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A Pin1/Mutant p53 Axis Promotes Aggressiveness in Breast Cancer

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SUMMARY

TP53 missense mutations dramatically influence tumor progression, however, their mechanism of action is still poorly understood. Here we demonstrate the fundamental role of the prolyl isomerase Pin1 in mutant p53 oncogenic functions. Pin1 enhances tumorigenesis in a Li-Fraumeni mouse model and cooperates with mutant p53 in Ras-dependent transformation. In breast cancer cells, Pin1 promotes mutant p53 dependent inhibition of the antimetastatic factor p63 and induction of a mutant p53 transcriptional program to increase aggressiveness. Furthermore, we identified a transcriptional signature associated with poor prognosis in breast cancer and, in a cohort of patients, Pin1 overexpression influenced the prognostic value of p53 mutation. These results define a Pin1/mutant p53 axis that conveys oncogenic signals to promote aggressiveness in human cancers.

INTRODUCTION

Mutations in the *TP53* tumor suppressor gene are among the most frequent genetic alterations in human cancers (Soussi and Wiman, 2007). In addition to sporadic tumors, inherited *TP53* mutations are causative of Li-Fraumeni syndrome, a disease characterized by an early onset of a broad tumor spectrum (Malkin et al., 1990). As a consequence of these mutations p53 loses the ability to bind wild-type p53 responsive elements on DNA, thus becoming defective for oncosuppressor functions, and can exert dominant-negative effects over wild-type p53. Yet, several p53 mutant proteins acquire novel activities, which can significantly contribute to various aspects of tumor progression, commonly described as gain of function properties (Oren and Rotter, 2010). In particular, compelling evidences have underscored the ability of mutant p53 to promote cell migration and metastasis (Adorno et al., 2009; Caulin et al., 2007; Muller et al., 2009; Terzian et al., 2008).

Significance

Accumulating evidence underscored the role of mutant p53 in promoting transformation and metastasis. We provide insights in the molecular basis of tumor progression showing how the prolyl isomerase Pin1 and mutant p53 become integrated into a molecular axis that promotes the acquisition of aggressive features by tumor cells. Pin1 transduces oncogenic signaling converging on mutant p53 to enhance its pro-migration and invasion activities both by neutralizing p63 and by activating a transcriptional program that correlates with poor clinical outcome in breast cancer. Our data imply that the combination of Pin1 overexpression and p53 mutation behaves as an independent prognostic factor of poor clinical outcome and that components of the Pin1/mutant p53 axis might be exploited as diagnostic and therapeutic tools.

Pivotal for mutant p53 gain of function is the formation of aberrant protein complexes perturbing the activity of the interacting partners. For instance, mutant p53 contributes to malignant phenotypes, such as migration and invasion or genomic instability, through binding and inactivating the antimetastatic factor p63 and Mre11, respectively (Adorno et al., 2009; Song et al., 2007). Although defective in sequence-specific DNA binding, mutant p53 exerts its gain of function also altering transcriptional regulation, based on aberrant interactions with several transcription factors, such as VDR, NF-Y, and E2F, and recruitment of transcriptional cofactors (Brosh and Rotter, 2009).

Evidence from in vivo studies highlighted that efficient gain of function of mutant p53 is associated with a transformed cellular environment (Song et al., 2007; Soussi and Béroud, 2001; Terzian et al., 2008), supporting the notion that oncogenic stress signaling could activate mutant p53 through mechanisms similar to those required to stimulate wild-type p53 (Song et al., 2007; Terzian et al., 2008). To investigate and dissect the mechanisms through which oncogenic signaling triggers mutant p53 activities, we focused on factors that might link cancer-related signaling with mutant p53 functions. An intriguing candidate for this role is the prolyl isomerase Pin1, that transduces phosphorylation signaling into conformational changes affecting the functions of its substrates (Lu and Zhou, 2007; Yeh and Means, 2007). We and others have previously identified this enzyme as a critical regulator of wild-type p53 activities in cells exposed to genotoxic stress (Mantovani et al., 2007; Zacchi et al., 2002; Zheng et al., 2002). Despite Pin1 supporting wild-type p53 functions, $Pin1^{-/-}$ mice do not develop tumors (Atchison et al., 2003). Instead, Pin1 is frequently overexpressed in human tumors (Bao et al., 2004) and has been shown to promote both Her2/Neu/Ras and Notch1 dependent transformation of breast cells (Rustighi et al., 2009; Wulf et al., 2004). Because several aspects of the regulation of wild-type and mutant p53 proteins are similar (Terzian et al., 2008), we reasoned that the role of Pin1 as a component of physiologic checkpoint mechanisms may be subverted during tumorigenesis, becoming a crucial amplifier of mutant p53 oncogenic functions.

