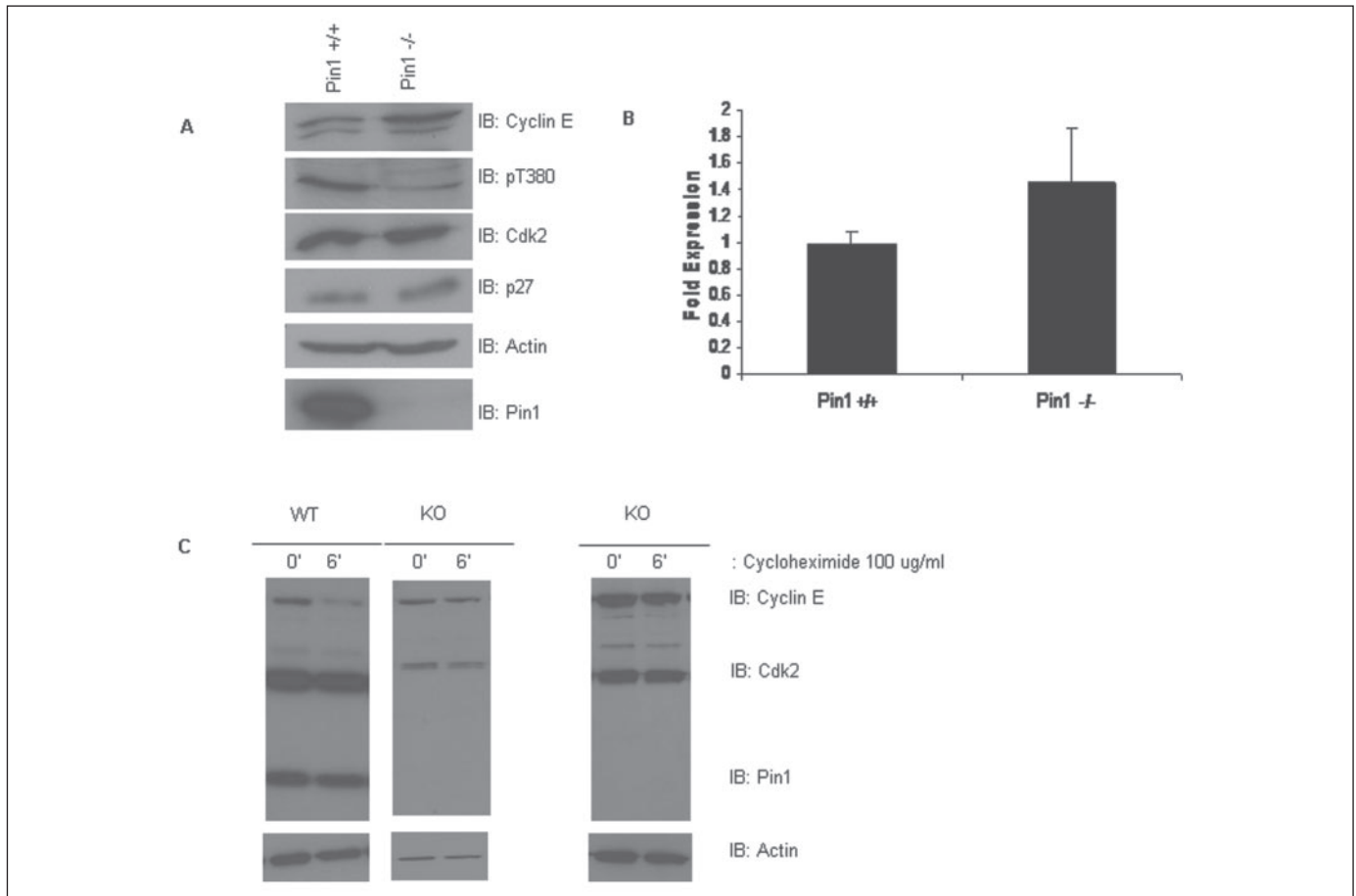


FIGURE 2. Pin1 influences the stability of cyclin E protein. *A*, steady-state levels of cyclin E protein are elevated in Pin1^{-/-} MEFs. Equal amounts of protein (50 μ g) from pin1^{+/+} and pin1^{-/-} MEFs were analyzed for protein content by Western blotting (IB). Protein levels were detected using anti-cyclin E (M20), anti-phospho-cyclin E T³⁸⁰, anti-Cdk2, anti-p27, anti-Pin1, and anti- β -actin as a loading control. Cyclin E levels were elevated 2-fold, and Thr(P)³⁸⁰ levels were markedly decreased in the absence of Pin1 as determined by densitometry and normalized to actin. Cdk2 and p27 levels were unchanged. Results are representative of five individual experiments. *B*, levels of cyclin E mRNA in the presence and absence of Pin1. Levels of cyclin E mRNA present in MEFs were quantified using real time PCR. Pin1^{-/-} MEFs displayed a slight increase in mRNA quantities, but this was not statistically significant. *C*, loss of Pin1 affects cyclin E protein stability. pin1^{+/+} and pin1^{-/-} MEFs were treated with 100 μ g/ml cycloheximide and harvested at 0 and 6 h. The middle panel represents a lower exposure of the right panel and was used for quantitation. Treatment with cycloheximide for 6 h led to a 67% decrease in cyclin E protein in pin1^{+/+} cells (left) but to only a slight decrease in cyclin E protein in pin1^{-/-} cells (middle) as determined by densitometry and normalized to actin. KO, knock-out.

