Prolyl Isomerase Pin1 Is a Conditional Tumor Suppressor

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Pharmacology and Cancer Biology
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2011

ABSTRACT

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Abstract

Pin1 specifically binds to and catalyzes the *cis-trans* isomerization of phosphorylated-Ser/Thr-Pro motifs, which modulate the stability, localization, and function of numerous Pin1 substrates involved in cell cycle and tumorigenesis. During cell cycle progression, the timely synthesis and degradation of key regulatory proteins is required to maintain genomic integrity. Previously, we determined that Pin1 binds to and promotes the degradation of the oncoprotein c-Myc. Pin1 and the SCF^{Cdc4} ubiquitin ligase recognize a "phosphodegron" sequence in c-Myc, which leads to its ubiquitination and degradation by the 26 S proteasome. Since cyclin E is another oncoprotein regulated by SCF^{Cdc4}, we hypothesized that Pin1 may also bind to and facilitate cyclin E turnover. Here we show that Pin1 binds to the cyclin E-Cdk2 complex in a manner that requires Ser384 of cyclin E, which is phosphorylated by Cdk2. The absence of Pin1 in mouse embryonic fibroblasts (MEFs) results in stabilization of cyclin E, and impairment of G1-S phase progression. Furthermore, deregulated cyclin E and c-Myc levels correlate with accelerated genomic instability in *Pin1*$^{-/-}$ MEFs, which results in sensitization of these cells to more aggressive Ras-dependent transformation and tumorigenesis.

However, the role of Pin1 in cancer is controversial as it has been proposed to conditionally promote or suppress tumorigenesis depending on the genetic context. In

human cancer, Pin1 protein levels are frequently altered in several cancers. Interestingly, the PIN1 gene is located on chromosome 19p13.2, which is a region subject to loss of heterozygosity in several tumors. Since Pin1 protein is frequently under-expressed in kidney cancer, we tested the hypothesis that it may have a tumor suppressive role in human clear cell renal cell carcinoma (ccRCC). Here we show evidence for PIN1 gene deletion and mRNA under-expression as a mechanism of Pin1 reduction in ccRCC tumors. We demonstrate that restoration of Pin1 in cell lines found to be deficient in Pin1 protein expression can attenuate the growth of ccRCC cells in soft agar and a xenograft tumor model. Moreover, this ability of Pin1 to negatively influence tumor growth in ccRCC cells may be dependent on the presence of functional p53, which is infrequently mutated in ccRCC. These observations suggest Pin1 may function as a conditional tumor suppressor.

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List of Acronyms and Abbreviations

Ala, A	Alanine
amp	Ampicillin
ANOVA	Analysis of variance
APC	Anaphase-promoting complex
Arg	Arginine
Asp	Aspartic acid
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
ccRCC	Clear cell renal cell carcinoma
Cdk	Cyclin-dependent kinase
CTD	C-terminal domain
Cys	Cysteine
DAB	3,3′-diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DAPK1	Death-associated protein kinase 1
DCC	Deleted in colorectal cancer

DD	Dominant negative
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Emi1	Early mitotic inhibitor-1
F	Forward
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gln	Glutamine
Gly, G	Glycine
GSA	Gene set analysis
GSK	Glycogen synthase kinase
GST	Glutathione S-transferase
hiFBS	Heat-inactivated fetal bovine serum
H&E	Hematoxylin and eosin
His	Histidine
hPin1	Human Pin1
hr	Hours
HRP	Horseradish peroxidase

Hygro	Hygromycin resistance
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactoside
Leu	Leucine
LOH	Loss of heterozygosity
Lys	Lysine
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MEF	Mouse embryonic fibroblast
mPin1L	Murine Pin1-like
Neo	Neomycin resistance
NIMA	Never in mitosis A
NSC	Non-silencing control
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
Phospho-	Phosphorylated
Pin1	Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1
PKA	Protein kinase A
PKC	Protein kinase C

PLK	Polo-like kinase
PML-RARα	Promyelocytic leukemia/retinoic acid receptor-α fusion
PPIase	Peptidyl-prolyl isomerase
Pro	Proline
Puro	Puromycin resistance
R	Reverse
Rb	Retinoblastoma
RCC	Renal cell carcinoma
RPTEC	Renal proximal tubule epithelial cells
RT	Reverse transcription
SCF	Skp1-Cul1-F-box
SDS	sodium dodecyl sulfate
shPin1	Short-hairpin RNA targeting Pin1
Ser, S	Serine
SNP	Single-nucleotide polymorphism
SRC-3	Steroid receptor coactivator 3
Stat3	Signal transducer and activator of transcription 3
TBS-T	Tris-buffered saline/0.2% Tween 20
TCGA	The Cancer Genome Atlas
TGFβ	Transforming growth factor β

Thr, T	Threonine
Topo	Topoisomerase
Trp, W	Tryptophan
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr, Y	Tyrosine
Val, V	Valine
WT	Wild type
xPin1	Xenopus Pin1

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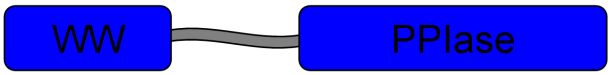
1. Introduction

1.1 Pin1: Structure and Function

1.1.1 Structure of Pin1

Pin1 is an evolutionarily conserved parvulin peptidyl-prolyl isomerase (PPIase), which catalyzes the *cis-trans* isomerization of proline amide bonds. However, Pin1 is distinct from other PPIases, in that it specifically recognizes and binds to phospho-Ser/Thr-Pro motifs (Yaffe et al 1997). Upon binding to such motifs, Pin1 can accelerate the *cis* to *trans* isomerization rate by 10,000-fold and the reverse rate by 1,000-fold (Pastorino et al 2006). In order to perform this activity, Pin1 has two functional domains (Figure 1), which are the N-terminal WW domain (residues 1-39) involved in substrate binding and C-terminal catalytic PPIase domain (residues 45-163).



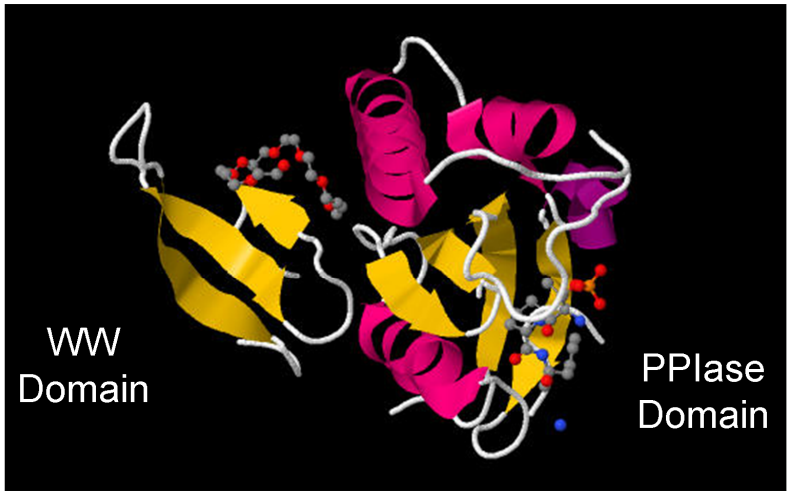


Figure 1: Structure of human Pin1.

Structure of human Pin1 (Zhang et al 2007) viewed using Jmol Viewer (www.pdb.org).

The WW domain ("WW" refers to its two conserved Trp residues) consists of a triple-stranded anti-parallel β sheet, which is stabilized through intradomain ionic (via Lys13) and hydrophobic (via Trp11) interactions (Behrsin et al 2007, Jäger et al 2001, Jäger et al 2007). This domain primarily functions as a protein-protein interaction module that allows Pin1 to bind its substrates. Several mechanistic details of how Pin1 binds to phospho-Ser/Thr-Pro motifs have been elucidated from Pin1 X-ray crystallographic structures and mutagenesis studies. In the crystal structure of Pin1 bound to RNA Polymerase II CTD phospho-peptide, Pin1's Ser16, Arg17, and Tyr23

form hydrogen bonds with the phosphate of phospho-Ser5 of the peptide (Verdecia et al 2000). Such interactions help provide the basis for the phosphorylation dependence of Pin1-substrate binding. Additional Pin1-substrate interactions occur through Tyr23 and Trp34, which form a clamp that holds the backbone atoms of Thr4 and phospho-Ser5, and the ring atoms of Pro6 of the CTD peptide (Verdecia et al 2000). The importance of Tyr23 or Trp34 to Pin1 function is demonstrated by the inability of $Pin1^{Y23A}$ and $Pin1^{W34A}$ to bind Pin1 substrates (Lu et al 1999). Thus, the WW domain is clearly involved in Pin1-substrate interaction, but one must also consider the contributions of the PPIase domain to Pin1's binding specificity and function.

A flexible linker connects Pin1's WW domain to its catalytic PPIase domain (Figure 1). The C-terminal PPIase domain consists of a four-stranded anti-parallel β sheet surrounded by four α helices (Ranganathan et al 1997). In the crystal structure of Pin1 in complex with Ala-Pro dipeptide and sulfate ion, the loop spanning residues 66 and 77 contains a cluster of basic residues (Lys63, Arg68, and Arg69) that binds sulfate, and thus forms a phosphate binding module (Ranganathan et al 1997). This loop structure confers additional phospho-specificity to Pin1's substrate binding and isomerase activity. Prolyl isomerization by Pin1 was originally presumed to occur through a covalent mechanism involving Cys113, His59, and His157, in which the Sγ of Cys113 participated in nucleophilic attack on the substrate carbonyl carbon (Ranganathan et al 1997). However, recent studies have called this proposed mode of

action into question, as mutational analysis revealed His59 and His157 may play structural rather than catalytic roles, and the catalytically important Cys113 could be functionally substituted by Asp (Bailey et al 2008, Behrsin et al 2007). Thus, it was postulated that Cys or Asp at position 113 may provide an electronegative environment sufficient to destabilize the double bond character of phospho-Ser/Thr-Pro bond, which is similar to the non-covalent mechanism used by other PPIases (Behrsin et al 2007). Although Pin1's catalytic activity may be more analogous to other PPIases than originally thought, Pin1 has structural features (e.g., phosphate binding modules) that impart a substrate binding specificity and function that are unique to Pin1.

1.1.2 Function of Pin1

Phosphorylation of certain Ser/Thr-Pro motifs in Pin1 substrates recruits Pin1 to alter their conformation via prolyl isomerization. Although in many cases the precise mechanism of Pin1 action is incompletely understood, Pin1 can modulate the stability, localization, and function of its substrates (Lu and Zhou 2007, Yeh and Means 2007). Thus, Pin1-mediated protein folding provides an additional level of regulation that collaborates with protein phosphorylation. Ser/Thr-Pro motifs are phosphorylated by several families of Pro-directed protein kinases, including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3 (GSK3), and polo-like kinases (PLKs) (Lu and Zhou 2007). These kinases are involved in key regulatory signal transduction pathways that control diverse cellular processes, such

as cell cycle regulation and stress response, which are relevant to human diseases like cancer. Therefore, several groups have investigated potential roles for Pin1 in regulating cellular function, which are discussed in greater detail below.

1.1.3 Regulation of Pin1

Preliminary clues about Pin1's function in a cell might be gleaned from how Pin1 itself is regulated. Pin1 can be phosphorylated on Ser16 (by PKA and PKC), Ser65 (by PLK1), and Ser71 (by death-associated protein kinase 1, DAPK1) (Eckerdt et al 2005, Lee et al 2011, Lu et al 2002). Both Ser16 and Ser71 reside in Pin1's phosphate binding modules; therefore, phosphorylation of these residues disrupts Pin1's ability to bind to and isomerize its substrates (Lee et al 2011, Lu et al 2002). In contrast, phosphorylation of Ser65 does not interfere with Pin1's catalytic activity, but rather increases Pin1's stability by preventing its ubiquitination (Eckerdt et al 2005). Interestingly, the phosphorylation status of Pin1 seems to be cell cycle dependent, where Ser16 becomes dephosphorylated and Ser65 becomes phosphorylated as the cell progresses towards mitosis, which correlates with increasing Pin1 levels and activity. Pin1 phosphorylation can also affect Pin1 subcellular localization, which is determined by Pin1-substrate interaction (i.e., many Pin1 substrates localize to the nucleus) and a Pin1 nuclear localization signal (residues 61-70) (Lu et al 2002, Lufei and Cao 2009). In addition, the cell cycle dependent expression of Pin1 is controlled by E2F-mediated transcriptional activity (Ryo et al 2002). Considering the fact that many cell cycle effectors reside in the

nucleus, it seems probable that the cell cycle dependent up-regulation of Pin1 may be involved in cell cycle progression.

1.2 Pin1 and Cell Cycle

Cell division is a tightly regulated and well ordered process (Figure 2). Mitogenic stimulation activates signal transduction pathways, like the Ras-Raf-MEK-ERK cascade, which induces immediate early gene expression (e.g., c-Fos and c-Jun) as well as other important events that are reviewed elsewhere (Manning and Cantley 2007, Meloche and Pouyssegur 2007). c-Fos and c-Jun form AP1 complexes that regulate the transcription of key cell cycle regulators, such as cyclin D1 (Shaulian and Karin 2002). Cyclin D1 binds to and activates CDK4 to phosphorylate retinoblastoma, or Rb (the canonical gatekeeper of the G1-S phase transition) (Sherr 1995). The inactivating phosphorylation of Rb allows E2F transcription factors to turn on the cellular machinery required for the timely and accurate replication of the genome prior to mitosis (DeGregori et al 1995, Hiebert et al 1992). Since this well orchestrated sequence of events is escalated and regulated in large part by phosphorylation of proteins on Ser/Thr-Pro motifs (Lu and Zhou 2007), a growing number of studies demonstrate the importance of Pin1 in regulating cell cycle.

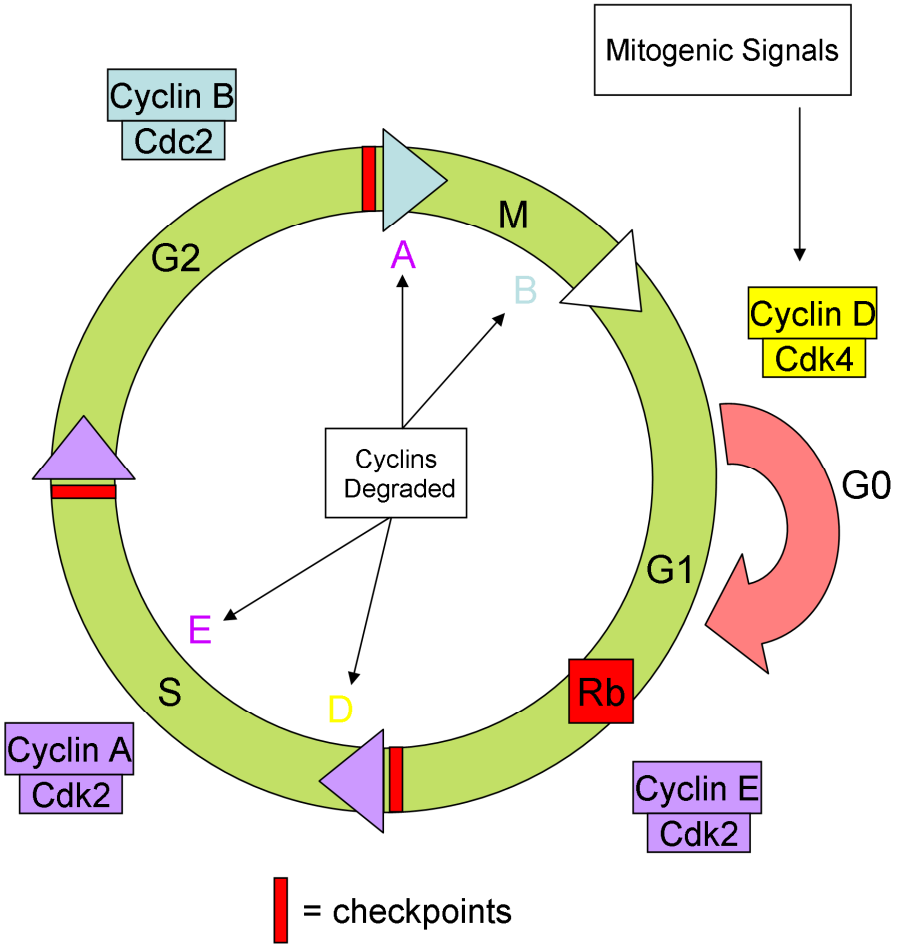


Figure 2: Cell cycle model.

1.2.1 Pin1 in G0/G1-S phase

Previous research using *Pin1*$^{-/-}$ mice from a mixed genetic background indicates that Pin1 may play a key role in G0/G1-S phase transition. Indeed, the phenotypes exhibited by these *Pin1*$^{-/-}$ mice are strikingly similar to those found in cyclin D1-null mice, including reduced body weight, testicular and retinal atrophy, and impaired mammary epithelial cell expansion during pregnancy (Liou et al 2002). Moreover, Pin1

binds to cyclin D1 at phospho-Thr286-Pro, and increases the post-translational stability and nuclear localization of cyclin D1 in mouse embryonic fibroblasts (MEFs) (Liou et al 2002). Further studies in cancer cell lines suggest Pin1 can also regulate cyclin D1 at the transcriptional level by binding to and increasing the activity and/or stability of c-Fos, c-Jun, and β-catenin (Monje et al 2005, Ryo et al 2001, Wulf et al 2001). Given the important role of these factors in G0/G1-S phase, it is unsurprising that *Pin1*$^{-/-}$ MEFs show defects in re-entering the cell cycle from G0 arrest (Fujimori et al 1999, You et al 2002). In addition, the loss of Pin1 in primordial germ cells from isogenic C57BL6 mice or the depletion of PINA from *Aspergillus nidulans* results in the prolongation of G1-S transition in these cells (Atchison et al 2003, Joseph et al 2004). Thus, Pin1 is involved in promoting G0/G1-S phase progression.