RESULTS

Pin1 Enhances Tumorigenesis in a Mutant p53 Knockin Mouse Model

To understand the impact of Pin1 on mutant p53 in tumorigenesis, we crossed mutant p53 knockin mice, bearing one knockin allele for p53R172H ($p53^{M/+}$) (Lang et al., 2004), with $Pin1^{+/-}$ mice (Atchison et al., 2003). Two cohorts of compound mice, having either one or two knockin alleles with wild-type or knock-out Pin1 genotypes ($p53^{M/+}Pin1^{+/+}$, $p53^{M/+}Pin1^{-/-}$, and $p53^{M/M}Pin1^{+/+}$, $p53^{M/M}Pin1^{-/-}$), were generated and monitored. Survival data for $p53^{M/M}Pin1^{+/+}$ and $p53^{M/+}Pin1^{+/+}$ mice were consistent with published results (Lang et al., 2004). Mice lacking Pin1 displayed an increased median tumor-free survival in both cohorts (Figures 1A and 1B). Although in the $p53^{M/M}$ cohort absence of Pin1 caused variations only in the frequency of some tumor types (see Table S1 available online), in the $p53^{M/+}Pin1^{-/-}$ cohort, as compared to $p53^{M/+}Pin1^{+/+}$ mice, we observed a markedly reduced tumor frequency, a reduced number of lymphomas and notably a complete absence of carcinomas (Figure 1C; Table S2).

We noticed a more pronounced effect due to lack of Pin1 in the median survival time of the $p53^{M/+}$ compared with the $p53^{M/M}$ cohorts (7 weeks versus 4 weeks), which could be ascribed to a contribution of Pin1 in the dominant negative activity of mutant p53 over the remaining wild-type allele. However, in this model of Li-Fraumeni syndrome, this activity is not evident, because the survival curves of $p53^{M/+}$ compared to $p53^{+/-}$ mice are indistinguishable (Lang et al., 2004). Moreover, albeit Pin1 enhances complex formation between wild-type and mutant p53, this does not abolish wild-type p53 transcriptional activity (data not shown), likely due to a concomitant effect of Pin1 in potentiating the function of unbound wild-type p53 (Zacchi et al., 2002; Zheng et al., 2002). We also analyzed cohorts of $p53^{+/-}Pin1^{+/+}$ and $p53^{+/-}Pin1^{-/-}$ mice and failed to observe any difference in tumor-free survival, tumor frequency or spectrum (Figures S1A and S1B and Table S2). Therefore, absence of Pin1 reduces spontaneous tumorigenesis exclusively in mice expressing mutant p53, suggesting that their cooperation in tumorigenesis relies mainly on mutant p53 gain of function properties.

The genetic interaction between Pin1 and mutant p53 revealed above, prompted us to dissect the underlying mechanisms. Notably, Pin1 coimmunoprecipitated with p53 in tumor samples from $p53^{M/+}Pin1^{+/+}$ mice (Figure S1C). Given that Pin1 specifically recognizes phosphorylated S/T-P motifs on its substrates, our observation suggests that in tumor cells Pin1 might bind mutant p53 in response to oncogenic signaling-mediated phosphorylation to regulate its function. To demonstrate this hypothesis, we analyzed the effect of Pin1 on the ability of mutant p53 to enhance Ras-induced transformation of primary cells (Lang et al., 2004). Mouse embryo fibroblasts (MEFs) obtained from $p53^{M/M}Pin1^{+/+}$, $p53^{M/M}Pin1^{-/-}$, $p53^{-/-}Pin1^{+/+}$, or $p53^{-/-}Pin1^{-/-}$ embryos were tested for anchorage-independent growth as well as for their tumorigenic potential in vivo on retroviral transduction of $H\text{-}Ras^{V12}$. Notably, $p53^{M/M}Pin1^{+/+}$ cells formed 2-fold more colonies in soft agar (Figure 1D, left) and when injected subcutaneously into immunocompromised mice, they developed tumors double in size as compared with either $p53^{M/M}Pin1^{-/-}$ or $p53^{-/-}$ cells (Figure 1D, right; Figure S1D). In $H\text{-}Ras^{V12}$-transduced $p53^{M/M}Pin1^{+/+}$ MEFs, coimmunoprecipitation (CoIP) experiments allowed us to confirm the mutant p53-Pin1 interaction (Figure S1E). In these cells, overexpression of $H\text{-}Ras^{V12}$ caused upregulation of mutant p53 and Pin1 (Figure S1F), in agreement with previous observations (Ryo et al., 2002). We next immunoprecipitated mutant p53 from cellular lysates, normalized for p53 levels, and phosphorylation was probed with a phospho-S/T-P specific antibody (MPM2). As shown in Figure 1E (left), $H\text{-}Ras^{V12}$ overexpression increased phosphorylation of mutant p53 on putative Pin1 binding motifs and, accordingly, its interaction with Pin1 (Figure 1E, right).