might be attributed to increased cyclin E mRNA. We performed real time PCR on total RNA isolated from either pin1^{+/+} or pin1^{-/-} MEFs and found that, although there appeared to be a slight increase in cyclin E mRNA in pin1^{-/-} cells, the increase was not statistically significant (Fig. 2*B*). We next probed the immunoblots for two components that are known to interact with cyclin E to regulate its level and/or activity in the cell. As shown in Fig. 2*A*, neither the level of Cdk2 nor the cyclin-dependent kinase inhibitor p27 was changed in pin1^{-/-} cells. Finally, we tested whether cyclin E is appropriately degraded in the absence of Pin1. Either Pin1 WT or Pin1 KO cells were treated with cycloheximide for 6 h. Fig. 2*C* shows that whereas inhibition of translation led to a ~70% decrease in cyclin E protein in the wild type cells, cyclin E turnover was impaired in the pin1^{-/-} cells and showed only a small decrease (<20%) 6 h after the addition of cycloheximide (Fig. 2*C*). Based on these results, we conclude that the primary way by which Pin1 regulates cyclin E is likely to be at the protein rather than the mRNA level.

The Absence of Pin1 in MEFs Results in Cell Cycle Defects—pin1^{-/-} MEFs contain increased amounts of cyclin E (this study) and c-Myc (5), and deregulation of either cyclin E or c-Myc results in defects in the cell cycle (1). Cyclin E protein overexpression leads to an accelerated progression of G₀/G₁ to S phase coupled with an overall increase in the length of the cell cycle, possibly due to an increase in the duration of S phase (13, 14, 16, 38). Since cyclin E and c-Myc are deregulated in

pin1^{-/-} MEFs, we questioned whether cell cycle progression through S phase might be compromised. First, as shown in Fig. 3*A*, the doubling time of pin1^{-/-} MEFs is considerably slower than that of WT MEFs. This result is similar to that reported by others using MEFs isolated from Pin1 null mice of a different genetic background than ours (28, 40). Second, to evaluate whether this slower cell cycle progression might reflect changes in S phase entry or progression, we pulse-labeled asynchronously growing populations of MEFs with BrdUrd and quantified its presence by immunocytochemical analysis of both pin1^{+/+} and pin1^{-/-} MEFs. As shown in Fig. 3*B* (which represents three collective experiments with each experiment containing $n \geq 300$ /cell type), the absence of Pin1 in MEFs results in a statistically significant ($p = 0.0037$ evaluated by Student's *t* test) decrease in the percentage of cells that incorporate BrdUrd. This outcome could be explained by either a decrease in the number of cells entering S phase during the time of the BrdUrd pulse or an inability of cells that enter S phase to synthesize DNA efficiently. To distinguish between these possibilities, we subjected asynchronously growing cells to single parameter DNA profiling to determine the percentage of cells in G₁, S, and G₂/M phases. Fig. 3*C* (left and middle bars) shows that a higher percentage of pin1^{-/-} cells are in G₁ and S phases relative to WT cells. On the other hand, we found a concomitant decrease in the percentage of pin1^{-/-} cells in G₂/M as seen in Fig. 3*C* (right bars). Thus, progression through the G₁/S phases