In addition, Pin1 is able to enhance mitogenic signaling by increasing the phosphorylation of MEK and ERK in response to growth factors (Khanal et al 2010). The activation of MEK/ERK is known to cause hyper-phosphorylation of Raf-1, as a potential negative feedback mechanism (Dougherty et al 2005). However, Pin1 facilitates the dephosphorylation of hyper-phosphorylated/desensitized Raf-1 by protein phosphatase PP2A to allow reactivation of the Raf-MEK-ERK pathway (Dougherty et al 2005), which would be necessary for G0 exit. Pin1 also increases the insulin-induced phosphorylation of $p70^{S6K}$ leading to enhanced ERK and AP1 activation (Lee et al 2009). These studies suggest that Pin1 participates in a feed forward mechanism involving multiple signaling

pathways to promote cell cycle progression. Moreover, the subsequent induction and activation of G1 cyclin/CDK complexes might be influenced by Pin1's ability to affect the levels of CDK inhibitor $p27^{Kip1}$ (Brenkman et al 2008, Zhou et al 2009). Collectively, these observations suggest Pin1 is a key modulator of G0/G1-S transition.

1.2.2 Pin1 in G2-M phase

A role for Pin1 in mitosis was first discovered through a screen that identified Pin1 as a protein that interacts with mitotic kinase NIMA (Lu et al 1996), which is an activity that Pin1 was named after, i.e., peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1. Pin1 attenuates NIMA's mitosis promoting activity in a manner that can prevent NIMA-induced mitotic catastrophe in yeast (Lu et al 1996). On the other hand, depletion of the yeast Pin1 ortholog Ess1 from yeast results in mitotic arrest (Hanes et al 1989), which suggests a complex role for Pin1 in mitosis. Follow-up studies using *Xenopus* extracts revealed Pin1 can interact with several other mitotic phospho-proteins, including Cdc25 and Wee1, which are critical regulators of cyclin B/CDC2 (Shen et al 1998). Although Pin1 does not bind to cyclin B directly (as it does with cyclin D1), Pin1 is presumed to regulate cyclin B/CDC2 through the modulation of Cdc25 and Wee1 activity (Crenshaw et al 1998, Okamoto and Sagata 2007, Stukenberg and Kirschner 2001, Zhou et al 2000). In addition, Pin1-mediated stabilization of early mitotic inhibitor-1 (Emi1), which inhibits anaphase-promoting complex (APC) activity, may promote cyclin B accumulation in G2-M phase (Bernis et al 2006).

In mammalian cell systems, the depletion or over-expression of Pin1 can perturb G2-M phase progression (Lu et al 1996, Rippmann et al 2000). It was recently shown that Pin1 is necessary for mitotic chromosome condensation in HELA cells, which may involve Pin1's ability to enhance the phosphorylation and DNA binding activity of topoisomerase (topo)-IIα (Xu and Manley 2007). Indeed, the addition of purified Pin1 and cyclin B/CDC2 is sufficient to induce chromosome condensation in S phase nuclei (Xu and Manley 2007). In S phase, Pin1 participates in centrosome duplication, which is necessary for chromosome segregation in mitosis (Suizu et al 2006). Furthermore, Pin1 interacts with mitotic phospho-protein Cep55 to regulate cytokinesis, such that Pin1-depleted HELA cells and *Pin1*$^{-/-}$ MEFs exhibit defects in cytokinesis (Van Der Horst and Khanna 2009). Hence, Pin1 exerts a diverse and complex role in G2-M phase as well as in G0/G1-S phase progression.

1.2.3 Pin1 as a molecular timer

Considering the plethora of cell cycle effectors regulated by Pin1, it is somewhat surprising that Pin1 is not essential in metazoans, like it is in *Saccharomyces cerevisiae* and *Aspergillus nidulans* (Yeh and Means 2007). However, it is interesting that only a very small amount (~400 molecules) of Pin1/Ess1 is required for the growth of yeast (Gemmill et al 2005). Thus, one explanation for the viability of *Pin1*$^{-/-}$ mice is complementation by other PPIases. Indeed, a murine Pin1 isoform (mPin1L) has been identified that is 3-fold less efficient than Pin1 (Zhu et al 2007). Also, parvulin Par14 is up-regulated 3-fold in

Pin1$^{-/-}$ MEF (Uchida et al 1999). Moreover, Pin1 appears to serve as a modulator of cell cycle proteins as opposed to an absolute "on-or-off switch". As a result, Pin1 is able to coordinate the timing of cell cycle events, and thus has been termed a "molecular timer" for the cell cycle (Atchison et al 2003, Yeh and Means 2007). However, the mistiming or deregulation of cell cycle can still have deleterious effects on a cell, which can lead to genomic instability.

1.3 Pin1 and Genomic Instability

During the cell cycle a cell grows, replicates its DNA, and divides into two daughter cells with each containing a full complement of the cellular genome. In particular, DNA synthesis must occur in a timely and accurate manner. Nonetheless, many factors such as ultraviolet or ionizing radiation, chemicals, and reactive oxygen species induce genotoxic stress. In order to deal with DNA damage, eukaryotic cells have developed cell cycle checkpoints (Figure 2) and DNA damage repair pathways, which block cell cycle progression until the damage is repaired or induce apoptosis if the damage is irreparable. The ATM/ATR kinases along with their downstream effectors, including Chk1/2 and p53, are important mediators of genotoxic stress response (Khanna and Jackson 2001).

In response to genotoxic insults, Pin1 binds to and stabilizes phosphorylated p53 and p73 (a p53 family member) (Mantovani et al 2004, Zacchi et al 2002, Zheng et al 2002). Whereas Pin1 stabilizes p53 by disrupting its interaction with ubiquitin ligase

MDM2, Pin1 stabilizes p73 by increasing its interaction with p300 (Mantovani et al 2004, Zacchi et al 2002). Pin1 also stimulates the p300-mediated acetylation of both p53 and p73 (Mantovani et al 2004, Mantovani et al 2007), which may contribute to Pin1's ability to enhance p53 and p73 transcriptional activity towards genes involved in DNA damage checkpoints or apoptosis. As a result, *Pin1*$^{-/-}$ MEF display impaired cell cycle arrest and apoptosis in response to DNA damage (Zacchi et al 2002, Zheng et al 2002), which suggests Pin1 is essential for genotoxic stress responses mediated by p53 family members.

Moreover, Pin1 facilitates the ATM-induced degradation of Bcl-6, a transcriptional repressor that can suppress p53-dependent and -independent responses in germinal center B cells, which undergo extensive genome-remodeling (Phan et al 2007). This highlights the possibility that Pin1 may have a broader role in ATM/ATR-mediated response to genotoxic stress. Indeed, Pin1-depletion from *Xenopus* extracts mimics the effects of ATM/ATR inhibition by caffeine treatment, which impairs G2-M checkpoint activation in response to unreplicated DNA (Winkler et al 2000). Incomplete DNA replication or improperly repaired DNA damage can lead to genomic instability and aneuploidy, which are hallmarks of human cancer. Since Pin1 modulates pathways responsible for genotoxic stress response and cell cycle regulation, Pin1 may play a role in genomic instability and cancer.

1.4 Pin1 and Cancer

1.4.1 Pin1 as a tumor promoter

Previous studies support a role for Pin1 in tumor progression. In fact, Pin1 is prevalently over-expressed in human tumors, including those from prostate, breast, liver, lung, and colon (Bao et al 2004). In some cases, Pin1 over-expression correlates with poor prognosis and clinical outcomes (Ayala et al 2003, Kuramochi et al 2006, Tan et al 2010), which suggests Pin1 may promote tumorigenesis. Several factors already discussed in Chapter 1.2.1 may contribute to Pin1-mediated tumor progression; however, a number of studies associate this with Pin1's ability to positively regulate cyclin D1 (Li et al 2006, Wulf et al 2001). Indeed, high levels of Pin1 correlate with cyclin D1 over-expression in several human cancers (Kuramochi et al 2006, Miyashita et al 2003, Nakashima et al 2004, Pang et al 2004). Notably, the reduction of Pin1 over-expression in hepatocellular carcinoma cells results in decreased cyclin D1 and β-catenin levels, and the reversal of their tumorigenic phenotype (Pang et al 2006). Additionally, Pin1 ablation in mammary epithelial cells prevents Ras/Neu-induced cyclin D1 expression and cellular transformation (Ryo et al 2002, Wulf et al 2004).

The effects of Pin1 over-expression in cancer cells are certainly not limited to cyclin D1 up-regulation. For example, high levels of Pin1 in breast cancer samples correlates with centrosome amplification (Suizu et al 2006). NIH3T3 cells that express ectopic Pin1 also exhibit centrosome amplification, which results in increased genomic

instability (Suizu et al 2006). Furthermore, Pin1 binds to and increases the stability and/or activation of several other oncogenic proteins, including Neu, Akt, Stat3, NF-κB and Mcl-1 (Ding et al 2008, Lam et al 2008, Liao et al 2009, Lufei et al 2007, Ryo et al 2003). Collectively, these results suggest Pin1 is a tumor promoter and might be an attractive therapeutic target.

1.4.2 Pin1 as a conditional tumor suppressor

Although the evidence presented above seems to suggest a causal relationship between Pin1 and cancer, the role of Pin1 is complex and enigmatic. As discussed in Chapter 1.3, Pin1 increases the protein stability and transcriptional activity of p53 and p73 in response to genotoxic stress. The fact that Pin1 can enhance the function of these classic tumor suppressors seems to suggest Pin1 may have some tumor suppressive activities in cells. Further support for this notion is found in previous studies that reveal Pin1 can also destabilize oncoproteins, including Bcl-6, PML-RARα, SRC-3 and c-Myc (Gianni et al 2009, Phan et al 2007, Yeh et al 2004, Yi et al 2005). Our group's contribution to this effort included the elucidation of how Pin1 negatively regulates c-Myc, which is discussed in Chapter 3.1.

Several other observations are also supportive of a tumor attenuating role for Pin1 in cancer. In fact, human chromosome 19p13.2 (the genomic location containing the PIN1 gene) undergoes frequent loss of heterozygosity (LOH) in breast tumor samples (Oesterreich et al 2001, Yang et al 2004), which suggests Pin1 may be under-expressed in

several tumors. Indeed, the Pin1 protein is prevalently under-expressed in human tumors from kidney, testis, adrenal gland and stomach (Bao et al 2004). Although the relevance of Pin1 under-expression in human cancer has not been investigated, the deletion of Pin1 from p53-null C57BL6 mice results in accelerated thymic hyperplasia due to increased levels of the intracellular form of Notch1 (Takahashi et al 2006). Perhaps more strikingly, these *Pin1*$^{-/-}$ mice can spontaneously develop lymphoma in the absence of a second transforming mutation (Takahashi et al 2006). We also observe infrequent spontaneous tumor formation in *Pin1*$^{-/-}$ C57BL6 mice, which does not occur in age-matched wild type (WT) mice[1]. Taking into account the tumor promoting and suppressing functions of Pin1 in cancer, we hypothesize that Pin1 can serve as a "conditional" tumor suppressor.

In order to better understand the conditions that influence Pin1 to behave as a tumor promoter or suppressor, the goal of the research described herein was to identify mechanisms that contribute to Pin1's ability to mediate tumor suppression. As discussed in this introduction, Pin1 plays a diverse role in all phases of the cell cycle and in the checkpoints that maintain genomic integrity and prevent cancer. Hence, we investigated the effects of Pin1 depletion on cell cycle progression, genomic instability and cancer development. Toward this end, we first identify cyclin E as a novel Pin1 substrate. Similar to c-Myc, Pin1 promotes cyclin E turnover via SCF^{Cdc4}. Using C57BL6 *Pin1*$^{-/-}$ MEF,

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[1] Unpublished observation: Theresa Barberi, Brian Teng, and Anthony Means

we demonstrate the absence of Pin1 can promote defects in cell cycle progression, and accelerate genomic instability and tumorigenesis. Second, we test the hypothesis that Pin1 can serve as a conditional tumor suppressor in human cancer. We determine genomic deletion may be a mechanism for Pin1 reduction in kidney/renal cancer. Using renal cell carcinoma (RCC) cell lines that exhibit Pin1 under-expression, we show the restoration of Pin1 levels attenuates their soft agar and tumor growth. In addition, Pin1-mediated tumor suppression appears to require functional p53. Therefore, the role of Pin1 in cancer is highly dependent on the genetic context and can serve as a conditional tumor suppressor, which has important implications for Pin1's utility as a therapeutic target or prognostic factor for cancer.

2. Materials and Methods[1]

2.1 Reagents

Table 1: DNA Plasmids

Plasmid Name	Source/Generation	Use
pGEX2T	Amersham (Piscataway, NJ)	GST protein expression
pGEX2T-xPin1	Winkler et al 2000	GST protein expression, GST-Pin1 pull-down
pCS2-myc-Cyclin E	Gift from J. Wade Harper (Harvard Medical School, Boston, MA)	Mammalian expression
pCS2-myc-Cyclin E^{T62A}	Gift from J. Wade Harper	Mammalian expression
pCS2-myc-Cyclin $E^{T62/T380A}$	Gift from J. Wade Harper	Mammalian expression
pCS2-myc-Cyclin E^{S372A}	Gift from J. Wade Harper	Mammalian expression
pCS2-myc-Cyclin E^{S384A}	Gift from J. Wade Harper	Mammalian expression
pBABE-hygro-$p53^{DD}$	Gift from William Hahn (Dana Farber Research Institute, Boston, MA)	Retroviral transduction
pBABE-puro	Gift from Christopher Counter (Duke University, Durham, NC)	Retroviral transduction
pBABE-puro-$p53^{DD}$	Transferred insert from pBABE-hygro-$p53^{DD}$ with BamHI/EcoRI	Retroviral transduction
pBABE-puro-H-Ras^{G12V}	Gift from Christopher Counter	Retroviral transduction
pCL-10A1	Gift from Christopher Counter	Retrovirus production
pSUPER.Mamm-X	Oligoengine (Seattle, WA)	Retroviral transduction
pSUPER.shPin1	Sequence targeting 5'-CTGCCACCGTCA-CACAGTA-3' inserted into pSUPER	Retroviral transduction, Pin1 RNA interference
pBABE-neo	Gift from Christopher Counter	Retroviral transduction
pBABE-neo-Pin1	PCR hPin1 from pGEX2TK-hPin1, ligated into EcoRI/SalI sites of pBABE-neo	Retroviral transduction, Pin1 over-expression
pBABE-neo-$Pin1^{W34A}$	Mutation by GenScript (Piscataway, NJ)	Retroviral transduction

[1] Previously described in Yeh et al 2006, Teng et al 2011.

Table 2: DNA Primers

Primer Name	Sequence (5′ to 3′)	Use
pSUPER.shPin1-F	GATCCCCCTGCCACCGTCACACAGTATTCA AGAGATACTGTGTGACGGTGGCAGTTTTTA	Plasmid construction
pSUPER.shPin1-R	AGCTTAAAAACTGCCACCGTCACACAGTAT CTCTTGAATACTGTGTGACGGTGGCAGGGG	Plasmid construction
pBABE-Pin1-EcoRI-F	TTGAATTCATGGCGGACGAGGAGAAG	Plasmid construction
pBABE-Pin1-SalI-R	TTGTCGACTCACTCAGTGCGGAGGAT	Plasmid construction
pSUPER-F	GGAAGCCTTGGCTTTTG	Sequencing
pBABE-F	CTTTATCCAGCCCTCAC	Sequencing
pBABE-R	ACCCTAACTGACACACATTCC	Sequencing
SV40-F	TATTTATGCAGAGGCCGAGG	Sequencing
Oligo-dT	TTTTTTTTTTTTTTTT	Reverse transcription
Cyclin E-F	ACGGACCACAGCAACATGAA	Real-time PCR
Cyclin E-R	AAACACGGCCACATTTGCCT	Real-time PCR
$p53^{DD}$-F	GCTCACTCCAGCTACCTGAA	RT-PCR
$p53^{DD}$-R	TTCTGCCTGCTGGGGAG	RT-PCR
GAPDH-F	GAAGGTGAAGGTCGGAGTC	RT-PCR
GAPDH-R	GAAGATGGTGATGGGATTTC	RT-PCR

Table 3: Antibodies

Antibody	Source	Use
Pin1	Winkler et al 2000	Immunoblot, Immunohistochemistry
Cyclin E (M20)	Santa Cruz (Santa Cruz, CA)	Immunoblot
Cyclin E (HE12)	Upstate (Waltham, MA)	Immunoblot
Cyclin E (pT380)	Santa Cruz	Immunoblot
CDK2 (M2)	Santa Cruz	Immunoblot
Actin (AC-15)	Sigma-Aldrich (St. Louis, MO)	Immunoblot
c-Myc (9E10)	Santa Cruz	Immunoblot, Immunoprecipitation
p27 (C19)	Santa Cruz	Immunoblot
p53 (FL393)	Santa Cruz	Immunoblot
BrdU	Roche (Indianapolis, IN)	Immunofluorescence
Ki67	Lab Vision (Fremont, CA)	Immunohistochemistry
Mouse IgG-FITC	Pierce Antibodies (Rockford, IL)	Immunofluorescence secondary
Mouse IgG-HRP	Jackson Immuno. (West Grove, PA)	ECL secondary
Rabbit IgG-HRP	Jackson Immuno.	ECL secondary
Mouse IgG-IRDye800	Rockland (Gilbertsville, PA)	LI-COR secondary
Rabbit IgG-Alexa680	Invitrogen (Carlsbad, CA)	LI-COR secondary

2.2 Cell Lines and Culture

MEF Preparation and Culture

Pin1$^{-/-}$ mice as previously described (Fujimori et al 1999), were obtained from Hoffmann-La Roche (Nutley, NJ). The *Pin1* gene deletion was transferred into the isogenic C57BL6 background using marker-assisted speed congenic breeding by Jackson Laboratory (Bar Harbor, ME). *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated from isogenic C57BL6 mice as previously described (Robertson 1987). MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (hiFBS; Atlanta Biologicals, Lawrenceville, GA). MEFs were maintained at 37°C in a 5% CO_2 humidified incubator.

Human Cell Culture

Human embryonic kidney 293T cells, renal cell carcinoma A498 and ACHN cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Mediatech, Manassas, VA) containing 10% hiFBS (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin-streptomycin (Mediatech). Cells were maintained at 37°C in a 5% CO_2 humidified environment.