Modulation of Pin1 levels in these cells and in thymic lymphomas of the same genotypes did not affect mutant p53 protein accumulation (data not shown). This observation does not exclude, however, that a contribution of Pin1 in mutant p53 stabilization could be relevant during early steps of tumorigenesis.

All together these findings suggest that Pin1 is necessary for the ability of mutant p53 to promote both cell transformation and

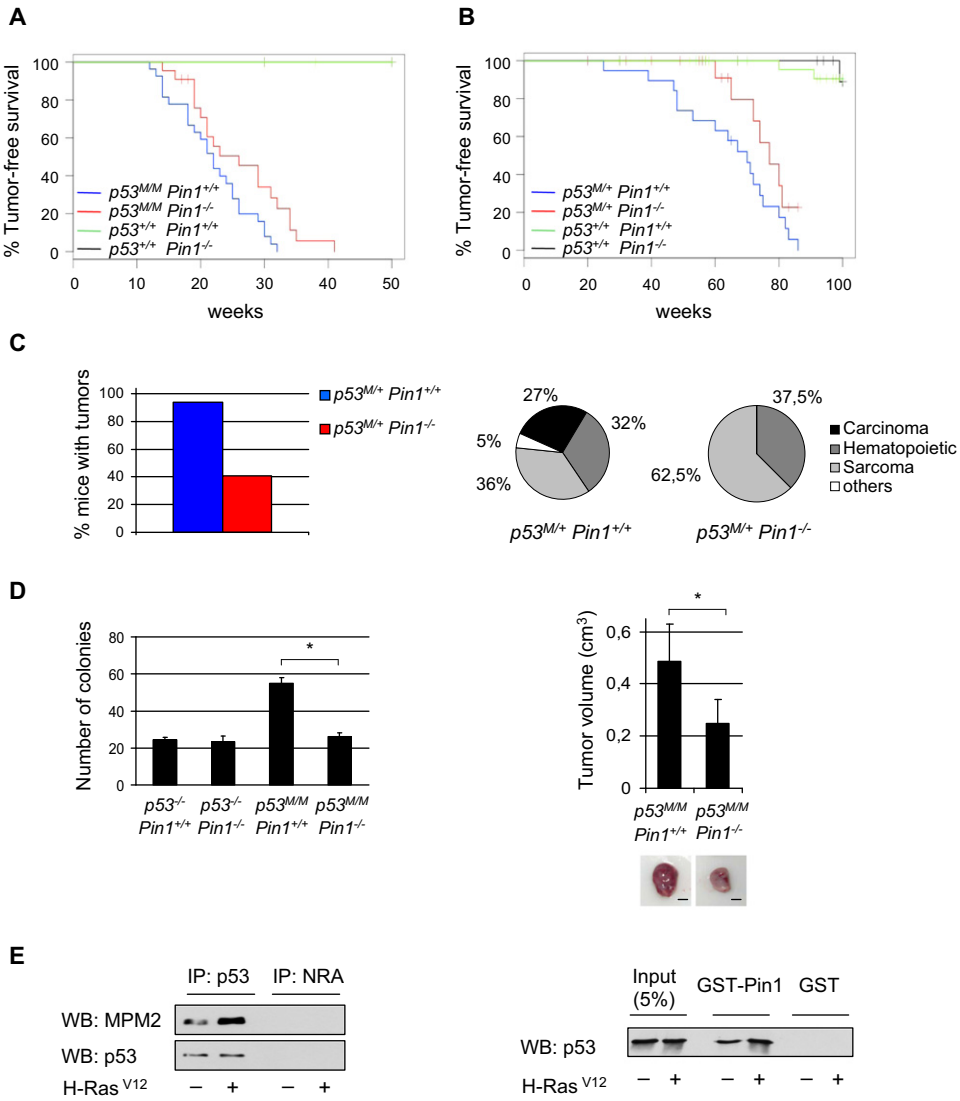


Figure 1. Pin1 Enhances Tumorigenesis in Mutant p53 Knockin Mice

(A) Tumor-free Kaplan-Meier survival curves of $p53^{M/M}Pin1^{+/+}$ (n = 27) and $p53^{M/M}Pin1^{-/-}$ (n = 22) mice cohorts ($\chi^2 = 4$, p = 0.02, log rank Mantel-Haenszel test). As a control, tumor-free Kaplan-Meier survival curves of $p53^{+/+}Pin1^{+/+}$ (n = 22) and $p53^{+/+}Pin1^{-/-}$ (n = 18) mice cohorts are shown.

(B) Tumor-free Kaplan-Meier survival curves of $p53^{M/+}Pin1^{+/+}$ (n = 19) and $p53^{M/+}Pin1^{-/-}$ (n = 17) mice cohorts ($\chi^2 = 5.4$, p = 0.045, log rank Mantel-Haenszel test). As a control, tumor-free Kaplan-Meier survival curves of $p53^{+/+}Pin1^{+/+}$ (n = 22) and $p53^{+/+}Pin1^{-/-}$ (n = 18) mice cohorts are shown.

(C) Left: bar graph showing the proportion of mice of the indicated genotypes developing tumors. Right: pie graphs representing the proportion of tumor types in $p53^{M/+}Pin1^{+/+}$ and $p53^{M/+}Pin1^{-/-}$ mice relative to tumor totals.