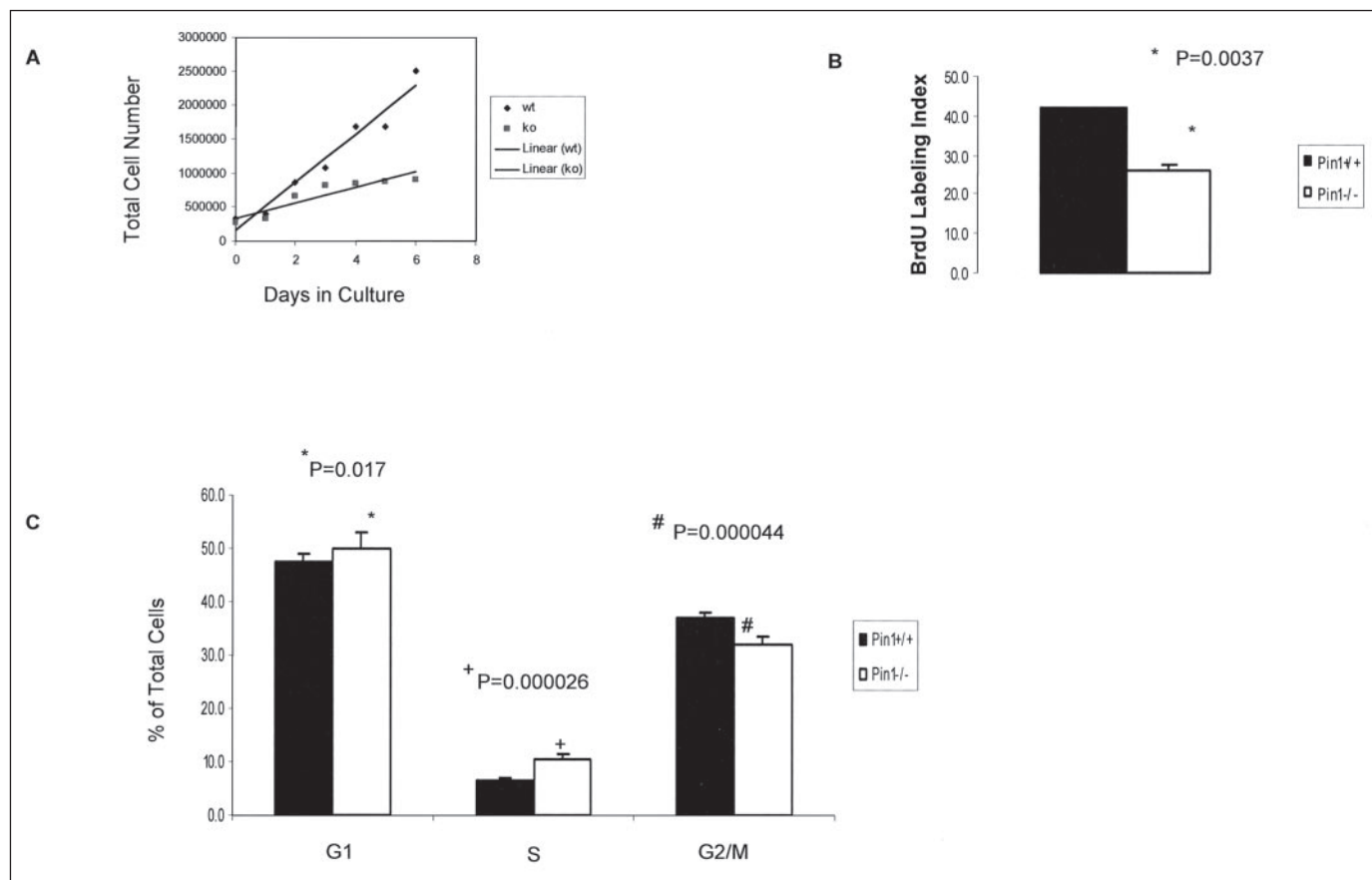


FIGURE 3. The absence of Pin1 in MEFs results in cell cycle defects. A, Pin1 null MEFs have longer doubling times compared with wild type. Equal numbers of $pin1^{+/+}$ and $pin1^{-/-}$ MEFs were plated into 6-well dishes. Cells from duplicate wells per each cell type were trypsinized and counted every 24 h for 6 days. The graph is representative of five similar independent experiments. $pin1^{-/-}$ cells display a growth deficiency. B, Pin1 null cells undergo DNA synthesis more slowly as measured by BrdUrd incorporation. Cells were plated on glass coverslips in equal numbers. Cells were pulsed with BrdUrd prior to fixation and staining with anti-BrdUrd antibody to assess the number of cells which have incorporated BrdUrd. Cells lacking Pin1 incorporated less BrdUrd. This experiment was performed three times, counting $n \geq 300$ per cell type per experiment, and is statistically significant as analyzed by the Student's *t* test ($p = 0.0037$). C, $pin1^{-/-}$ MEFs progress more slowly through G_1/S phases. Asynchronous populations of $pin1^{+/+}$ and $pin1^{-/-}$ MEFs were trypsinized and fixed for single parameter fluorescence-activated cell sorting analysis. Cells were resuspended in PBS containing RNase and stained with propidium iodide to analyze DNA content. This experiment represents three individual experiments using ≥ 3 replicates/cell type/experiment and is statistically significant as analyzed by Student's *t* test (see right panel for *p* value). The graph represents the total percentage of cells in G_1 , S, and G_2/M phases. The $pin1^{-/-}$ MEFs show a greater percentage of cells in G_1 and S phases than $pin1^{+/+}$ MEFs, and a concomitant decrease in G_2/M occurs in the $pin1^{-/-}$ population. *ko*, knock-out.

of the cell cycle is impaired in $pin1^{-/-}$ cells. These data are representative of three individual experiments, which each used ≥ 3 replicate samples/cell type, and are highly statistically significant (see Fig. 3C). The results in Fig. 3, B and C, are entirely consistent with data obtained by Ekholm-Reed *et al.* (24), who overexpressed cyclin E in human cells and support the concept that the absence of Pin1, at least in part due to the deregulation of cyclin E, compromises the ability of cells to synthesize DNA and to progress through S phase.

Immortalization of $Pin1^{-/-}$ MEFs with a Dominant Negative p53 Results in a More Rapid Progression toward Genomic Instability—In addition to cell cycle defects involving G_1/S progression, cyclin E and c-Myc protein deregulation is involved in the generation of chromosomal instability (16, 20, 22, 25, 27, 41–50) and correlates with tumor formation (18–22, 25, 41, 51–64). Because c-Myc and cyclin E levels are increased in $pin1^{-/-}$ cells, we evaluated the $pin1^{-/-}$ cells for markers of genomic instability. We first quantified the number of micronucleated cells in early passage (passage 3) primary MEFs of the $pin1^{+/+}$ and $pin1^{-/-}$ genotypes. As shown in the left pair of bars in Fig. 4B, micronuclei occur in about 5% of the WT MEFs, and, although the Pin1 null cells show only about a 2% increase in this percentage (to about 7%), the difference is highly statistically significant ($p = 0.0000158$, Student's *t* test, $n = 1000$). These data show that even at passage 3, primary MEFs

form micronuclei and that the absence of Pin1 exacerbates this defect. To determine whether these DNA changes result in aneuploidy, we subjected asynchronously cycling populations of passage 3 primary $pin1^{+/+}$ and $pin1^{-/-}$ cells to single parameter DNA profiling but found that both populations of primary cells displayed normal distribution in each phase of the cell cycle as measured by fluorescence-activated cell sorting (Fig. 4C, top panels; data represent three individual experiments performed with ≥ 3 replicates/cell type in each experiment).