Retrovirus Preparation and Transduction

Retrovirus production and transduction were performed following established methods (O'Hayer and Counter 2006). Briefly, 293T cells were transfected with 3 μg

pCL-10A1 and 3 μg pBABE/pSUPER plasmid using FuGENE-6 (Roche). Retrovirus-containing media was filtered through a 0.45 μm Millex-HA syringe filter unit (Millipore, Billerica, MA), then supplemented with 4 μg/ml hexadimethrine bromide (Sigma-Aldrich). MEFs were infected at passage 3 and then subjected to polyclonal selection using 10 μg/ml hygromycin (Calbiochem, Gibbstown, NJ) or 0.5 μg/ml puromycin (Calbiochem). Retrovirally transduced A498 cells were stably selected using 0.5 mg/ml G418 (Invitrogen), and ACHN cells with 1 mg/ml G418 or 2 μg/ml puromycin (Sigma-Aldrich) for 7-10 days.

2.3 Protein Interactions

Protein Expression

For bacterial protein expression, GST and GST-Pin1 recombinant proteins were prepared by transforming BL21(DE3) competent cells (Stratagene, La Jolla, CA) with pGEX2T plasmids, then plating them onto 100 μg/ml ampicillin (amp; Sigma-Aldrich) LB-agar plates. Single colonies were picked for 50 ml LB-amp starter cultures that were incubated with shaking for 18 hr at 37°C. A 500 ml culture was inoculated with the starter culture and grown until OD600 = 0.6. Protein expression was induced by adding 1 mM IPTG (Sigma-Aldrich) and incubating for 5 hr. Bacteria were resuspended in 1x phosphate-buffered saline (PBS) containing 10 mM EDTA, 1 mM DTT, and 0.1 mg/ml Pefabloc (Roche), then flash frozen in liquid nitrogen. Lysis was performed by sonication and addition of 1% Triton X-100. Protein lysates were clarified by centrifugation; then

GST and GST-Pin1 were bound to glutathione sepharose beads (Amersham) and stored at 4°C.

For mammalian protein expression of myc-tagged-cyclin E and its mutants, 10^6 293T cells were seeded per 100-mm dish, then transfected the next day with 2.5 μg of plasmid DNA using Lipofectamine (Invitrogen). Transfected cells were incubated for 12-18 hr prior to lysis or roscovitine (Calbiochem) treatment.

GST Pull-down

293T cells expressing cyclin E were lysed in binding buffer (1x PBS, 10 mM EDTA, 1 mM DTT, 10mM NaF, 0.1 mg/ml Pefabloc and 1% Triton X-100). Binding was carried out by incubating 10 μg GST proteins with 500 μg clarified protein lysate for 1 hr at 4°C. Beads were washed four times with binding buffer. Bound proteins were denatured in 2x protein sample buffer and analyzed by immunoblotting.

Immunoprecipitation (IP)

293T cells expressing cyclin E were lysed in IP buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 5 mM DTT, 10mM NaF, 0.1 mg/ml Pefabloc and 1% Triton X-100). IP was performed by incubating 2 μg anti-Myc with 500 μg clarified/pre-cleared protein lysate for 14-18 hr at 4°C, then adding protein A/G sepharose beads (Amersham). Beads were washed four times with IP buffer. Bound proteins were denatured in 2x protein sample buffer and analyzed by immunoblotting.

2.4 Immunoblotting

Equal amounts of protein sample were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to Immobilon-P or Immobilon-FL membranes (Millipore).

Detection by ECL

Immobilon-P membranes were blocked in 5% milk dissolved in Tris-buffered saline/0.2% Tween 20 (TBS-T) for 1 hr at room temperature. Primary antibodies were diluted 1:1000 in blocking buffer, except anti-Pin1 and anti-β-actin, which were diluted 1:10,000. After overnight incubation at 4°C with primary antibody, the membranes were washed three times with TBS-T for 5 min each wash. Primary antibodies were detected with the appropriate HRP-conjugated secondary antibody using ECL reagent (Amersham).

Quantitative Detection by Fluorescent Imaging

Quantitative immunoblotting was performed using procedures established by LI-COR Biosciences (Lincoln, NE). Briefly, Immobilon-FL membranes were blocked in 0.1% casein (MP Biomedicals, Solon, OH) and 0.2% fish gelatin (Sigma-Aldrich) dissolved in 0.2x PBS for 1 hr at room temperature. Anti-Pin1 was diluted 1:10,000 and anti-β-actin was diluted 1:50,000 in blocking buffer containing 0.2% Tween 20. After overnight incubation at 4°C with primary antibody, the membranes were washed four

times with TBS-T for 5 min each wash. Primary antibodies were detected with the appropriate infrared dye-conjugated secondary antibody diluted in primary antibody buffer containing 0.02% SDS. Immunoblots were washed, scanned, and quantified using the LI-COR Odyssey Infrared Imaging System.

2.5 Real-Time PCR and RT-PCR

RNA was isolated from MEFs using TRIzol (Invitrogen), or from human cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Equal amounts of isolated RNA were reverse-transcribed using either Superscript III SuperMix (Invitrogen) for murine RNA or Omniscript RT Kit (Qiagen) for human RNA. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Ectopic expression of $p53^{DD}$ was verified by qualitative RT-PCR as previously described (Adam and Counter 2008), except the PCR was performed using JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich). The sequences of all PCR primers used are provided in Table 2.

2.6 Cell Cycle Analysis

Proliferation Assay

Cell proliferation was assessed by seeding 2.5 x 10^5 cells per 60-mm dish and counting the cells every 24 hr using a Beckman Coulter particle counter (Miami, FL).

Bromodeoxyuridine (BrdU) Incorporation Assay

One hundred thousand cells were seeded onto coverslips in 6-well plates and grown until 70-80% confluent. Cells were pulsed with 10 μM BrdU for 30 min then fixed

in cold methanol for 5 min at -20°C. After rehydration in PBS, cells were treated with 2 M HCl/0.5% Triton X-100 for 30 min at room temperature. Coverslips were washed once with 0.1 M sodium tetraborate (pH 8.5) and twice with PBS for 5 min each wash. Cells were blocked in 1% bovine serum albumin (BSA)/PBS for 30 min. To perform staining, anti-BrdU diluted 1:100 in 0.1% BSA/PBS was added to each coverslip and incubated at 4°C overnight. Primary antibody was detected by anti-mouse-FITC. Cells were counterstained with DAPI and analyzed by fluorescence microscopy (Zeiss Axioskop microscope, Oberkochen, Germany).

Cell Cycle Analysis by Flow Cytometry

Cells were grown until 70-80% confluent in 6-well plates, harvested by trypsinization, and then fixed with cold 70% ethanol. At this time, cells were either stored at -20°C until used or kept on ice for 2 hr prior to staining. DNA staining was performed by resuspending cells in PBS containing propidium iodide (5 μg/ml) and RNase A (100 U/ml). Cell cycle profiles were determined by fluorescence-activated cell sorting (Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource, Durham, NC).

2.7 Soft Agar Assay

Cellular transformation was evaluated by soft agar assay as previously described (O'Hayer and Counter 2006). Briefly, 5 x 10^4 MEF cells per 35-mm gridded dish were seeded in 0.3% BD Difco Noble top agar supplemented with culture media. MEFs were

fed with 1 ml of agar-media mixture once every week for 3 weeks. For A498 and ACHN cell lines, 2.5 x 10^4 or 5 x 10^4 cells were seeded in each well of a 6-well plate and fed every 3-4 days with 0.2 ml of culture media for 21 days. Colonies greater than 30 cells were counted under a light microscope. Images were acquired with a Sony DSC-W50 (New York, NY) attached to a Zeiss Axiovert 10 Microscope using a CamAdapter Kit (The Microscope Store, Wirtz, VA).

2.8 Xenograft Tumor Model

For MEF xenograft model, immunodeficient (SCID) mice were injected with a single subcutaneous injection of 10^7 cells per mouse. All procedures and care methods were in compliance with National Institutes of Health and Duke Institutional guidelines on the use of laboratory and experimental animals.

For ACHN xenograft model, 0.5 x 10^6 or 10^6 ACHN cells were subcutaneously injected into each flank of athymic nude mice using protocols approved by the University of North Carolina Institutional Animal Care and Use Committee. Tumor dimensions were measured every 3-4 days with calipers until largest tumors reached 0.8 cm in length, after which the animals were sacrificed. Xenograft tumors were fixed with formalin and embedded in paraffin for tissue sectioning.

2.9 Immunohistochemistry

Immunohistochemical staining was performed as previously published (Chen et al 2010). TUNEL assay was performed using ApopTag Peroxidase *In Situ* Apoptosis

Detection Kit (Millipore). High resolution images were captured using an Infinity 2-3 Camera (Lumenera Corporation, Ottawa, ON, Canada) affixed to an Olympus CX41 Microscope (Center Valley, PA). Average percent DAB-positive staining was quantified from 10 different fields per tumor using ImmunoRatio (Tuominen et al 2010) or blinded manual cell counting.

2.10 Bioinformatics

Copy Number Analysis

SNP6.0 array data corresponding to gene expression data deposited in Gene Expression Omnibus GSE17818 (Dalgliesh et al 2010) were obtained from the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). Raw allele intensities were imported into Partek Genomics Suite software, version 6.5 (St. Louis, MO). Based on the PCA plot, one outlying tumor and corresponding normal were excluded, batch effect was removed, and copy number values were created from allele intensities. Genomic segmentation was performed with segmentation parameters of a minimum of 10 genomic markers, p-value threshold of 0.001, and a signal to noise ratio of 0.3. Identified regions were visualized using the Plot Chromosome function within Partek Genomic Suite.

Gene Expression Analysis

PIN1 gene expression levels were examined from previously described gene expression data (Brannon et al 2010), which was re-downloaded with 44 additional new

tumor samples from the UNC Microarray Database in log2 Lowess-normalized sample/reference format (median), filtered for 70% of probes present above background levels and annotated using the 20101031 Agilent annotation release. The data was imported into Partek Genomics Suite software and batch effect was removed through Partek's batch effect removal algorithms. All human studies were approved by the University of North Carolina Biomedical Institutional Review Board and Office of Human Research Ethics.

Gene Set Analysis (GSA)

Pathway analysis was performed on previously described gene expression data (Dalgliesh et al 2010) downloaded from Gene Expression Omnibus (GSE17818). RMA normalization was used to create summarized expression values, three outlying tumors were removed, and the median of redundant probes was computed. To identify pathways whose expression was associated with Pin1 gene expression levels, SAM-GSA (http://www-stat.stanford.edu/~tibs/SAM/) was subsequently performed using the MSigDB curated gene sets, version 3.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Heat maps were created using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and visualized using Java TreeView (http://jtreeview.sourceforge.net/).

2.11 Statistical Analysis

Bar graphs show mean ± standard error of the indicated number of experiments or samples. Statistical analyses were performed using Microsoft Excel and StatView. Cutoff for statistical significance was arbitrarily set at $P<0.05$. * denotes significant and ** denotes highly significant, with specific P values indicated in the figure legends.

3. Pin1 Modulates Cyclin E Stability, Genomic Instability, and Cellular Transformation in C57BL6 MEF[1]

3.1 Preface

Mitogenic stimuli initiate a sequence of events that result in the entry of quiescent cells into S phase (McGowan 2003). Critical for this important transition is the ordered synthesis and degradation of transcription factors such as c-Jun and c-Myc, and cyclins (Figure 2) such as cyclin D and cyclin E (McGowan 2003). 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) group of ubiquitin enzymes in which the F-box component, which serves as the ubiquitin E3 ligase, is Cdc4 (SEL-10, Fbw7, Ago) (Nakayama and Nakayama 2005). SCF^{Cdc4} binds to a component of each protein that has been termed the "phosphodegron" (Orlicky et al 2003) to promote the ubiquitylation and degradation of these proteins via the 26 S proteasome. c-Jun and c-Myc have an additional binding

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[1] This research was originally published in the Journal of Biological Chemistry. Yeh et al. The Loss of PIN1 Deregulates Cyclin E and Sensitizes Mouse Embryo Fibroblasts to Genomic Instability. *J Biol Chem*. 2006; 281:241-251. © the American Society for Biochemistry and Molecular Biology.

protein in common, namely Pin1, which binds and isomerizes prolyl bonds in the context of phospho-Ser/Thr-Pro motifs (Wulf et al 2001, Yeh et al 2004).

Previously, we described the mechanism by which Pin1 promotes c-Myc degradation (Yeh et al 2004). The Cdc4 phosphodegron of c-Myc is present in a domain termed Myc box 1, containing the sequence Leu-Pro-pThr-Pro-Pro-Leu-pSer-Pro (where pThr represents phospho-threonine and pSer represents phospho-serine), in which the two phosphorylation events occur sequentially and are catalyzed by ERK (Ser62) and GSK3β (Thr58), respectively (Welcker et al 2004b, Yada et al 2004). 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 (Yeh et al 2004). Ubiquitylated c-Myc is only phosphorylated on Thr58 (Yeh et al 2004), suggesting that either dephosphorylation of Ser62 occurs before Cdc4 can bind to c-Myc or before ubiquitylation can occur. Regardless of the precise mechanism involved, inhibition of PP2A or the absence of Pin1 results in stabilization of c-Myc (Yeh et al 2004). c-Myc can also be stabilized by mutating Thr58 to Ala, and this mutation renders c-Myc oncogenic in a primary human cell transformation assay (Yeh et al 2004). Since residues in the c-Myc phosphodegron, including Thr58 and Pro57, are frequently mutated in lymphomas (Gregory and Hann 2000, Hemann et al 2005, Pulverer et al 1994, Salghetti et al 1999), deregulation of c-Myc due to mutations of this region of the protein can play a role in human cancer (Salghetti et al 1999).

The phosphodegron of cyclin E, Leu-Leu-pThr-Pro-Pro-Gln-pSer-Gly, is remarkably similar to that of c-Myc (Welcker et al 2003). In the case of cyclin E, the phosphorylation events are catalyzed by Cdk2 (Ser384) and GSK3β (Thr380) (Hemann et al 2005), respectively, and x-ray structural studies have shown that phospho-Thr380 plays a crucial role in the binding of cyclin E by Cdc4 (Orlicky et al 2003, Welcker et al 2003). As is the case for c-Myc, deregulation of cyclin E can result in cell cycle defects (Ohtsubo and Roberts 1993, Rajagopalan et al 2004, Resnitzky et al 1994, Spruck et al 1999, Wimmel et al 1994) that predispose cells to oncogenesis (Akli and Keyomarsi 2004, Bedrosian et al 2004, Hubalek et al 2004, Hwang and Clurman 2005, Willmarth et al 2004, Yamada et al 2004). Such defects include aberrant DNA replication and the loss of genomic integrity. Indeed, cyclin E deregulation is associated with many human cancers including breast (Akli and Keyomarsi 2004, Akli et al 2004, Ekholm-Reed et al 2004, Strohmaier et al 2001, Willmarth et al 2004), ovarian (Bedrosian et al 2004), and bladder cancers (Kawamura et al 2004), 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 c-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 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.

3.2 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 (Clurman and Groudine 1997, Clurman et al 1996, Koepp et al 2001, Strohmaier et al 2001, Welcker et al 2003, Ye et al 2004). At least four of these sites are implicated in its degradation (Clurman and Groudine 1997, Clurman et al 1996, Koepp et al 2001, Strohmaier et al 2001, Welcker et al 2003, Ye et al 2004) (Figure 3A). 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 (Strohmaier et al 2001, Welcker et al 2003, Ye et al 2004). Next, we transfected 293 cells with 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 Figure 3B, GST-Pin1 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 Figure 3A. Surprisingly, mutation of Thr62, Thr380, or the combination of Thr62 and Thr380 does not affect Pin1 binding (Figure 3B, lanes 3–8). On the other hand, mutation of Ser384 nearly abolishes Pin1 binding (Figure 3B, lanes 9 and 10). Since Ser384 is phosphorylated by the Cdk2 component of the cyclin E-Cdk2 heterodimer (Welcker et al 2003), 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 Figure 3C–E. First, when Pin1 is associated with WT endogenous or Myc-tagged cyclin E, immunoblot analysis reveals the presence of both cyclin E and Cdk2 (Figure 3C-D, lanes 1 and 2, and Figure 3E, 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 (Figure 3D, lanes 3 and 4). Although roscovitine is an inhibitor of Cdk2 activity, it may also affect Cdk2 and/or cyclin E protein levels (Alessi et al 1998). Thus, we evaluated Cdk2 and cyclin E levels by immunoblotting extracts derived from cells that had been treated with either vehicle (DMSO) or 30 μm roscovitine. As shown in Figure 3D (right panel), treatment with 30 μm 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 c-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 (Welcker et al 2003) and is a potential phospho-Ser-Pro binding site for Pin1. As shown in the left panel of Figure 3E, 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 immunoblot) and of each cyclin E protein (based on Coomassie staining) was immunoprecipitated (Figure 3E, left panel). In addition, each of the whole cell extracts contained similar amounts of Cdk2, Pin1, and the appropriate cyclin E protein (Figure 3E, right panel). 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.