(D) Left: histogram of anchorage-independent growth of H-Ras^{V12} transduced MEFs. Error bars indicate standard deviation (SD) (n = 3), *P* relative to $p53^{M/M}Pin1^{+/+}$ MEFs < 0.01 in all cases (two-tailed t test). Right: bar graph representing the volume of tumors originated by the indicated H-Ras^{V12}-transduced MEFs injected in SCID NOD mice measured on sacrifice. The graph shows the average volume and SD of tumors from five mice injected for each cell type. p < 0.01 (two-tailed t test). Representative images of tumors are shown below. Scale bar represents 0.2 cm.

(E) Left: western blot analysis of phosphorylated S-P or T-P sites (MPM-2 antibody) of normalized levels of immunoprecipitated p53R172H from $p53^{M/M}Pin1^{+/+}$ MEFs infected with H-Ras^{V12} (+) or empty vector (–). NRA, not related antibody. Right: western blot analysis of pull-down assays with recombinant GST-Pin1 using cell lysates normalized as above. See also Figure S1 and Tables S1 and S2.

tumorigenicity and reveal a role for Pin1 as a key transducer of oncogenic signaling, thus activating mutant p53 gain of function.

Pin1 Potentiates Mutant p53-Dependent Cell Migration and Invasion of Human Tumor Cell Lines

To establish whether Pin1 could sustain mutant p53 oncogenic function also in human cancer cells, we tested the ability of Pin1 to bind to endogenous mutant p53 in human breast cancer cell lines MDA-MB-231 and SK-BR-3. In these cells, mutant p53 is constitutively phosphorylated on some Pin1 binding sites (S33 and S46) (Zacchi et al., 2002; Zheng et al., 2002) and able to interact with Pin1 in a phosphorylation-dependent manner as judged by CoIP after treatment with λ phosphatase (Figures S2A–S2C). To verify whether H-Ras^{V12}