Inactivation of p53 will immortalize mouse cells and lead to progressive genomic instability (27, 65, 66). To determine whether this p53 inactivation-dependent process would also be sensitized by the absence of Pin1, we infected the passage 3 $pin1^{+/+}$ and $pin1^{-/-}$ MEFs with a retrovirus encoding a dominant-negative p53 that expresses a truncated version of the p53 protein termed p53_{DD} (Fig. 4A). p53_{DD} is the C-terminal portion of p53 containing amino acids 302–390 and functions to prevent transcription by inhibiting p53 tetramer formation (67). Upon stable expression of p53_{DD}, we obtained cell lines that continue to grow for greater than 50 passages (data not shown), which is many more than the 4–6 passages number that is typical of primary MEFs. Since Pin1 generally functions in the timing of events that occur during cell proliferation (30, 68, 69) and we found an increase in micronuclei formation even in passage 3 primary MEFs, we wanted to evaluate the effect of Pin1

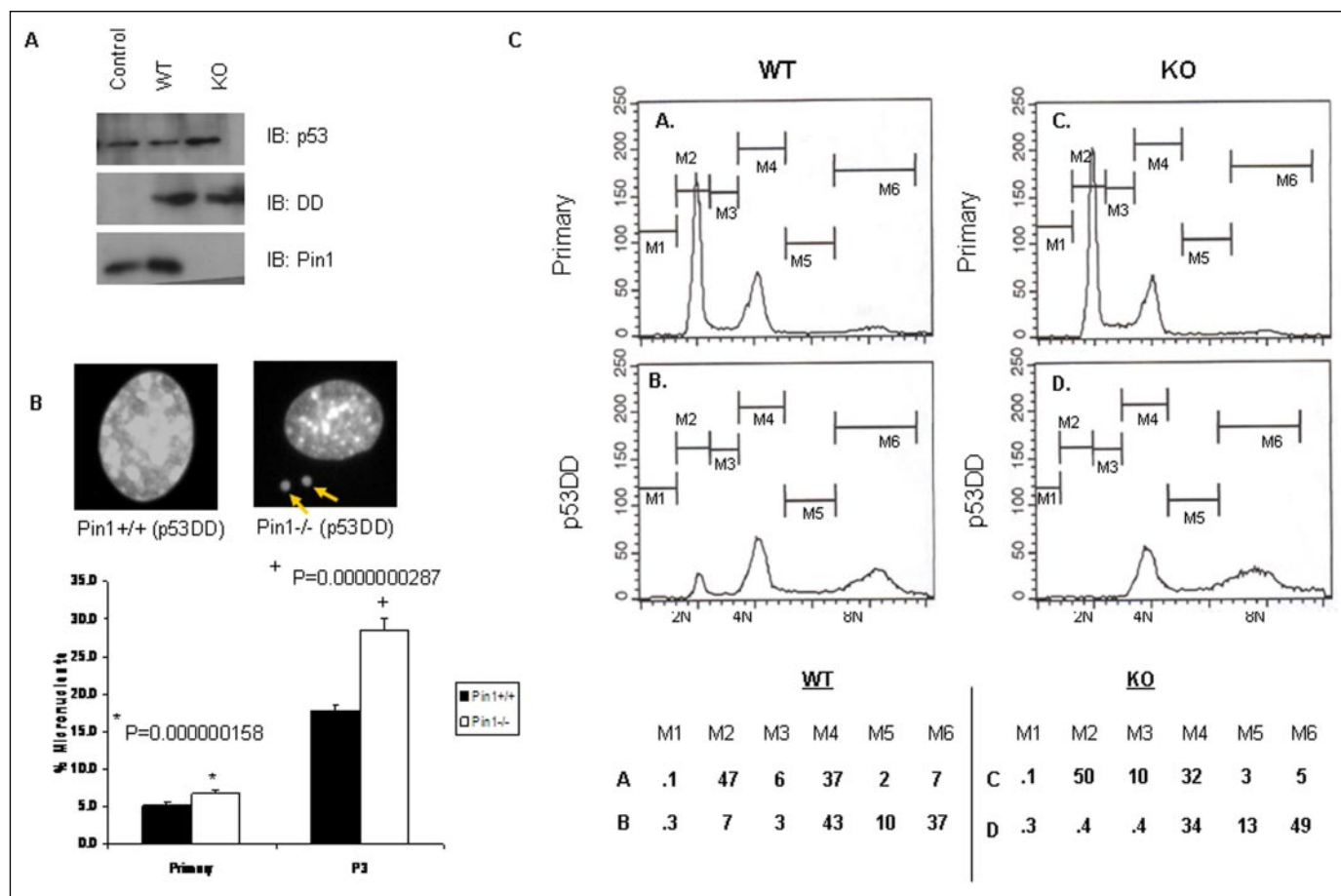


FIGURE 4. Immortalization of $Pin1^{-/-}$ MEFs with a dominant negative p53 results in a more rapid progression toward genomic instability. A, creation of stable cell line expressing p53_{DD}. Primary MEFs were infected with retrovirus containing the dominant negative form of p53, p53_{DD}. Cell lysates were analyzed by Western blotting (IB) and probed for the expression of p53_{DD} using anti-p53 antibody. p53_{DD} is expressed as a truncated form of p53 protein and runs at a lower *M_r* (middle) than native p53 protein (top). B, Pin1 null cells show increased formation of micronuclei. Cells were plated on coverslips prior to methanol fixation and staining with 4',6-diamidino-2-phenylindole. Cells were visualized by fluorescence microscopy and scored by counting total number of cells (≥ 300 cells counted per cell type in three individual experiments; see figure for *p* value) compared with the number of cells containing micronuclei. C, immortalization of $Pin1^{-/-}$ MEFs with a dominant negative p53 results in a more rapid progression toward aneuploidy. Primary MEFs (passage 3) and p53_{DD} MEFs (passage 3 after the addition of p53_{DD}) were fixed and stained with propidium iodide prior to analysis by single parameter flow cytometry to determine DNA content. Primary $pin1^{+/+}$ and $pin1^{-/-}$ MEFs did not display aneuploidy, whereas immortalized cells of both genotypes resulted in a higher number of cells containing 4 N DNA content indicating aneuploidy. $pin1^{-/-}$ cells contained the highest percentage of cells with >4 N DNA content (compare rows B and D, M5 and M6). KO, knock-out.