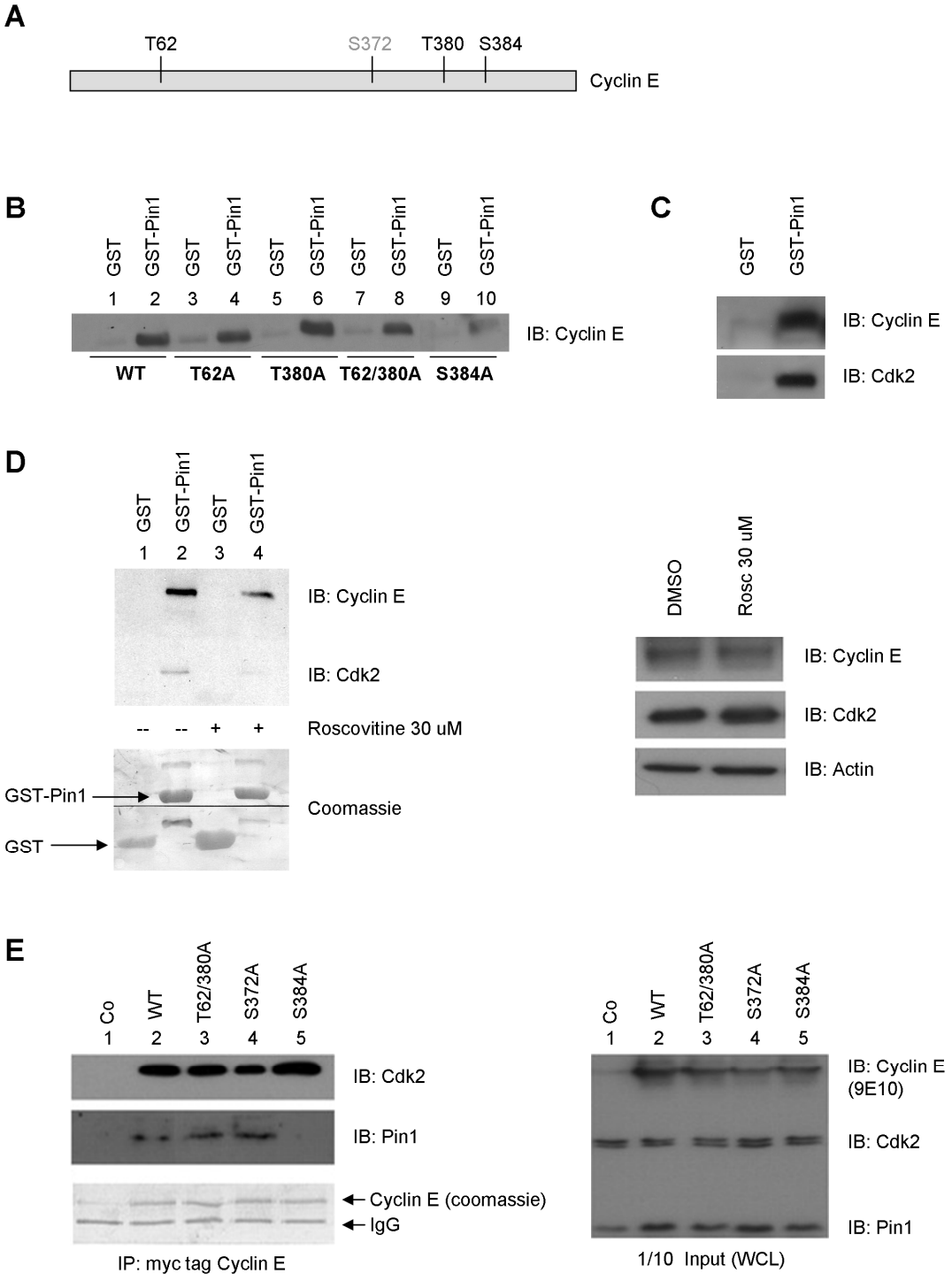


Figure 3: Pin1 binds to cyclin E.

(A) Diagram of the phosphorylation sites on cyclin E protein that are involved in cyclin E turnover (not to scale).
(B) Pin1 binds to cyclin E, but not when Ser384 is mutated. 293T cells were transfected with Myc-tagged cyclin E^{WT}, cyclin E^{T62A}, cyclin E^{T380A}, cyclin $E^{T62/T380A}$, 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 immunoblot using anti-9E10 antibody for the Myc tag.
(C) Pin1 interacts with endogenous cyclin E-Cdk2 complex. Nontransfected 293T cells were lysed and subjected to GST pull-down using GST alone or GST-Pin1.
(D) Inhibition of Cdk2 impairs cyclin E-Cdk2 complex interaction with Pin1. 293T cells were transfected with Myc-tagged cyclin E and treated with either DMSO or 30μm roscovitine. Left panel, GST pull-down was performed as in (C). Right panel, whole cell lysates were immunoblotted using the anti-HE12 antibody to detect cyclin E. No significant changes in the amounts of cyclin E or Cdk2 proteins were observed between the DMSO and 30μm roscovitine treatments.
(E) Cdk2 remains associated with cyclin E^{S384A} though Pin1 binding is impaired. 293T cells were mock-transfected (lane 1) or transfected with Myc-tagged cyclin E^{WT}, cyclin $E^{T62A/T380A}$, cyclin E^{S372A}, or cyclin E^{S384A} (lanes 2–5) prior to IP using anti-9E10 antibody. Bound (left panel) or input (right panel) proteins were analyzed by immunoblot using indicated antibodies or coomassie stain. IB, immunoblot; IP, immunoprecipitation.

Since the phosphorylation of Thr380 and Ser384 on cyclin E are important for regulating the Cdc4-mediated turnover of cyclin E protein (Strohmaier et al 2001, Welcker et al 2003, Ye et al 2004), 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 immunoblotting and found that steady-state levels of cyclin E were up-regulated 2-fold in the absence of Pin1 (Figure 4A). 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 might be attributed to increased cyclin E mRNA levels. We performed real time PCR on total RNA isolated from WT 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 (Figure 4B). 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 Figure 4A, neither the level of Cdk2 nor the cyclin-dependent kinase inhibitor p27^{Kip1} was changed in *Pin1*$^{-/-}$ cells. Finally, we tested whether cyclin E is appropriately degraded in the absence of Pin1. Either WT or *Pin1*$^{-/-}$ cells were treated with cycloheximide for 6 hr. Figure 4C 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 hr after the addition of cycloheximide (Figure 4C). 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.

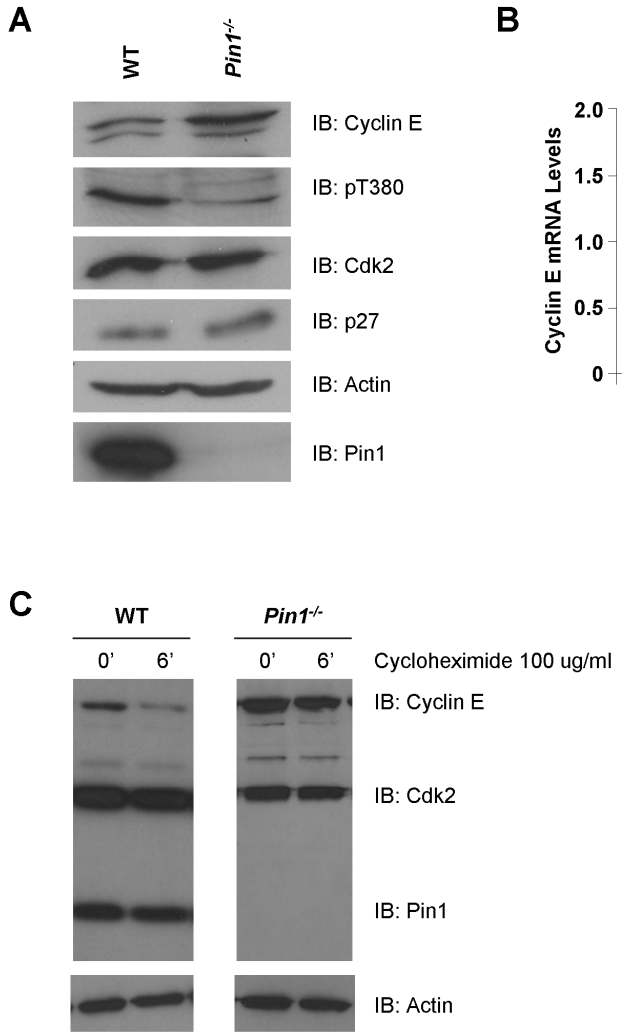


Figure 4: Pin1 influences the stability of cyclin E protein.

(A) Steady-state levels of cyclin E protein are elevated in *Pin1*$^{-/-}$ MEFs. WT and *Pin1*$^{-/-}$ MEF cell lysates were analyzed by immunoblot with the indicated antibodies. β-actin served as a loading control. Results are representative of five individual experiments.
(B) Levels of cyclin E mRNA in the presence and absence of Pin1 was quantified by real time PCR. Results were obtained from two independent experiment performed in duplicate.
(C) Loss of Pin1 results in increased cyclin E protein stability. WT and *Pin1*$^{-/-}$ MEFs were treated with 100 μg/ml cycloheximide and harvested at 0 and 6 hr, then analyzed by immunoblot.

The absence of Pin1 in MEFs results in cell cycle defects

Pin1$^{-/-}$ MEFs contain increased amounts of cyclin E (this study) and c-Myc (Yeh et al 2004), and deregulation of either cyclin E or c-Myc results in defects in the cell cycle (McGowan 2003). Cyclin E protein over-expression 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 (Ohtsubo et al 1995, Rajagopalan et al 2004, Resnitzky et al 1994, Wimmel et al 1994). 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 Figure 5A, 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 (Fujimori et al 1999, You et al 2002). 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 BrdU and quantified its presence by immunocytochemical analysis of WT and *Pin1*$^{-/-}$ MEFs. As shown in Figure 5B, the absence of Pin1 in MEFs results in a statistically significant decrease in the percentage of cells that incorporate BrdU. This outcome could be explained by either a decrease in the number of cells entering S phase during the time of the BrdU 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. Figure 5C (left and middle bars) shows 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 Figure 5C (right bars). Thus, progression through the G1/S phases of the cell cycle is impaired in *Pin1*$^{-/-}$ cells. The results in Figure 5B and C, are entirely consistent with previous research showing cyclin E over-expression in human cells impairs S phase progression (Ekholm-Reed et al 2004), which supports the concept that the deregulation of cyclin E in *Pin1*$^{-/-}$ MEFs may at least in part contribute to the compromised ability of these cells to synthesize DNA and progress through S phase.

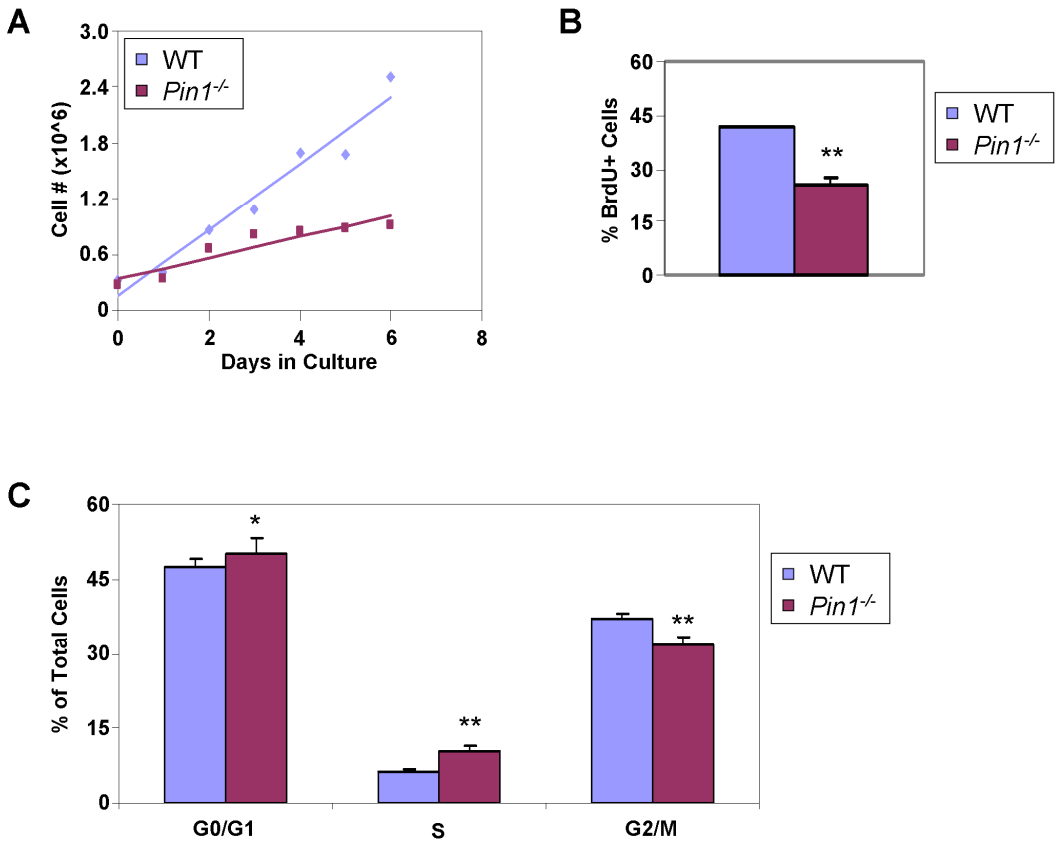


Figure 5: The absence of Pin1 in MEFs results in cell cycle defects.

(A) *Pin1*$^{-/-}$ MEFs have longer doubling times compared to WT. Equal numbers of WT and *Pin1*$^{-/-}$ MEFs were seeded in 60-mm dishes. Cells were trypsinized and counted every 24 hr for 6 days. The graph is representative of five similar independent experiments.
(B) *Pin1*$^{-/-}$ MEFs exhibit defects in BrdU incorporation. Cells were pulsed with BrdU, fixed, and analyzed by immunocytochemistry. Results were obtained from three independent experiments, counting n ≥ 300 per cell type per experiment. **P<0.005 vs. WT, t-test.
(C) *Pin1*$^{-/-}$ MEFs progress more slowly through G1/S phases. Asynchronous populations of WT and *Pin1*$^{-/-}$ MEFs were fixed and stained with propidium iodide to analyze DNA content by flow cytometry. The graph represents the total percentage of cells in G1, S, and G2/M phases. Results were obtained from three independent experiments performed in triplicate. *P<0.05 or **P<0.0001 vs. WT, t-test.

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 G1-S progression, cyclin E and c-Myc protein deregulation is involved in the generation of chromosomal instability (Akli et al 2004, Felsher and Bishop 1999, Fest et al 2002, Hubalek et al 2004, Kawamura et al 2004, Li and Dang 1999, Mai et al 1996a, Mai et al 1996b, Mai and Mushinski 2003, Mushinski et al 1999, Rajagopalan et al 2004, Sheen and Dickson 2002, Willmarth et al 2004, Yin et al 1999) and correlates with tumor formation (Akli and Keyomarsi 2004, Akli et al 2004, Allen et al 1997, Bedrosian et al 2004, Brychtová et al 2004, Chen et al 1995, Chrzan et al 2001, Chung et al 2000, D'Cruz et al 2001, De Miglio et al 1999, Fan et al 2000, Felsher and Bishop 1999, Greco et al 2001, Hubalek et al 2004, Ioachim et al 2004, Latil et al 2000, McCormack et al 1998, Ozdek et al 2004, Rodrigo et al 1996, Willmarth et al 2004, Yamada et al 2004). Because c-Myc and cyclin E levels are increased in *Pin1*$^{-/-}$ MEFs, we evaluated these cells for markers of genomic instability. We first quantified the number of micronucleate cells in early passage (passage 3) primary MEFs of the WT and *Pin1*$^{-/-}$ genotypes. As shown in the left pair of bars in Figure 6B, micronuclei occur in about 5% of the WT MEFs, and though the Pin1-null cells show only about a 2% increase in this percentage (to about 7%), the difference is highly statistically significant. 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 WT and *Pin1*$^{-/-}$ MEFs 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 flow cytometry (Figure 6C, top panels).

Inactivation of p53 will immortalize mouse cells and lead to progressive genomic instability (Kawamura et al 2004, Tainsky et al 1995, Yamamoto et al 1997). To determine whether this p53 inactivation-dependent process would also be sensitized by the absence of Pin1, we infected the passage 3 WT and WT and *Pin1*$^{-/-}$ MEFs with a retrovirus encoding a dominant-negative p53 that expresses a truncated version of the p53 protein termed p53^{DD} (Figure 6A). p53^{DD} is the C-terminal portion of p53 containing amino acids 302–390 and functions to prevent transcription by inhibiting p53 tetramer formation (Bowman et al 1996). 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 (Atchison et al 2003, Joseph et al 2004, Winkler et al 2000) and we found an increase in micronuclei formation even in passage 3 primary MEFs, we wanted to evaluate the effect of Pin1 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. Figure 6A shows that $p53^{DD}$ is expressed to a similar extent in WT 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 WT and *Pin1*$^{-/-}$ cells, though the absence of Pin1 increased this from about 17.5 to 28% of the cells (Figure 6B, right pair of bars). This increase in the percentage of micronucleate cells is mirrored by a change in the DNA profiles of WT and *Pin1*$^{-/-}$ cells expressing $p53^{DD}$ (Figure 6C, bottom panels). 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 >4N DNA content in WT and *Pin1*$^{-/-}$ cells, respectively; Figure 6C, bottom panels). 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.