absence as early as possible after the stable integration of p53_{DD}. This cell selection and verification of protein expression required three cell passages, so MEFs at passage 3 after infection of the retrovirus expressing p53_{DD} (or a total of six passages altogether) were used in all subsequent experiments. Fig. 4A shows that p53_{DD} is expressed to a similar extent in both $pin1^{+/+}$ and $pin1^{-/-}$ MEFs and that this does not alter the expression of endogenous p53. Expression of p53_{DD} caused a marked increase in micronuclei formation in both Pin1-replete and -null cells, although the absence of Pin1 increased this from about 17.5 to 28% of the cells ($p = 0.000000287$, Student's *t* test, $n = 1000$) (Fig. 4B, right pair of bars). This increase in the percentage of micronucleated cells is mirrored by a change in the DNA profile in both $pin1^{+/+}$ and $pin1^{-/-}$ cells expressing p53_{DD} (Fig. 4C, bottom panels; data represent three individual experiments performed with ≥ 3 replicates/cell type in each experiment). Aneuploidy is evident in both cell populations but is considerably more advanced in the cells null for Pin1 (e.g. 47% versus 62% of cells with >4 N DNA content in $pin1^{+/+}$ and $pin1^{-/-}$ cells, respectively; Fig. 4C, lanes M5 and M6, compare rows B and D). These results support our conjecture that the absence of Pin1 promotes genomic instability in cells in a more accelerated fashion than in the presence of Pin1 and that the loss of Pin1 acts in concert with the loss of p53 function to promote this effect.

The Absence of Pin1 Sensitizes Cells to More Aggressive Transformation and Tumorigenesis in Vivo—Studies from the cyclin E knock-out mouse indicate that the loss of cyclin E protects mouse cells from Ras-mediated transformation (70). Because cyclin E is up-regulated in $pin1^{-/-}$ cells and these cells show increased genomic instability at early passage after immortalization, we hypothesized that deletion of Pin1 might lead to changes in the genome that are sufficient to promote more advanced cellular transformation of MEFs. In support of this hypothesis, we have previously reported that c-Myc is stabilized in the absence of Pin1 (5). Since Myc and Ras collaborate to transform rodent cells (47), it seemed plausible that the addition of oncogenic Ras alone to $Pin1^{-/-}$ primary MEFs might induce spontaneous transformation. However, expression of Ras^{G12V} alone in $pin1^{-/-}$ cells caused the early passage primary MEFs to senesce, similar to what we found to occur in wild type MEFs (data not shown).

Previous reports indicate that whereas cooperating oncogenes such as Myc and Ras can spontaneously transform rodent cells, several lines of evidence suggest that the expression of these oncogenes co-selects for the loss of p53 and INK4a (71, 72). Boehm *et al.* (73) recently demonstrated that p53_{DD} collaborates with Myc and Ras to efficiently transform MEFs. These authors demonstrated that whereas p53_{DD} and Ras alone can result in transformation of wild type MEFs, the transforma-

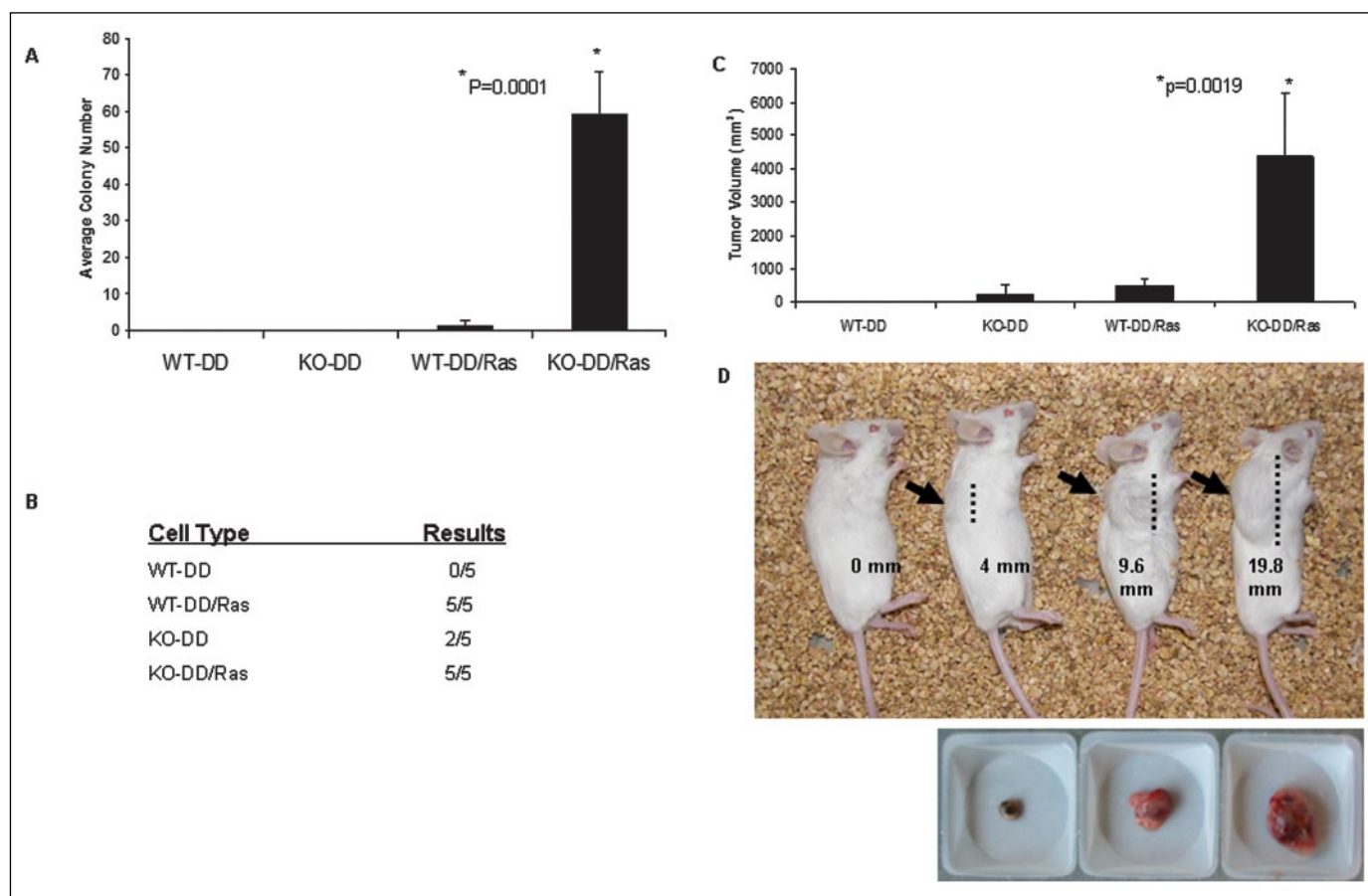


FIGURE 5. Pin1 depletion sensitizes cells to transformation and can initiate tumorigenesis *in vivo*. A, Pin1-null cells are more readily transformed in the presence of Ras. *pin1*^{+/+} or *pin1*^{-/-} cells were stably infected with p53_{DD} alone or p53_{DD} and Ras^{G12V} retrovirus as indicated. 50,000 cells were plated in 0.3% agar, incubated for 21 days, and assessed for colony growth in soft agar. The mean and S.D. represent two independent experiments performed in triplicate ($p = 0.0001$, Student's *t* test). The *pin1*^{-/-} p53_{DD} Ras cells formed colonies in soft agar to a much greater extent than *pin1*^{+/+} p53_{DD} Ras cells. B, summary of *in vivo* tumor formation. Mice were injected with *pin1*^{+/+} and *pin1*^{-/-} MEF expressing p53_{DD} alone and *pin1*^{+/+} and *pin1*^{-/-} MEF expressing p53_{DD} and Ras^{G12V} ($n = 5$ mice for each cell type). Injections were performed subcutaneously at the shoulder. C, mice injected with *pin1*^{-/-} MEF expressing p53_{DD} and Ras^{G12V} formed more aggressive tumors than *pin1*^{+/+} MEF expressing p53_{DD} and Ras^{G12V}. Tumor volume analysis represents the average tumor volume calculated from the five mice injected per cell type ($p = 0.0019$; compare *pin1*^{+/+} and *pin1*^{-/-} MEF expressing p53_{DD} and Ras^{G12V} by Student's *t* test). The volumes (in mm³) of tumors derived from *pin1*^{-/-} MEFs are significantly larger than tumors derived from *pin1*^{+/+} MEFs. D, the tumors derived from *pin1*^{-/-} MEFs were markedly larger than the tumors derived from *pin1*^{+/+} MEFs. Images are representative of tumor size (indicated with average diameter; top) prior to and after excision (bottom).