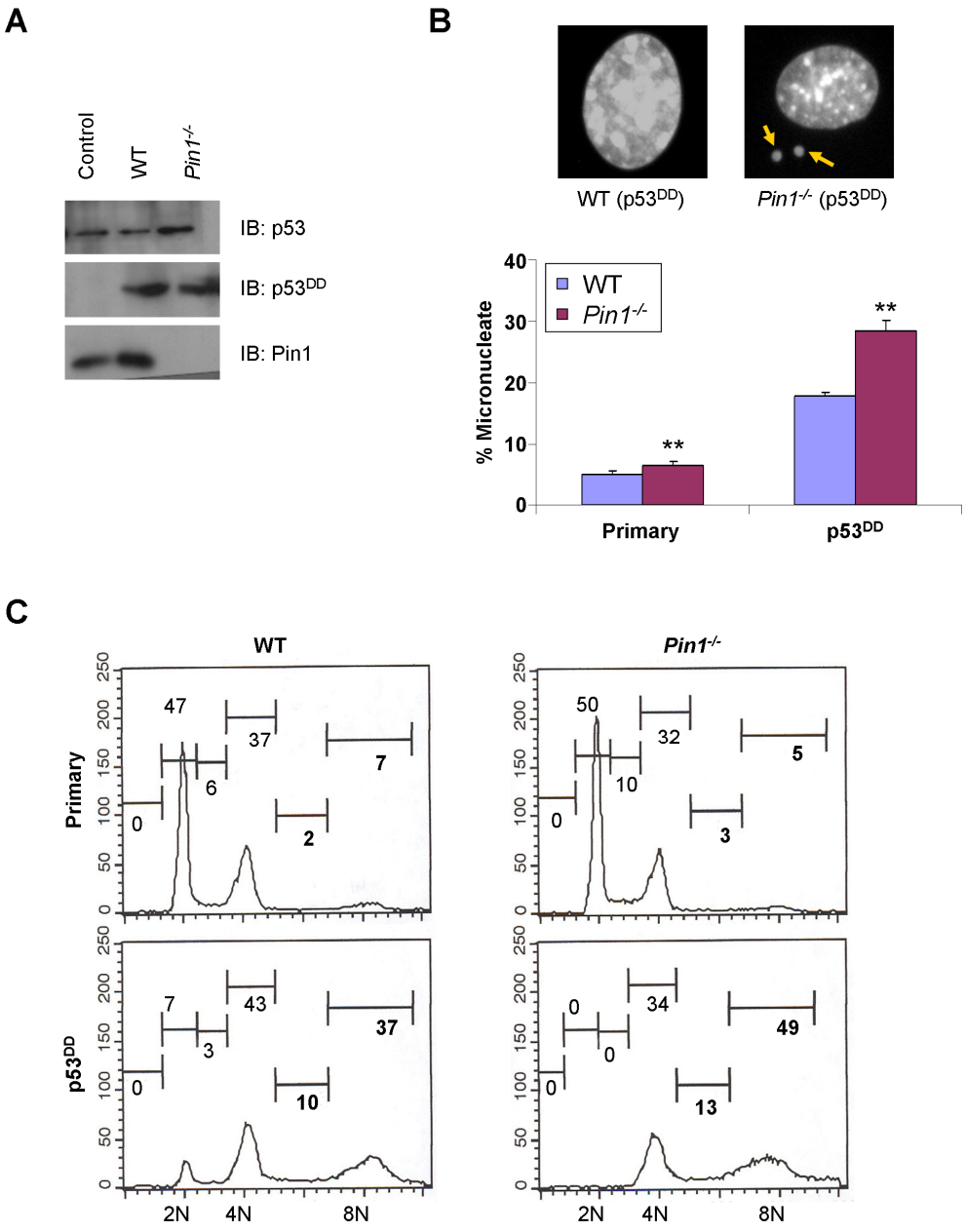


Figure 6: Immortalization of *Pin1*$^{-/-}$ MEFs with a dominant negative p53 results in a more rapid progression toward genomic instability.

(A) Creation of p53^{DD} immortalized MEF cell lines. Primary MEFs were transduced with the dominant negative form of p53, p53^{DD}. Expression of p53^{DD} was confirmed by immunoblot using anti-p53 antibody. The truncated form of p53 protein runs at a lower M_r (middle) than native p53 protein (top).

(B) *Pin1*$^{-/-}$ MEFs exhibit accelerated formation of micronuclei. Cells were fixed on coverslips and stained with DAPI. Cells were visualized by fluorescence microscopy (arrows indicate micronuclei). Results were obtained from three independent experiments (≥300 cells counted per cell type per experiment). **P<0.0001 vs. WT, t-test. (C) Immortalization of *Pin1*$^{-/-}$ MEFs with a p53^{DD} results in a more rapid progression toward aneuploidy. Primary and p53^{DD}-transduced MEFs were fixed, stained with propidium iodide, and analyzed by flow cytometry to determine DNA content. Immortal *Pin1*$^{-/-}$ cells contained the highest percentage of cells with >4N DNA content. Data is representative of six independent FACS analyses.

The absence of Pin1 sensitizes cells to more aggressive transformation and tumorigenesis in vivo

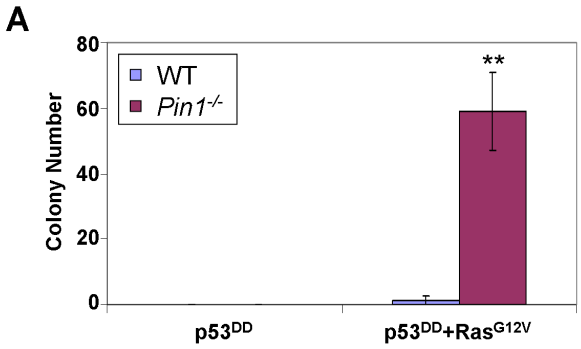
Studies from the cyclin E knock-out mouse indicate that the loss of cyclin E protects mouse cells from Ras-mediated transformation (Geng et al 2003). Because cyclin E is up-regulated in *Pin1*$^{-/-}$ cells and these cells show increased genomic instability at early passage after immortalization, we 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 (Yeh et al 2004). Since c-Myc and H-Ras^{G12V} collaborate to transform rodent cells (Mushinski et al 1999), it seemed plausible that the addition of oncogenic Ras alone to *Pin1*$^{-/-}$ primary MEFs might induce spontaneous transformation. However, expression of H-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 c-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 (Finlay et al 1989, Kamijo et al 1997). It was recently demonstrated that $p53^{DD}$ collaborates with c-Myc and oncogenic Ras to efficiently transform MEFs (Boehm et al 2005). These authors demonstrated that whereas $p53^{DD}$ and Ras alone can result in transformation of wild type MEFs, the transformation of these cells can be accelerated by the addition of another oncoprotein, such as c-Myc. Thus, MEFs expressing $p53^{DD}$ and Ras formed significantly fewer colonies in soft agar than MEFs containing $p53^{DD}$, Ras, and c-Myc (Boehm et al 2005). These results suggest that the serial introduction of oncogenic components ($p53^{DD}$, Ras, c-Myc) can result in an additive effect on transformation. In Figure 7A, we demonstrate that the expression of oncogenic Ras results in the ability of $p53^{DD}$-transduced *Pin1*$^{-/-}$ MEFs to form colonies in soft agar than WT MEFs expressing the same oncogenes. The fact that these latter cells would form colonies to a markedly lower extent is consistent with previous research (Boehm et al 2005). These results show that deletion of Pin1 can sensitize cells to a more aggressive Ras-induced transformation relative to WT 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 H-Ras^{G12V}, WT MEFs expressing $p53^{DD}$ alone, and WT MEFs expressing $p53^{DD}$ and H-Ras^{G12V}. 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 WT MEFs expressing $p53^{DD}$ and H-Ras^{G12V} and *Pin1*$^{-/-}$ MEFs expressing $p53^{DD}$ and H-Ras^{G12V} supported tumor formation in immunocompromised mice (Figure 7B), the tumors that developed from the *Pin1*$^{-/-}$ MEFs expressing $p53^{DD}$ and H-Ras^{G12V} were significantly larger (Figure 7C) than their WT counterparts just 1 week post-injection. In fact, two of the five SCID mice injected with *Pin1*$^{-/-}$ MEFs expressing $p53^{DD}$ alone formed tumors (Figure 7B). 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 c-Myc overexpression. Thus, oncogenic 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 c-Myc, has a profound effect on proliferation, genomic instability, and tumorigenesis of MEFs.



B

Cell Type	# Tumors Formed
WT+p53^{DD}	0/5
Pin1$^{-/-}$+ p53^{DD}	2/5
WT+p53^{DD}+Ras^{G12V}	5/5
Pin1$^{-/-}$+p53^{DD}+Ras^{G12V}	5/5

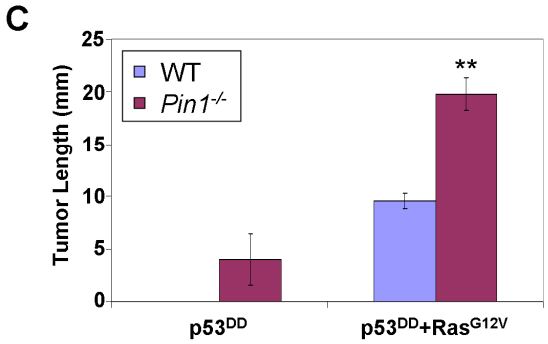


Figure 7: Pin1 deletion sensitizes immortalized MEFs to Ras-induced transformation and tumorigenesis.

(A) Immortalized *Pin1*$^{-/-}$ MEFs are more readily transformed by H-Ras^{G12V}. WT or *Pin1*$^{-/-}$ MEFs were transduced with the indicated oncogenes and assayed for colony formation in soft agar. Results were obtained from two independent experiments performed in triplicate. **P<0.0005 vs. WT, t-test.
(B) Summary of *in vivo* tumor formation. SCID mice were injected with WT and *Pin1*$^{-/-}$ MEFs expressing indicated oncogenes (n=5).
(C) SCID mice injected with *Pin1*$^{-/-}$ MEF expressing p53^{DD} and H-Ras^{G12V} formed more aggressive tumors than WT counterpart. Graph represents average final tumor length (n=5, **P<0.005 vs. WT, t-test).

3.3 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 phospho-Ser384 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 (Welcker et al 2003) and this phosphorylation is required for SCF^{Cdc4}-dependent degradation of cyclin E (Clurman et al 1996, Welcker et al 2003, Ye et al 2004), 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 c-Myc is a phosphorylation-dependent Pin1-binding protein, the loss of Pin1 increased the steady state level of c-Myc in MEFs, and the deregulation of c-Myc protein was due to a decreased rate of its ubiquitylation and degradation (Yeh et al 2004). Since both cyclin E and c-Myc are degraded by SCF^{Cdc4} (Koepp et al 2001, Welcker et al 2004a,

Welcker et al 2004b, Yada et al 2004, Ye et al 2004) 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 c-Myc may be different. In the case of c-Myc, the binding of Pin1 required phosphorylation of Thr58, which has been proposed to constitute the primary Cdc4 recognition site (Welcker et al 2004a, Welcker et al 2004b, Yada et al 2004). Pin1 binding resulted in a conformational change in the phosphodegron that presented phospho-Ser62 as a substrate for PP2A (Yeh et al 2004). Since ubiquitylated c-Myc contained only phospho-Thr58, we concluded that Pin1/PP2A-mediated dephosphorylation of Ser62 was important for ubiquitylation and/or degradation (Yeh et al 2004). We show in the current study that Pin1 binding does not require phosphorylation of Thr380 in cyclin E, which is equivalent to Thr58 in c-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 (Orlicky et al 2003). 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 c-Myc. Regardless of these differences, the outcome of Pin1 binding to a phosphorylated form of cyclin E and c-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-S phase transition, and many of its proposed targets are deregulated in human cancer (Ayala et al 2003, Bao et al 2004, Crenshaw et al 1998, Dougherty et al 2005, Liou et al 2002, Lu 2003, Lu 2004, Miyashita et al 2003, Nakashima et al 2004, Oberst et al 2005, Ryo et al 2001, Ryo et al 2002, Wulf et al 2003, Wulf et al 2004, Wulf et al 2001, Wulf et al 2002, Yeh et al 2004). Because cyclin E and c-Myc levels are increased in MEFs null for Pin1 (Yeh et al 2004) and deregulation of cyclin E and c-Myc have been linked to processes involved in tumorigenesis (Akli et al 2004, Felsher and Bishop 1999, Fest et al 2002, Hubalek et al 2004, Kawamura et al 2004, Li and Dang 1999, Mai et al 1996a, Mai et al 1999, Mai and Mushinski 2003, Rajagopalan et al 2004, Willmarth et al 2004), such as hyper-proliferation and genomic instability, we evaluated whether Pin1-null MEFs would be more susceptible to Ras-induced 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, the loss of Pin1 sensitizes MEFs to more extensive and aggressive transformation and tumorigenesis.

Contrary to our results, loss of Pin1 has been suggested to decrease susceptibility to cancer (Lu 2004). It would follow that mice null for Pin1 might be "protected" from oncogene-mediated tumorigenesis. Wulf et al tested this hypothesis by breeding FVB mice expressing Neu or H-Ras (under the MMTV promoter) with Pin1-null mice maintained in a SvJae/129/C57BL6 mixed genetic background (Wulf et al 2004). MMTV-Neu and MMTV-Ras transgenic mice have previously been shown to form spontaneous breast tumors, resulting in decreased survival (Hundley et al 1997, Lucchini et al 1992). Wulf et al 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, cells from the Pin1-null mice maintained in the SvJae/129/C57BL6 background demonstrated decreased protein levels of cyclin D1 and β-catenin (Liou et al 2002, Ryo et al 2001). Therefore, it is difficult to contend that depletion of Pin1 cannot also decrease susceptibility to mammary cancer, at least in a FVB/129/SvJae/C57BL6 mixed genetic background.

How can one reconcile these apparently opposite roles for Pin1 in cancer? The differences in protein expression in the SvJae/129/C57BL6 (e.g., cyclin D1 and β-catenin) versus the isogenic C57BL6 background (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 help explain the conundrum of the role of Pin1 in tumorigenesis. This idea was clearly demonstrated by Reilly et al, who proposed that the susceptibility of mice to astrocytoma development is intimately linked to the genetic strain of mice in which the experiments are conducted (Reilly et al 2004). These authors engineered different strains of mice (namely 129, 129/SvJae, 129/C57BL6, or C57BL6) that were mutant for *Nf1* or *Trp53* (Reilly et al 2004). 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, studies show the protein expression of deleted in colorectal cancer (DCC) is frequently lost in colorectal cancers, whereas forced expression of the DCC ligand, netrin-1, promotes intestinal tumor development (Mazelin et al 2004). 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 may 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. In Chapter 4, we determine Pin1 might also serve as a conditional tumor suppressor in the context of human cancer and evaluate other mechanisms that may contribute to Pin1’s tumor suppressive activity.

4. Pin1 Is a Conditional Tumor Suppressor in Human Kidney Cancer[1]

4.1 Preface

Pin1 induces conformational changes in its target phospho-proteins that can alter their biological function and/or stability (Lu and Zhou 2007). Several proteins important for cell cycle and cancer progression are regulated by Pin1, including β-catenin, c-Jun, c-Myc, cyclin D1, cyclin E, and p53 (Liou et al 2002, Ryo et al 2001, Wulf et al 2001, Yeh et al 2004, Yeh et al 2006, Zacchi et al 2002, Zheng et al 2002). Since these targets are key regulators of G1-S progression, Pin1 has been suggested to function as a molecular timer for the cell cycle (Yeh and Means 2007). Indeed, the loss of Pin1 in multiple cell types, such as *Pin1*$^{-/-}$ primordial germ cells and MEFs, leads to prolongation of G0/G1-S progression (Atchison et al 2003, Fujimori et al 1999, You et al 2002). Such a delay could be explained by Pin1's ability to promote the expression and stabilization of cyclin D1, underscoring the potential importance of Pin1 in cancer.

A number of studies report prevalent Pin1 over-expression in human cancers, including those of breast and prostate (Ayala et al 2003, Bao et al 2004, Wulf et al 2001). In breast cancer, Pin1 over-expression correlates with the deregulation of cyclin D1, which is likely the result of enhanced transcription of this gene by c-Jun and β-catenin as

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[1] This research was accepted for publication in Molecular Oncology. Teng et al. Tumor suppressive activity of prolyl isomerase Pin1 in renal cell carcinoma. *Mol Oncol*. 2011; doi:10.1016/j.molonc.2011.06.002. © Federation of European Biochemical Societies.

well as post-translational stabilization of the protein (Ryo et al 2001, Wulf et al 2001). Pin1 levels also correlate with poor clinical outcome and may have prognostic value in the case of prostate cancer (Ayala et al 2003). Based on these studies, one might conclude that increased levels of Pin1 promote tumor progression. However, and perhaps not surprising given the plethora of Pin1 targets, the role of Pin1 in human cancer is complex and context dependent.

In fact, several reports suggest Pin1 might inhibit tumor progression by destabilizing oncoproteins (e.g., cyclin E, c-Myc) or by activating tumor suppressors (e.g., p53, p73) (Yeh and Means 2007). Previously, we showed that cyclin E is stabilized in *Pin1*$^{-/-}$ C57BL6 MEF, which correlated with defective cell cycle progression and accelerated genomic instability (Yeh et al 2006). Moreover, *Pin1*$^{-/-}$ MEF cells transformed with p53^{DD} and/or H-Ras^{G12V} when grown as xenografts displayed reduced tumor free survival and increased tumor growth compared to wild type MEF, which suggests Pin1 may exhibit a tumor suppressive effect in these cells. Since Pin1 appears to play a tumor promoting or inhibitory role depending on the genetic context, Pin1 may function as a "conditional" tumor suppressor (Yeh and Means 2007). However, Pin1-mediated tumor suppressive effects in a human context have yet to be demonstrated.

Interestingly, Pin1 is prevalently under-expressed in some human cancers, including kidney cancer (Bao et al 2004). Although the relevance of Pin1 under-expression in kidney cancer has not been evaluated, the effect of differential Pin1 levels

on tumor progression likely depends on the specific repertoire of genetic mutations acquired by the cancer cells. In particular, although p53 is commonly mutated in many human cancers, p53 mutation is infrequent in renal cell carcinoma (RCC) (Dalgliesh et al 2010, Gurova et al 2004). Since Pin1 has been shown to positively regulate p53, one hypothesis is that Pin1 can exert a tumor suppressive role in a manner dependent on wild type p53. Herein, we evaluate the role of Pin1 in RCC and find support for this hypothesis.

4.2 Results

PIN1 gene is frequently deleted and under-expressed in human clear cell RCC (ccRCC)

Previous studies indicate that the Pin1 protein is prevalently under-expressed in kidney tumors compared to normal kidney tissue (Bao et al 2004). As shown in Figure 8A, we determined that Pin1 under-expression in kidney cancer may be in part due to deletion of the PIN1 locus from chromosome 19p13.2, and resultant haploinsufficiency, as copy number analysis revealed Pin1 loss in 24 of 86 (28%) ccRCC tumors. In order to evaluate the effective mRNA expression level of the PIN1 gene, we examined 18 renal tumor and normal kidney paired samples for relative PIN1 gene expression. We observed that the PIN1 gene was under-expressed in 4 of 18 (22%) ccRCC tumors relative to paired normal kidney tissue (Figure 8B), which correlates with the frequency of PIN1 deletion observed in ccRCC. Since genetic loss of function is a hallmark of tumor suppressors, we further assessed the relevance of Pin1 under-expression in ccRCC by

gene set analysis (GSA). GSA revealed low Pin1 levels correlate with reduced expression of genes involved in p53 stabilization as well as mitotic cell cycle pathways (Figure 9). In addition, depressed Pin1 levels are associated with increased expression of genes involved in metastasis, stem cell-related, and proliferation pathways (Figure 9). Collectively, these observations are compatible with the hypothesis that Pin1 may have tumor suppressive function in ccRCC.