tion of these cells can be accelerated by the addition of another oncoprotein, such as Myc. Thus, MEFs expressing p53_{DD} and Ras were less efficiently transformed compared with MEFs expressing p53_{DD}, Ras, and Myc, and the MEFs only containing p53_{DD} and Ras formed significantly fewer colonies in soft agar than MEFs containing p53_{DD}, Ras, and Myc (73). These results demonstrate that the serial introduction of oncogenic components (p53_{DD}, Ras, Myc) can result in an additive effect on transformation. In Fig. 5A, we demonstrate that expression of oncogenic Ras results in the ability of p53_{DD}, *pin1*^{-/-} MEFs to form colonies in soft agar to a higher extent than *pin1*^{+/+} MEFs expressing the same components (Fig. 5A). The fact that these latter cells would form colonies to a markedly lower extent is consistent with the conclusions of the study by Boehm *et al.* (73). These results show that deletion of Pin1 can sensitize cells to a more aggressive Ras-induced transformation relative to *pin1*^{+/+} cells. We postulate that the deregulation of cell cycle proteins, such as cyclin E and c-Myc, in *pin1*^{-/-} cells may be at least in part responsible for this difference.

In order to test the *in vivo* relevance of Pin1 depletion in tumor formation, we injected immunocompromised mice with four cell types: *pin1*^{-/-} MEFs expressing p53_{DD} alone, *pin1*^{-/-} MEFs expressing p53_{DD} and Ras, *pin1*^{+/+} MEFs expressing p53_{DD} alone, and *pin1*^{+/+} MEFs expressing p53_{DD} and Ras. Consistent with our demonstration that loss of Pin1 promotes a more aggressive type of transformation in

conjunction with loss of p53 function and oncogenic Ras when compared with WT cells, we found the same to be true in our tumorigenesis assay. Whereas both *pin1*^{+/+} MEFs expressing p53_{DD} and Ras and *pin1*^{-/-} MEFs expressing p53_{DD} and Ras supported tumor formation in immunocompromised mice (Fig. 5B), the tumors that developed from the *pin1*^{-/-} MEFs expressing p53_{DD} and Ras were significantly larger in volume (Fig. 5C) and size (Fig. 5D) than their WT counterparts just 1 week postinjection. In fact, two of the five SCID mice injected with *pin1*^{-/-} MEFs expressing p53_{DD} alone formed tumors (Fig. 5B). These data support the idea that cells can exhibit varying levels of oncogenic potency based on their ability to select for specific mutations, such as loss of p53 or Myc overexpression. Thus, Ras is more efficient in causing tumorigenesis when Pin1 null MEFs are immortalized by expression of p53_{DD} but not when cells contain normal levels of Pin1. Our study demonstrates that the loss of Pin1, perhaps due to a deregulation of Pin1 protein substrates such as cyclin E and Myc, has a profound effect on proliferation, genomic instability, and tumorigenesis of MEFs.

DISCUSSION

Here we show that cyclin E is a Pin1-binding protein and that the steady-state level of cyclin E protein is increased in Pin1-null MEFs isolated from mice in which the *pin1* gene deletion is maintained in an isogenic C57BL6 background. This increased protein level seems to be

predominantly due to a decreased rate of cyclin E turnover. Additionally, we show that Pin1 binds to the cyclin E-Cdk2 complex in a phosphorylation-dependent manner; the interaction requires an intact Ser³⁸⁴ and is decreased by inhibition of Cdk2 activity. Although antibodies that specifically recognize Ser(P)³⁸⁴ are not available, these data argue that Ser³⁸⁴ may need to be phosphorylated in order for Pin1 to efficiently bind the cyclin E-Cdk2 complex. The fact that Ser³⁸⁴ is phosphorylated by the Cdk2 component of the cyclin E-Cdk2 complex (12) and this phosphorylation is required for SCF^{Cdc4}-dependent degradation of cyclin E (12, 33, 35) but not for formation of the cyclin E-Cdk2 complex suggests the possibility that Pin1 binds to cyclin E and facilitates its ubiquitylation in preparation for its degradation via the 26 S proteasome. This hypothesis is consistent with our previous study, which demonstrated that Myc is a phosphorylation-dependent Pin1-binding protein, the loss of Pin1 increased the steady state level of Myc in MEFs, and the deregulation of Myc protein was due to a decreased rate of its ubiquitylation and degradation (5). Since both cyclin E and Myc are degraded by SCF^{Cdc4} (6, 7, 33, 36, 74) and the “phosphodegrons” recognized by Cdc4 and Pin1 are remarkably similar, our data support the idea that a mechanism with many common features controls the degradation of these two important cell regulatory proteins.