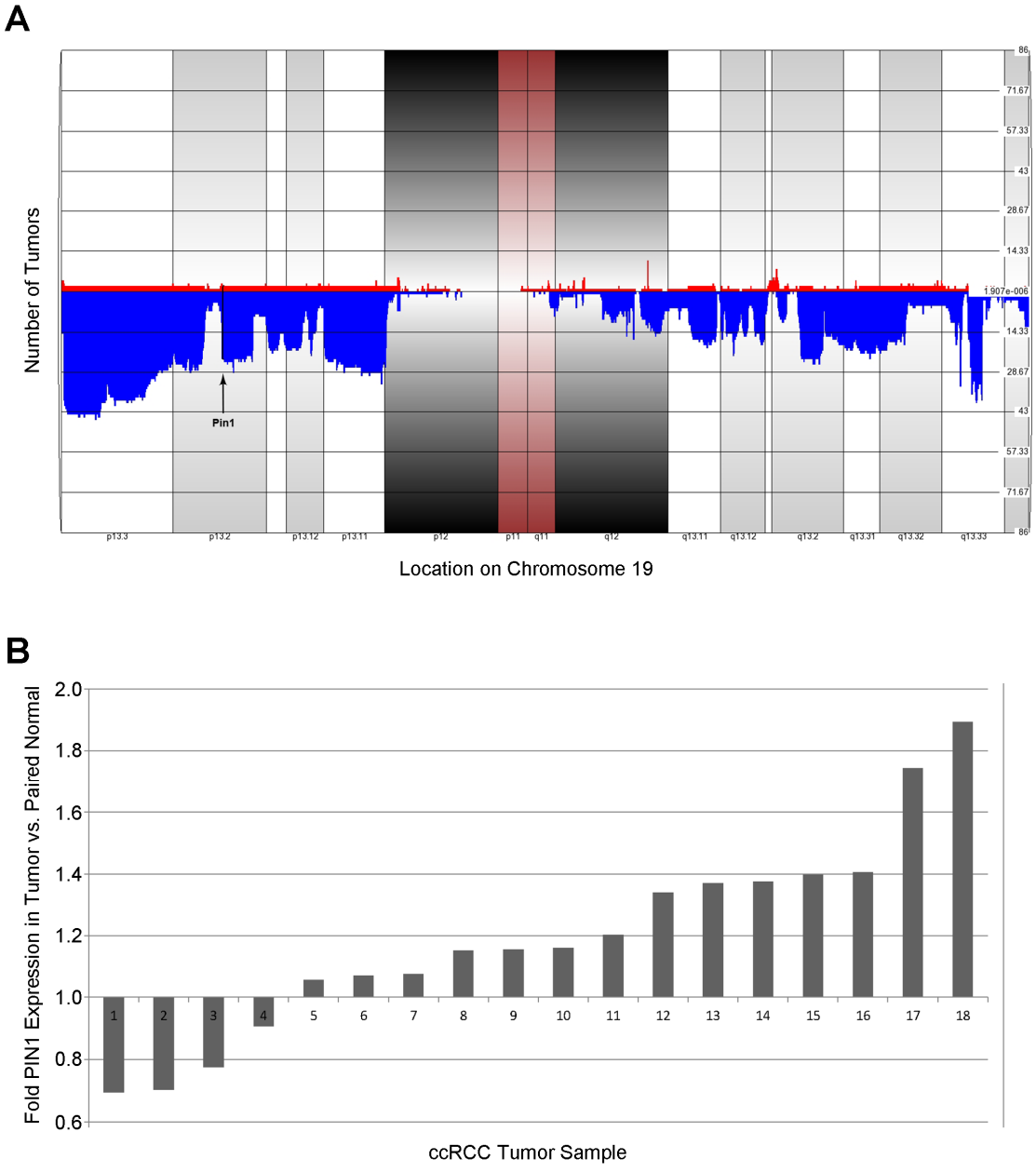

Figure 8: PIN1 gene is frequently deleted and under-expressed in human ccRCC tumors.

(A) Frequency of tumors containing deletion (blue) or amplification (red) of each region of chromosome 19. The PIN1 gene on 19p13.2 (9945999…9960358) is highlighted in black.

(B) Fold PIN1 gene expression in individual ccRCC tumor samples relative to matched normal kidney tissue, as determined by gene expression microarray.

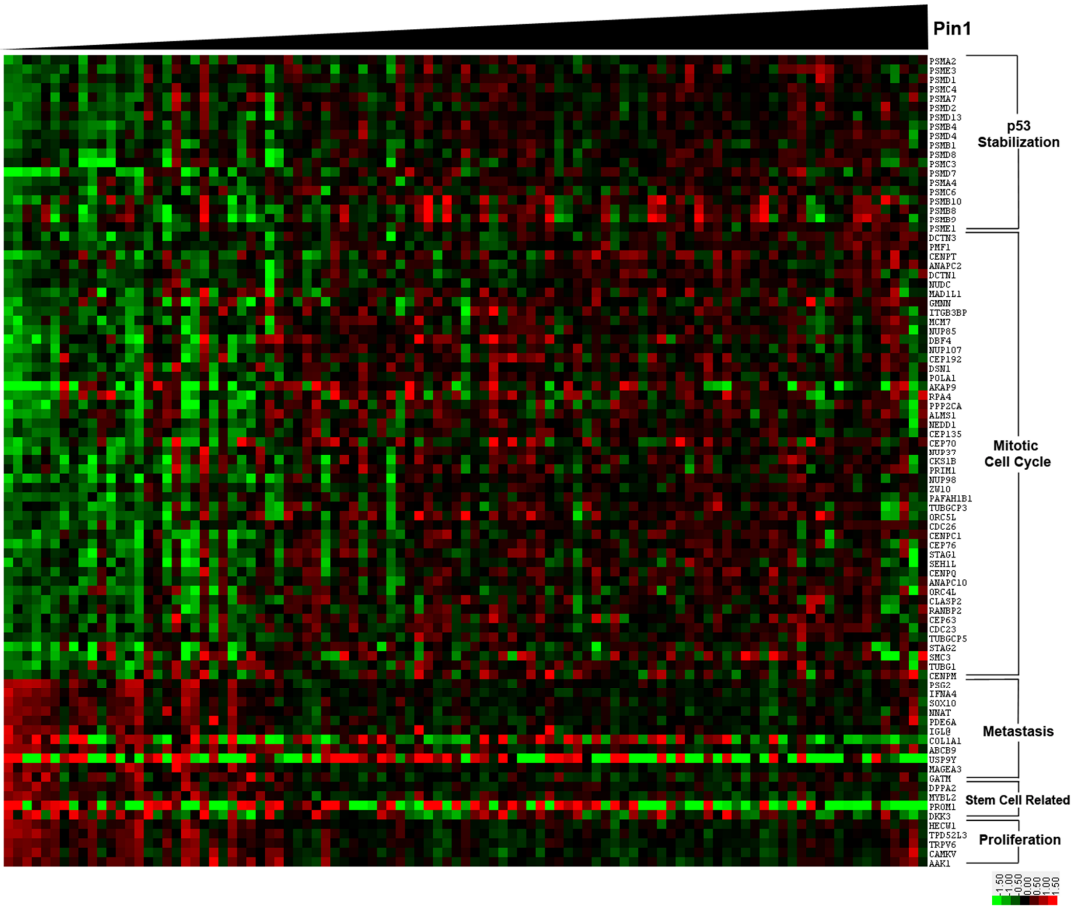

Figure 9: Gene set analysis performed on 99 human ccRCC samples.

Pathway analysis performed on 99 human ccRCC samples (Gene Expression Omnibus GSE17818) identifies gene sets whose expression levels are associated with Pin1 expression. Expression of genes involved in p53 stabilization and mitotic cell cycle positively correlate with Pin1 expression, while genes involved in metastasis, stem cell related, and proliferation pathways negatively correlate with Pin1 levels.

Pin1 attenuates growth of ccRCC cells in soft agar

To test if Pin1 could serve a tumor suppressive role, we selected two RCC cell lines that displayed reduced expression of Pin1 protein, ACHN and A498 (Figure 10A, Figure 11A). The rescue of reduced Pin1 levels to ~1.5-fold normal levels with ectopic expression of Pin1 was sufficient to modestly, but significantly inhibit the growth of both ACHN and A498 cells in soft agar (Figure 10, Figure 11A-C). Since A498 cells formed fewer and much smaller colonies, ACHN cells were chosen for further analysis of Pin1-mediated effects. To evaluate the importance of Pin1's WW-domain in tumor suppressive activity, ACHN cells were transduced with $Pin1^{W34A}$, a WW-domain mutant incapable of binding to and isomerizing Pin1 substrates (Lu et al 1999). Although $Pin1^{W34A}$ was expressed at a level similar to $Pin1^{WT}$ (Figure 11A), $Pin1^{W34A}$ was unable to diminish the growth of ACHN cells in soft agar (Figure 11B and C). This result suggests a functional WW-domain and canonical activity of Pin1 are essential for Pin1-mediated inhibition of anchorage independent growth. Conversely to ectopic Pin1 expression, further Pin1 depletion from ACHN cells by shRNA (Figure 11D) was associated with a 47% increase in soft agar colony formation (Figure 11E and F). These data demonstrate Pin1 can impede the growth of ccRCC cells in soft agar, which is consistent with tumor suppressive activity.

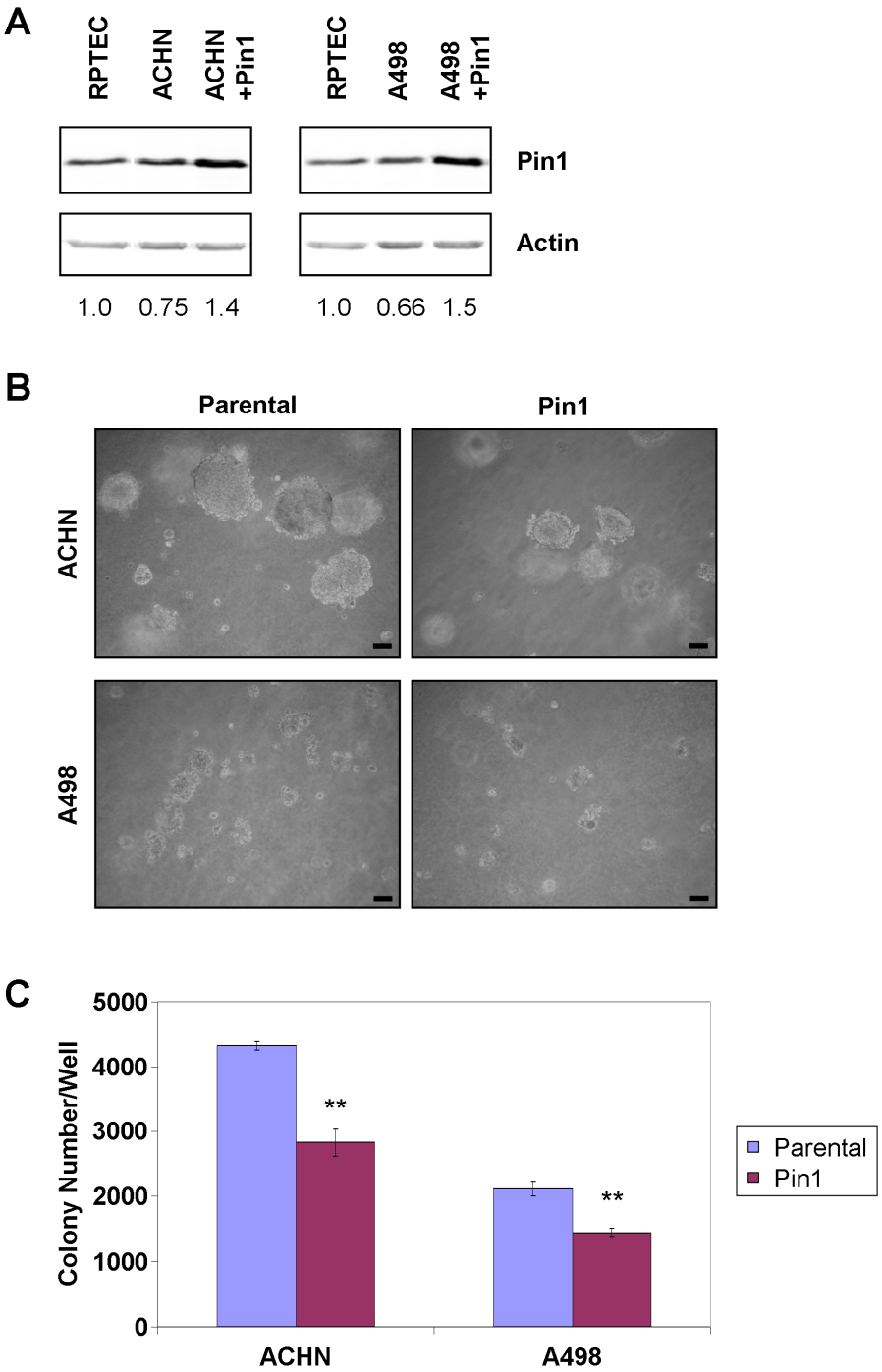

Figure 10: Pin1 attenuates growth of human renal cell carcinoma ACHN and A498 cells in soft agar.

(A) RPTEC, or ACHN and A498 cell lines (±Pin1 transduction) were analyzed by immunoblot. Average normalized Pin1 levels relative to RPTEC are below.
(B, C) ACHN±Pin1 and A498±Pin1 cells were grown in soft agar. After four weeks, colonies were quantified from two independent assays done in triplicate. Scale bars represent 75 μm. **$P<0.005$ vs. control, t-test.

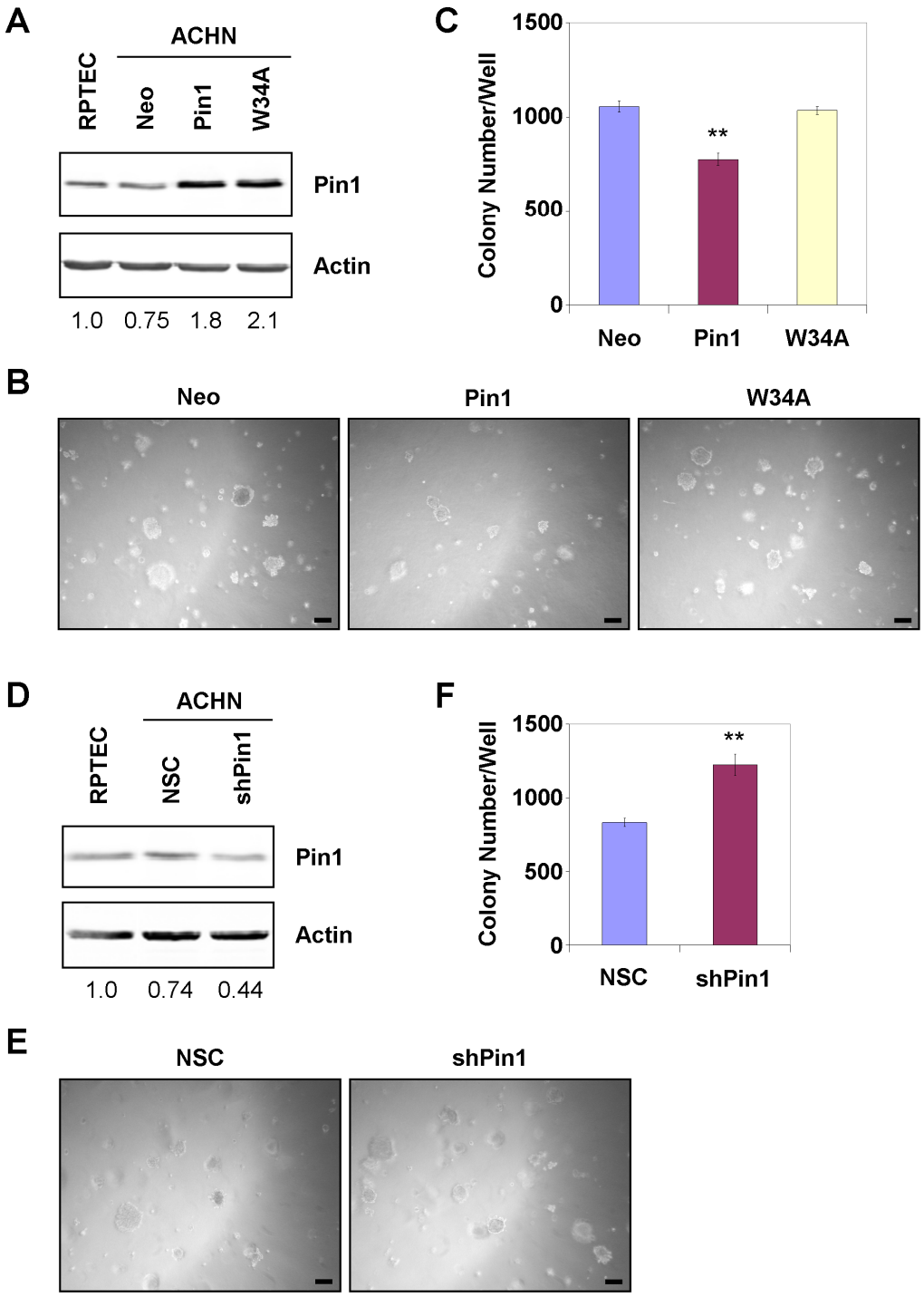

Figure 11: Ectopic Pin1 attenuates ACHN cell growth in soft agar in a WW domain-dependent manner, whereas Pin1 depletion increases colony formation.

(A) RPTEC or ACHN cells transduced with empty vector (Neo), Pin1 or Pin1^{W34A} were analyzed by immunoblot. Average normalized Pin1 levels relative to RPTEC are below.
(B, C) ACHN-Neo, ACHN-Pin1, and ACHN-W34A cells were grown in soft agar. After 21 days, colonies were quantified from three independent assays done in triplicate.
(D) RPTEC or ACHN cells transduced with non-silencing control shRNA (NSC) or Pin1 shRNA (shPin1) were analyzed by immunoblot as in (A).
(E, F) ACHN-NSC and ACHN-shPin1 cells were assayed for growth in soft agar as in (B, C), except results represent two independent experiments performed in triplicate.
Scale bars represent 125 μm. **P<0.001 vs. control, t-test.