On the other hand the precise way in which Pin1 participates in the Cdc4-dependent degradation of cyclin E and Myc may be different. In the case of Myc, the binding of Pin1 required phosphorylation of Thr⁵⁸, which has been proposed to constitute the primary Cdc4 recognition site (6, 7, 74). Pin1 binding resulted in a conformational change in the phosphodegron that presented Ser(P)⁶² as a substrate for PP2A (5). Since ubiquitylated Myc contained only Thr(P)⁵⁸, we concluded that Pin1/PP2A-mediated dephosphorylation of Ser⁶² was important for ubiquitylation and/or degradation (5). We show in the current study that Pin1 binding does not require phosphorylation of Thr³⁸⁰ in cyclin E, which is equivalent to Thr⁵⁸ in Myc and also constitutes the primary binding site for Cdc4 based on crystallographic analysis of Cdc4 in complex with a peptide mimic of the cyclin E phosphodegron (3). Rather, effective Pin1 binding to cyclin E is dependent on phosphorylation of Ser³⁸⁴, which is in a location in the phosphodegron equivalent to Ser⁶² in Myc. Regardless of these differences, the outcome of Pin1 binding to a phosphorylated form of cyclin E and Myc is the same, since this interaction promotes Cdc4-dependent degradation in each case. Clarifying the precise mechanism by which Pin1 facilitates the turnover of cyclin E will be an important future endeavor.

There is considerable information demonstrating a role for Pin1 in the G₀/G₁ to S phase transition, and many of its proposed targets are deregulated in human cancer (4, 5, 75–91). Because cyclin E and Myc levels (5) are increased in MEFs null for Pin1 and deregulation of cyclin E and Myc have been linked to processes involved in tumorigenesis (16, 20, 22, 25, 27, 41–44, 46, 92), such as hyperproliferation and genomic instability, we evaluated whether Pin1 null MEFs would be more susceptible to Ras-dependent transformation after they were immortalized by conditional inactivation of p53. Indeed, we found that the absence of Pin1 led to increased genomic instability, and in the presence of p53^{DD} these cells could be more aggressively transformed by oncogenic Ras than wild type cells. Thus, at least in a C57BL6 genetic background, loss of function of Pin1 sensitizes MEFs to more extensive and aggressive transformation and tumorigenesis.

Contrary to our results, loss of Pin1 has been suggested to decrease sensitivity to cancer (81). It would follow that mice null for Pin1 might be “protected” from oncogene-mediated tumorigenesis. Wulf *et al.* (89) tested this hypothesis by breeding FVB mice containing a transgene expressing MMTV-Neu or MMTV-H-Ras with Pin1-null mice main-

tained in SvJae/129/C57BL6 mixed genetic background. MMTV-Neu and MMTV-Ras have previously been shown to form spontaneous breast tumors, resulting in decreased survival (93, 94). Wulf *et al.* (67) hypothesized that the resulting *pin1*^{-/-}/*ras* or *pin1*^{-/-}/*neu* transgenic populations would be less susceptible to tumor formation and have an increased chance of survival. These authors found that the absence of Pin1 prevented oncogenic Neu or Ras from inducing breast cancer and attributed these effects to cyclin D deregulation due to the loss of Pin1. Correlating with these observations, MEFs from the Pin1 null mice maintained in the SVJae/129/C57BL6 background demonstrated decreased protein levels of cyclin D1, c-Jun, and β -catenin (4, 79, 88). Therefore, it is difficult to contend that depletion of Pin1 cannot also decrease susceptibility to mammary cancer, at least in a FBV/129/SvJae/C57BL6 mixed genetic background.

How can one reconcile these apparently opposite outcomes of the role for Pin1 in cancer? The differences in protein expression in the SVJae/129/C57BL6 mixed background (*e.g.* c-Jun, cyclin D1, and β -catenin) *versus* the isogenic C57BL6 (*e.g.* c-Myc and cyclin E) predict differential sensitivity of the two lines of mice to oncogenesis, and it is highly unlikely that these are the only differences that exist between cells derived from these two genetic backgrounds. It is certainly not surprising that identical mutations can have different biological effects when studied in distinct genetic backgrounds, which probably reflect differential expression of potential modifier genes, and this could explain the conundrum of the role of Pin1 in tumorigenesis. This idea was clearly demonstrated by Reilly *et al.* (95), who proposed that the susceptibility of mice to development of astrocytoma is intimately linked to the genetic strain of mice in which the experiments are conducted. These authors engineered different strains of mice (namely 129, 129/C57BL6, 129/SvJae, or C57BL6) to express mutant Nf1 or TP53 (95). The mutant mice maintained in the isogenic C57BL6 genetic background always formed astrocytomas. Conversely, the same mutations in mice maintained in 129, 129/C57BL6, or 129/SvJae backgrounds did not result in astrocytoma formation. These data provoked the authors to suggest that the study of specific gene mutations in mixed genetic backgrounds, especially those including 129, might conceal the functions of certain genes. Moreover, proteins other than Pin1 have also been reported to have conflicting roles in tumorigenesis. For example, transforming growth factor β has been described as functioning either to suppress or exacerbate invasive and/or metastatic behavior of tumor cells (39, 96, 97). Similarly, studies on the protein DCC demonstrate that the loss of DCC occurs frequently in colorectal cancers, whereas forced expression of the DCC ligand, netrin-1, promotes intestinal tumor development (31). As a result of these observations, it was suggested that DCC and netrin-1 be classified as “conditional” tumor suppressors to distinguish them from “classical” tumor suppressors, such as p53 or Rb, which always inhibit tumor formation due to their role in the cell cycle. Based on this analysis, it is tempting to speculate that, at least in the context of a C57BL6 genetic background, Pin1 can also function as a conditional tumor suppressor due in part to its role in regulating the timely Cdc4-dependent degradation of cyclin E and c-Myc.

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