Pin1 attenuates ACHN tumor growth in a xenograft model

To further examine the role of Pin1 in tumor growth, ACHN-Neo (vector control) and ACHN-Pin1 cells rescued with ectopic Pin1 expression were assayed for tumorigenicity in a xenograft model. Consistent with our soft agar assay data, Pin1 expression significantly improved tumor free survival and suppressed the tumorigenic growth of ACHN xenografts (Figure 12A and B). ACHN-Pin1 tumors were histologically similar to ACHN-Neo control tumors, which exhibited necrotic cores surrounded by proliferating cells (Figure 12C). In addition, Pin1 immunohistochemical analysis showed ACHN-Pin1 tumors retained elevated levels of Pin1 (Figure 12D), and thus did not grow as a result of escaping Pin1 over-expression.

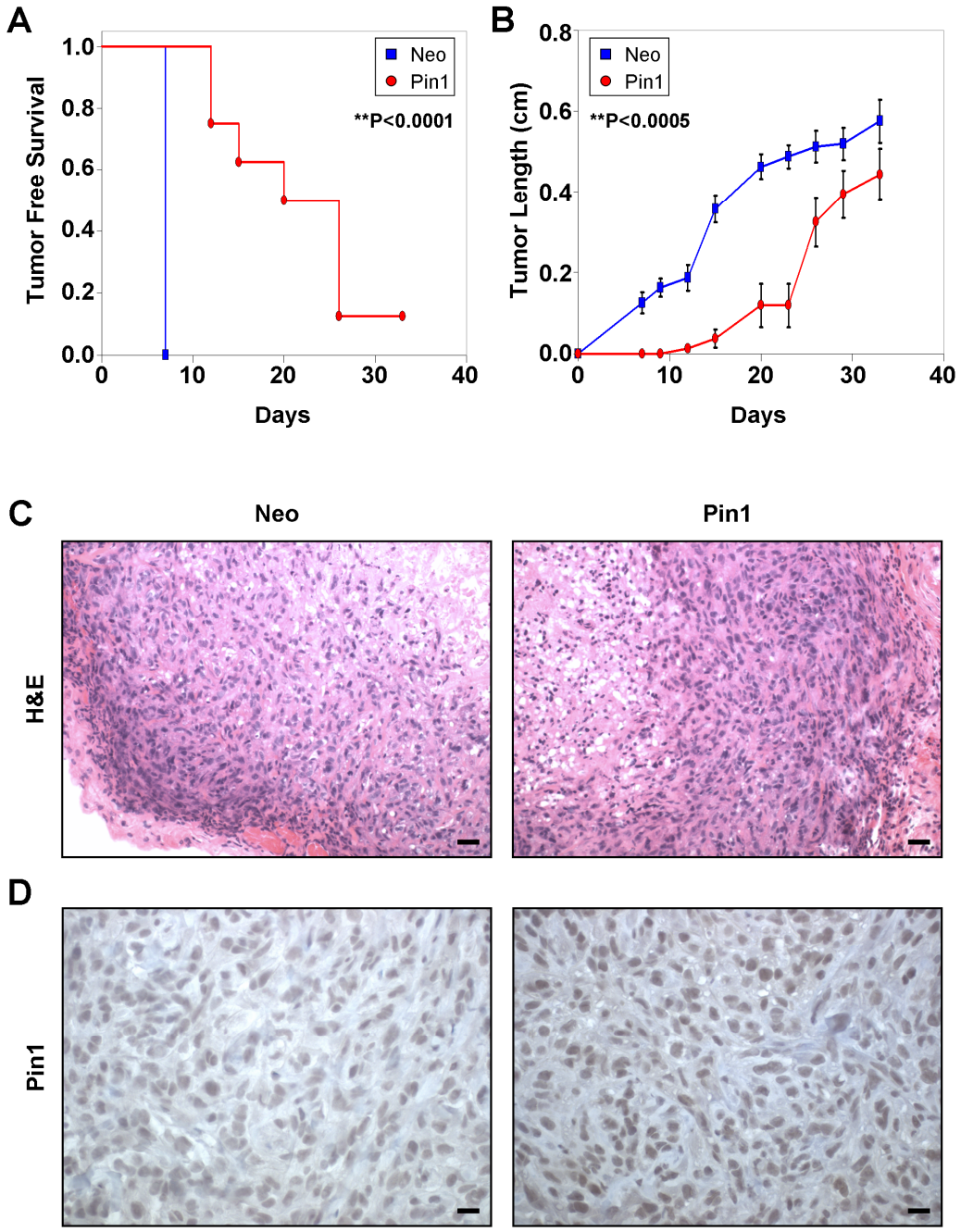

Figure 12: Pin1 attenuates tumor growth of ACHN cells in xenograft model.

ACHN-Neo and ACHN-Pin1 cells were subcutaneously injected into each flank of athymic nude mice.

(A) Tumor free survival of ACHN-Neo (n=8) and ACHN-Pin1 (n=8) xenografts was assessed by Kaplan-Meier survival analysis. P-value determined by log-rank test.
(B) Tumor growth was assessed from measurements of tumor length. P-value determined by repeated measures ANOVA.
(C) Hematoxylin and eosin (H&E) staining. Scale bars represent 50 μm.
(D) Pin1 was detected by DAB immunohistochemistry (IHC) then counterstained with hematoxylin. Scale bars represent 25 μm.

To explore the means by which Pin1 expression impeded xenograft growth, we compared rates of proliferation and apoptosis in the xenografts. Ectopic Pin1 expressing tumors displayed significantly reduced Ki67 staining, indicating that Pin1 contributes to decreased RCC tumor growth by inhibiting cellular proliferation (Figure 13A). Indeed, ACHN-Pin1 tumors showed a 29% decrease in the number of Ki67-positive nuclei (Figure 13C), which is comparable to the impaired growth of ACHN-Pin1 cells in soft agar (Figure 11C), and is consistent with our GSA showing that Pin1 negatively correlates with genes involved in proliferation (Figure 9). Intriguingly, ectopic rescue of Pin1 also led to increased apoptosis in the xenograft tumors as measured by TUNEL staining (Figure 13B and D). Thus ectopic Pin1 expression attenuates ACHN tumor growth through effects on both proliferation and apoptosis.

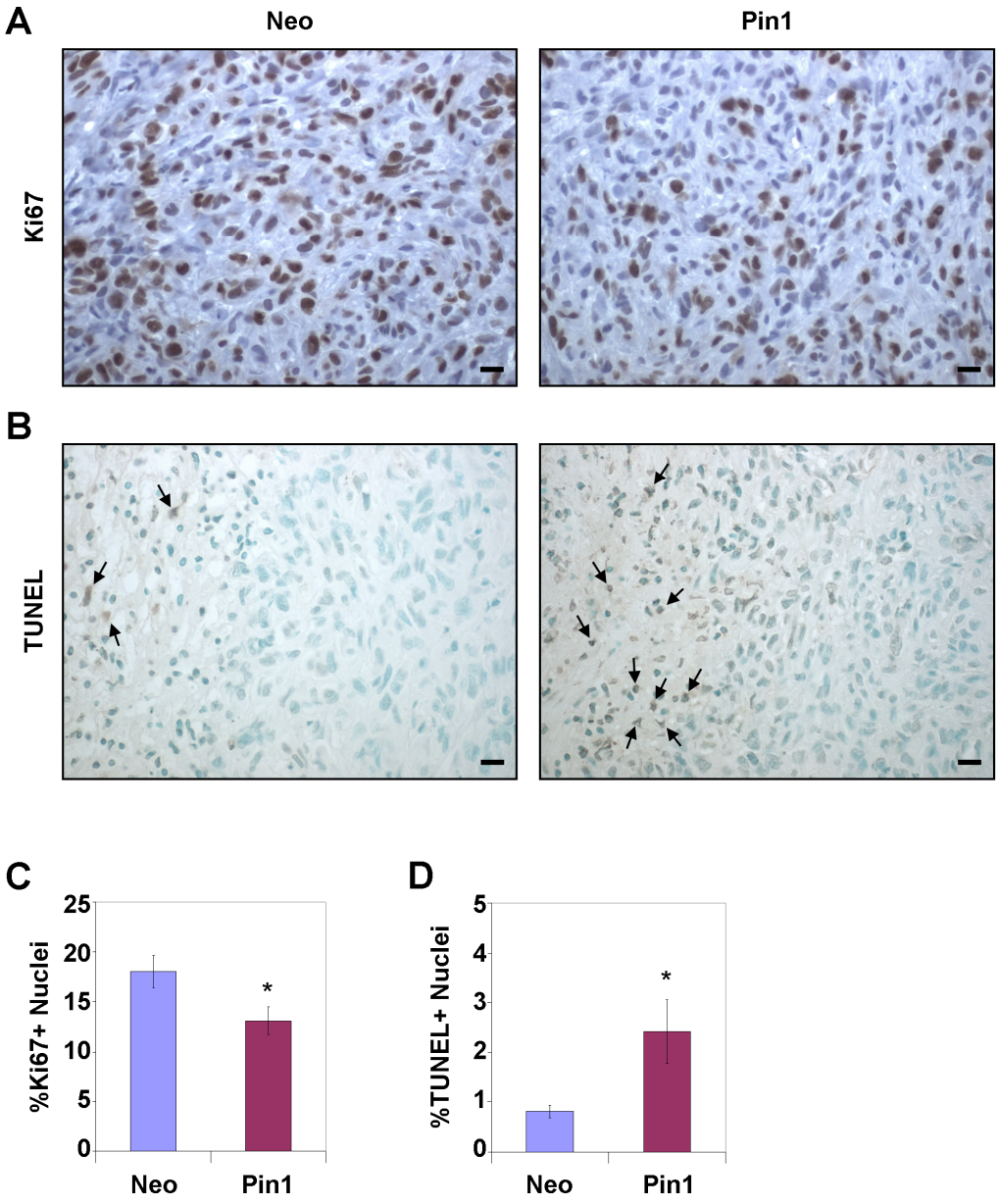

Figure 13: Pin1 attenuates ACHN xenograft tumor growth through effects on proliferation and apoptosis.

(A) Proliferation was assessed by IHC analysis of Ki67.
(B) Apoptosis was evaluated by TUNEL assay. Black arrows point to examples of DAB-positive nuclei.

(C, D) Average percent DAB-positive nuclei was quantified for (A) Ki67 and (B) TUNEL stains respectively. Data represent ACHN-Neo (n=8) and ACHN-Pin1 (n=7) with *P<0.05 vs. control, t-test. Scale bars represent 25 μm.

Repression of p53 activity can prevent Pin1-mediated tumor inhibitory activity

Since previous research suggests Pin1 can enhance p53 function in order to inhibit cell proliferation and induce apoptosis in response to genotoxic stress (Zacchi et al 2002, Zheng et al 2002), we hypothesized that the Pin1-mediated growth inhibitory effect on tumors might require functional p53, which is known to be wild type in ACHN cells (O'Connor et al 1997, Warburton et al 2005). To test this hypothesis, p53 was specifically inhibited in ACHN-Pin1 cells by a dominant negative p53 ($p53^{DD}$) mutant that we have previously utilized (Yeh et al 2006). Expression of $p53^{DD}$ and Pin1 was verified by RT-PCR or immunoblot, respectively (Figure 14A and B). As shown in Figure 14C-E, as expected, the inhibition of p53 by $p53^{DD}$ enhanced growth in soft agar and in xenograft; however, ectopic Pin1 rescue in this setting failed to suppress soft agar colony formation or xenograft tumor growth. Therefore Pin1-mediated tumor inhibitory activity in RCC appears to require functional p53.

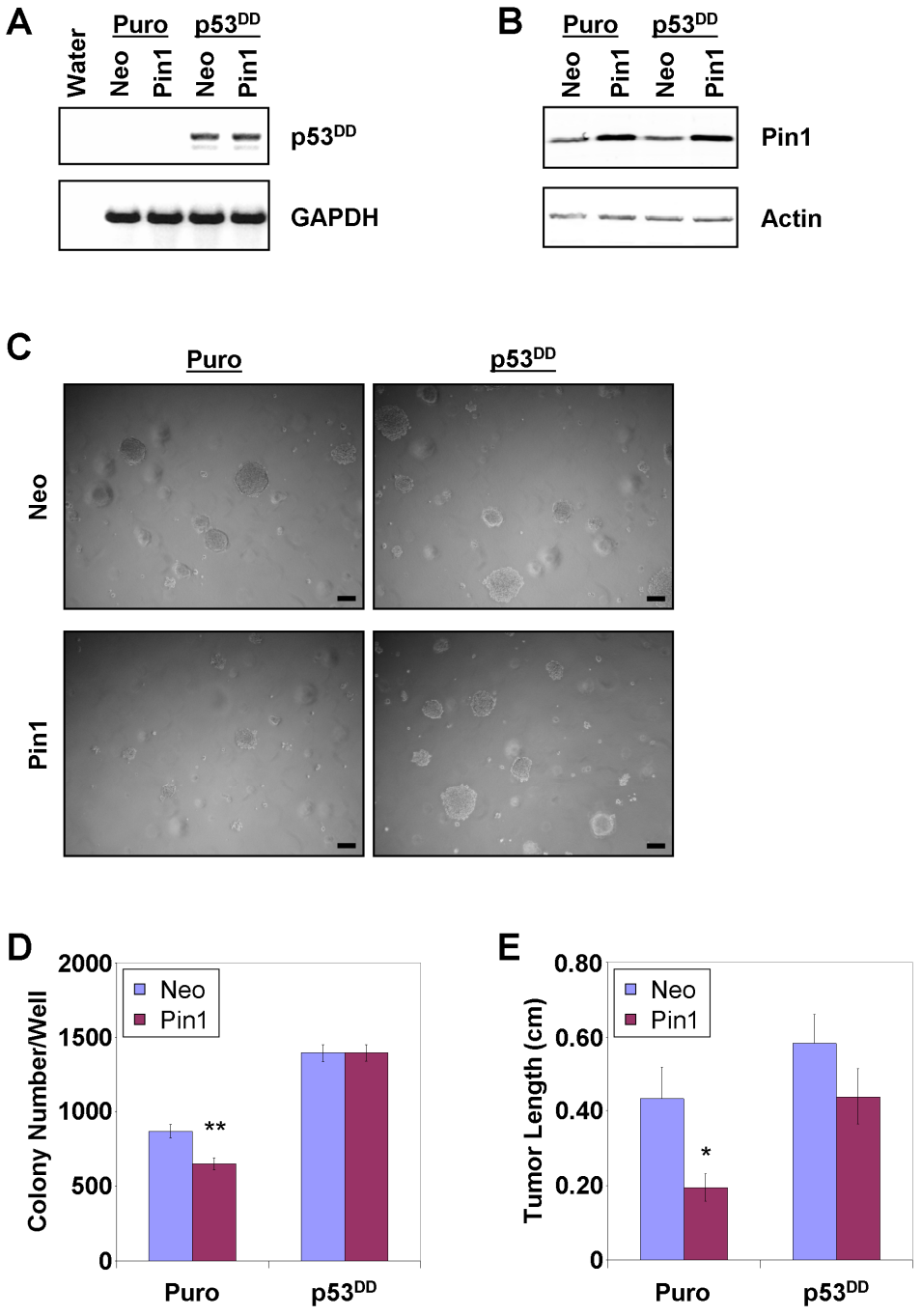

Figure 14: Repression of p53 blocks Pin1-mediated tumor suppressive activity in ACHN cells.

(A) ACHN cells transduced with the indicated combinations of vector (Neo, Puro), Pin1, and $p53^{DD}$ were analyzed for $p53^{DD}$ expression by RT-PCR.

(B) Ectopic Pin1 expression in cell lines from (A) was confirmed by immunoblot.
(C, D) ACHN cells from (A, B) were assayed for growth in soft agar with results quantified from two independent experiments performed in triplicate. Scale bars represent 125 μm.
(E) Final tumor length of ACHN xenograft tumors (n=8) grown for four months.
*P<0.05 or **P<0.005 vs. corresponding Neo control, t-test.

4.3 Discussion

Here we provide the first demonstration of Pin1-mediated tumor inhibitory activity in human cancer cells. The restoration of Pin1 expression in Pin1-deficient ccRCC cells produced reductions in tumor cell growth in soft agar, as well as in xenograft model growth. These effects although modest, were significant, and would be expected to have substantial consequences over the long natural history of renal cell carcinoma. Because tumorigenicity was not ablated, we have referred to Pin1 as a protein with tumor inhibitory activity, rather than a nascent tumor suppressor. Moreover, Pin1's tumor inhibitory effects in renal carcinoma cells seem to require functional p53, as p53 inhibition was sufficient to prevent Pin1-mediated tumor suppressive activity in these cells. This result suggests the possibility that Pin1 acts to attenuate tumor cell growth in conditions in which p53 signaling is intact, and is consistent with the physiologic role of Pin1 in positively regulating p53 biological function, which has been previously documented (Berger et al 2005, Mantovani et al 2007, Zacchi et al 2002, Zheng et al 2002). As the PIN1 gene is frequently deleted in ccRCC tumors and Pin1 exhibits tumor attenuating activity in p53 wild type ccRCC cell

lines, but not those in which p53 is inhibited, we surmise that Pin1 may play an important role in cancer biology to conditionally inhibit growth in some scenarios while exerting tumor promoting activities in other contexts.

Certainly, the concept of a protein functioning with conditional tumor suppressive activity is not novel. Indeed, there are several proteins reported to possess tumor suppressing or promoting activity depending on the genetic context, including deleted in colon cancer (DCC), netrin-1 and transforming growth factor β (TGFβ) (Massagué 2008, Mazelin et al 2004). In "normal" contexts, TGFβ mediates tumor suppression through mechanisms that regulate cytostasis, apoptosis, and differentiation. However, TGFβ can alternatively promote the growth and metastasis of tumors containing certain genetic mutations or deletions that disable TGFβ's tumor suppressive function. The example of TGFβ interestingly seems to share some parallels with what has been observed for Pin1 in cancer. Since Pin1 has been shown to function to either decrease or enhance tumor growth, we speculate that mutations that disable Pin1's tumor suppressing activities (e.g., p53 mutation) may enhance Pin1's tumor promoting activities (e.g., cyclin D1 over-expression) in these contexts. Notably, frequent Pin1 over-expression correlating with high cyclin D1 expression has been shown to occur in human cancers where p53 is frequently mutated, including breast and liver cancers (Pang et al 2004, Wulf et al 2001).

On the other hand, frequent Pin1 under-expression has been most commonly observed in cancers where p53 is frequently wild type, including kidney and testicular cancers (Bao et al 2004, Gurova et al 2004, Heimdal et al 1993). Based on these observations, the role of Pin1 as an inhibitor of human tumorigenesis seems to be highly dependent on the tumor cell context. In ccRCC which harbors infrequent mutations of p53 and other common mediators of tumorigenesis, Pin1 appears to play a mild tumor suppressive role, and further investigation is warranted to ascertain if Pin1 could be a useful prognostic factor or therapeutic target in human cancer.

5. Conclusions and Future Directions

5.1 Conclusions

Pin1 plays a diverse and complex role in cell cycle regulation, genomic instability, and cancer as reviewed in Chapter 1 and elsewhere (Lu and Zhou 2007, Yeh and Means 2007). Despite the growing number of studies to elucidate Pin1's function in the cell, there are still unresolved issues. Indeed, many of Pin1's proposed functions are seemingly at odds with one another. For example, Pin1 can up-regulate oncoproteins (e.g., β-catenin and cyclin D1) or destabilize them (e.g., c-Myc and cyclin E). How is Pin1 able to mediate stabilization versus destabilization? One answer is likely to be that protein-specific differences cause this dichotomy, e.g., different degradation pathways. Even in the case of c-Myc and cyclin E, though Pin1 mediates their degradation via a SCF^{Cdc4} (Yeh et al 2004, Yeh et al 2006), the mechanism by which Pin1 accomplishes this task is protein specific (Chapter 3.3). This was further confirmed by recent research that suggests Pin1 isomerizes a non-canonical Pro381-Pro382 bond to facilitate cyclin E degradation (van Drogen et al 2006).

Another factor that may determine how Pin1 regulates these proteins appears to be the genetic background in which the studies are performed. In fact, unlike Pin1-null MEFs from a mixed genetic background, we do not observe any changes in the levels of

cyclin D1 or β-catenin in isogenic C57BL6 *Pin1*$^{-/-}$ MEFs[1], and as a potential consequence the cycle re-entry phenotype displayed by our cells is relatively mild[2]. However, C57BL6 *Pin1*$^{-/-}$ primordial germ cells exhibit a profound G1-S phase defect (Atchison et al 2003), which suggests that other cell cycle regulators may be regulated by Pin1 in this isogenic background. Given the role of Pin1 in cell cycle regulation, in Chapter 3, we further explored the effects of Pin1 deletion on cell cycle, genomic instability, and tumorigenesis in the C57BL6 genetic background. First, we identified a novel Pin1 substrate cyclin E, whose levels were increased in the absence of Pin1. This deregulation of cyclin E correlated with a specific impairment in the S phase progression of *Pin1*$^{-/-}$ MEFs. Second, we showed the loss of Pin1 results in accelerated genomic instability and aneuploidy, particularly when the cells are immortalized by p53 repression. Finally, immortalized C57BL6 *Pin1*$^{-/-}$ MEFs exhibited increased sensitivity to oncogenic Ras-induced transformation and tumorigenesis. These results provide the first evidence for Pin1 involvement in genomic instability and tumor suppression.

On the other hand, Suizu et al demonstrated Pin1 over-expression in cells derived from a mixed genetic background can induce genomic instability and oncogenesis (Suizu et al 2006). Also consistent with these findings, Pin1 deletion from these mice can prevent Ras/Neu-induced breast cancer formation (Wulf et al 2004). Thus,

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[1] Unpublished observation: Elizabeth Yeh, Brian Teng, and Anthony Means.
[2] Unpublished observation: Brian Teng and Anthony Means.

our results are apparently contrary to studies that have utilized mice from a mixed genetic background. However, as discussed in Chapter 3.3, genetic background can greatly influence the tumor phenotypes observed for a given genetic mutation, which has been previously reported (Donehower et al 1995, Freeman et al 2006, Reilly et al 2004). This certainly suggests the presence of modifiers in each genetic background that predispose Pin1 to behaving as a tumor promoter or suppressor. One interesting question that remains is: how could one use these two systems to gain insights into how Pin1 is functioning in a given cell? Perhaps the direct comparison of the two by genomic and proteomic approaches could identify the modifiers responsible for determining Pin1's behavior, which is an idea that has been proposed for discovering tumor susceptibility genes (Balmain 2002). The findings from such studies may be particularly relevant to resolving the conundrum of Pin1's role in human cancer.

Next, we sought to determine if Pin1 could function as a conditional tumor suppressor in human cancer. As discussed in Chapter 1.4, there is considerable evidence to suggest Pin1 may function as either a tumor promoter or suppressor; however, no evidence has been provided (up until the work described in this dissertation) to support the latter case in a human context. Based on the fact that the Pin1 protein is frequently under-expressed in kidney cancer samples (Bao et al 2004), in Chapter 4, we evaluated if Pin1 might serve a tumor suppressive function in the most common form of kidney cancer, namely ccRCC. First, we identified PIN1 gene deletion and mRNA under-

expression as potential mechanisms that contribute to Pin1 reduction in ccRCC. Since gene deletion is a key characteristic of tumor suppressors, we also performed pathway analysis to find genes associated with Pin1 under-expression, and found low levels of Pin1 to correlate with increased levels genes involved in proliferation and metastasis. Second, we identified ccRCC (A498 and ACHN) cell lines that exhibited Pin1 under-expression, and restored Pin1 levels via retroviral transduction. In a manner requiring Pin1's WW domain, ectopic Pin1 was able to attenuate the growth of ccRCC cells in soft agar. In addition, ectopic Pin1 suppressed the tumor growth of these cells in a xenograft model through effects on proliferation and apoptosis. Finally, we showed repression of p53 by $p53^{DD}$ can prevent Pin1's tumor suppressive activity in ccRCC cells grown in soft agar and xenograft, which is consistent with the notion that different genetic modifiers in a cell may influence Pin1's role in cancer. Collectively, our studies in mouse and human cells support the hypothesis that Pin1 is a conditional tumor suppressor.

5.2 Future Directions

Adding to the controversy already discussed in this dissertation, there is interesting clinical evidence that shows Pin1 over-expression in human cancers does not always lead to reduced survival of patients. Although Pin1 over-expression in prostate and lung cancers correlates with increased recurrence or reduced survival, respectively (Ayala et al 2003, Tan et al 2010), there are examples where this is not the case. For example, one study shows Pin1 over-expression in conjunction with high phospho-

Akt(Ser473) correlates with poor prognosis in breast cancer patients; however, neither Pin1 over-expression by itself nor in conjunction with low phospho-Akt(Ser473) affect survival (Liao et al 2009). Similarly, Pin1 over-expression or Mcl-1 over-expression by themselves do not correlate with decreased survival in breast cancer patients, but the co-expression of both does (Ding et al 2008). In the case of merkel cell carcinoma, Pin1 over-expression actually correlates with improved overall survival of these patients (Lill et al 2010). Although these studies only provide correlations and do not support causality, they do seem to support the notion of modifiers for Pin1 function and suggest Pin1 is a conditional tumor promoter.

What about the role of Pin1 under-expression in human cancer? This is an important question, but has been largely unaddressed. We show in Chapter 4 that Pin1 under-expression may promote kidney cancer progression, but were unable to identify clinical correlations between Pin1 under-expression and survival outcomes. This will certainly be an important future endeavor using appropriate data sets. Moreover, it will be necessary to evaluate the effect Pin1 under-expression in other cancers. We have initiated these studies and obtained some interesting results. A search for PIN1 using Oncomine[3] revealed PIN1 under-expression in brain cancer (Figure 15A). To verify these

[3] Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization.

findings, we obtained larger data sets from TCGA[4]. Our analysis shows 63 of 233 (27%) brain tumors exhibit deletion of the PIN1 gene (Figure 15B), which comparable to our findings in kidney cancer (Figure 8A). Consistent with PIN1 gene deletion, the PIN1 mRNA is prevalently under-expressed in glioblastoma samples compared to normal brain samples (Figure 15C). In Figure 16[5], we show using a data set produced at Duke (Rich et al 2005) that Pin1 under-expression may be associated with reduced survival of glioblastoma patients. Although more research will be required to validate these findings, the data further support our contention that Pin1 is a conditional tumor suppressor.

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[4] The results published here are in whole or part based upon data generated by The Cancer Genome Atlas Pilot Project established by the NCI and NHGRI. Information about TCGA and the investigators and institutions who constitute the TCGA research network can be found at http://cancergenome.nih.gov.
[5] Data kindly provided by Patrick Killela.

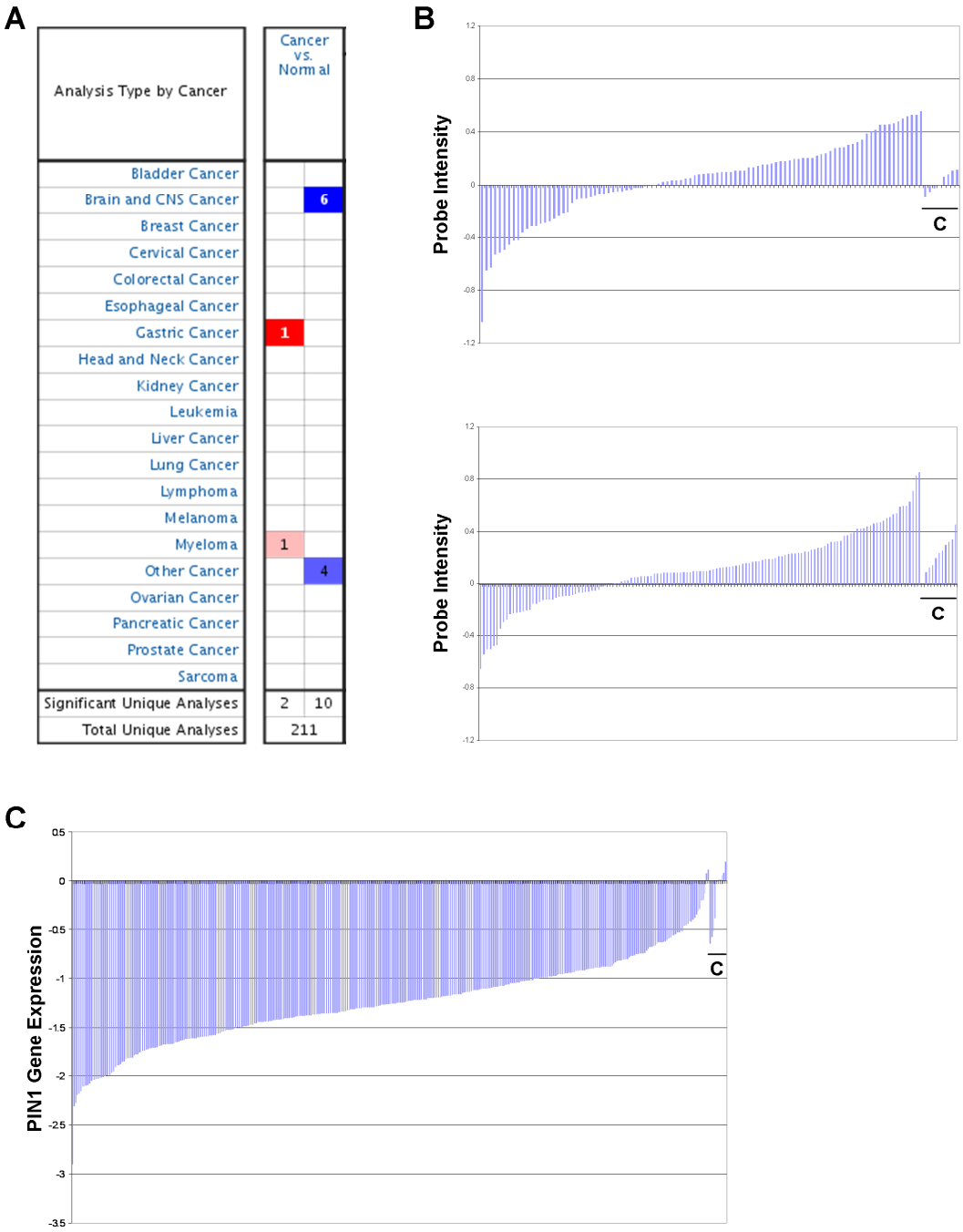

Figure 15: PIN1 gene is frequently deleted and under-expressed in human glioblastoma tumors.

(A) Oncomine summary table of PIN1 gene over-expression (red) or under-expression (blue) in cancer vs. normal. The "other cancer" where PIN1 is under-expressed is testicular cancer (see Chapter 4.3 for additional discussion).
(B) PIN1 copy number in glioblastoma vs. normal brain samples (denoted as C) was determined from the normalized signal intensity of probes on a single nucleotide polymorphism (SNP) array that cover the PIN1 gene. Top panel, data from Harvard Medical School (tumor N=98, normal N=8). Bottom panel, data from Memorial Sloan-Kettering Cancer Center (tumor N=135, normal N=11).
(C) Normalized PIN1 gene expression in glioblastoma vs. normal brain samples (denoted as C) from gene expression microarray data. Tumor N=371, normal N=10.

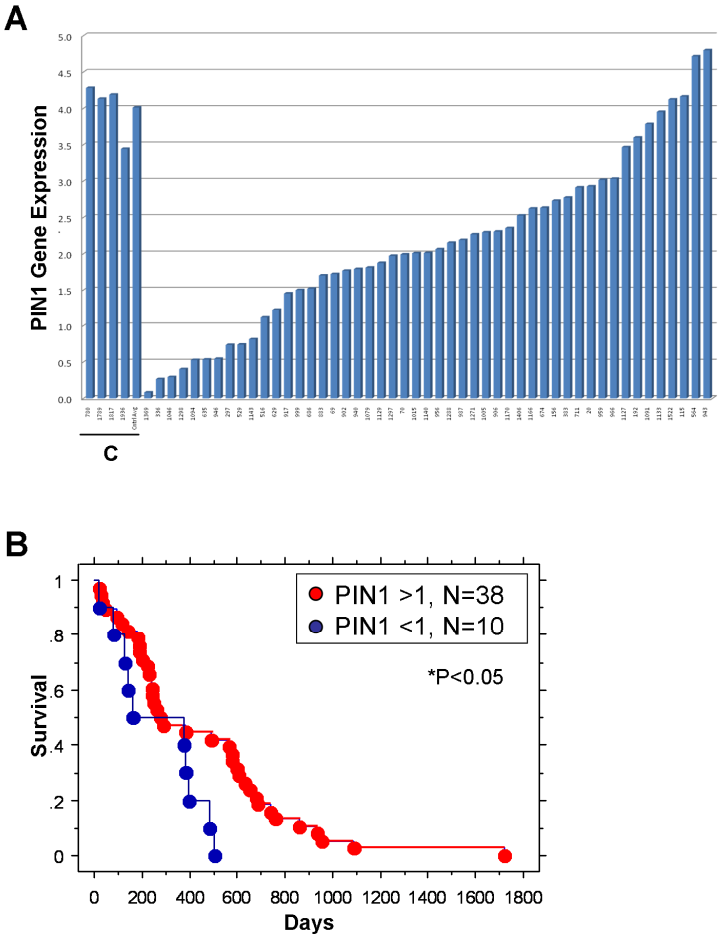

Figure 16: PIN1 gene under-expression correlates with reduced survival of glioblastoma patients.

(A) Normalized PIN1 gene expression in glioblastoma vs. normal brain samples (denoted as C) from gene expression microarray data. Tumor N=48, normal N=5.
(B) Survival of glioblastoma patients with PIN1 gene expression >1 (red) or <1 (blue) was assessed by Kaplan-Meier survival analysis. P-value determined by log-rank test.

One final question is: how might Pin1 be useful as a therapeutic target or prognostic factor in human cancer? Certainly, there is much work to be done before we can fully address this question. First, we need to know what modifiers of Pin1 function exist and how they do so. The research described in this dissertation suggests Cdc4 and p53 mutations could be relevant modifiers of Pin1 function. This is further supported by recent research that shows Pin1 enhances the oncogenic functions of missense mutant p53 (Girardini et al 2011). Second, we need a way to determine what modifiers are present in a given cancer cell and how they may interact with one another (e.g., dominant modifiers). This might be accomplished by genomics or proteomics; however, the issue of costs versus benefits must be evaluated. Finally, we need clinically useful Pin1 inhibitors[6] and to know when it would be appropriate to use them. For example, inhibiting deregulated Pin1 activity in certain prostate cancer patients may be beneficial (Ayala et al 2003, Ryo et al 2005), but perhaps not so in merkel cell carcinoma patients (Lill et al 2010). Therefore, based on the information presented here, the function of Pin1 is highly dependent on genetic context and "modifiers". The discovery and further

[6] Previously reviewed in Yeh and Means 2007.

elucidation of these Pin1 modifiers could aide the individualized treatment of patients suffering from cancer, which is a worthy endeavor.

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Biography

Brian L. Teng was born as Brian O. Lew in Tucson, AZ in December 1980. Brian attended the University of Arizona and graduated *summa cum laude* with a B.S. in Biochemistry and Molecular/Cellular Biology in December 2002. During his time at the University of Arizona, he was trained in pharmacology and basic methods of drug discovery by Josephine Lai. His growing interest in drug research prompted Brian to pursue a Ph.D. in Pharmacology at Duke University in the laboratory of Anthony Means. His work contributed to the publication of three peer-reviewed journal articles:

Teng, B.L., Hacker, K.E., Chen, S., Means, A.R., and Rathmell, W.K. (2011). "Tumor suppressive activity of prolyl isomerase Pin1 in renal cell carcinoma." *Mol Oncol* (10.1016/j.molonc.2011.06.002).

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Brian was awarded a Department of Defense Breast Cancer Research Program Pre-Doctoral Fellowship, which helped support his cancer training at Duke. Upon completion of his Pharmacology Ph.D. in September 2011, he intends to continue his career in drug discovery and development.