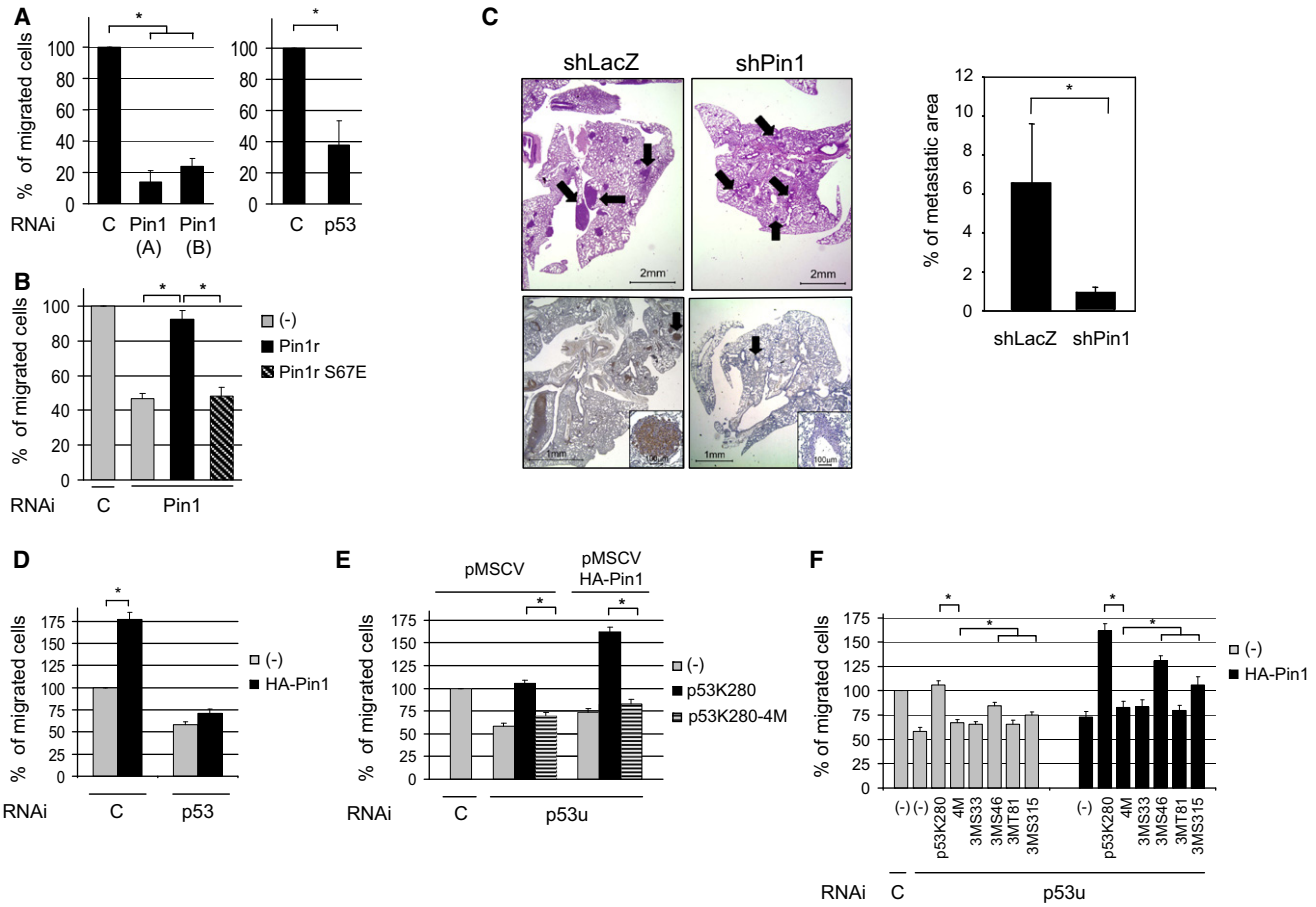


Figure 2. Pin1 Amplifies Mutant p53 Pro-Migration Function

(A) Transwell migration assays of MDA-MB-231 cells transfected with the indicated siRNAs (RNAi). Error bars indicate SD (n = 3, *p < 0.001, two-tailed t test). The effect on migration of two different Pin1 siRNA sequences (Pin1 [A] and Pin1 [B]) was analyzed. See Figure S2F for western blot analysis showing Pin1 and mutant p53 depletion.

(B) Transwell migration assays of MDA-MB-231 cells transfected with control (C) or Pin1 (A) siRNA on transduction with retroviral vectors expressing siRNA resistant HA-Pin1r, HA-Pin1r S67E (catalytically inactive) or empty vector (–). Error bars indicate SD (n = 3, *p < 0.001, two-tailed t test). See Figure S2F for western blot analysis of cell lysates.

(C) Lung colonization assays after tail vein injection of Pin1 depleted MDA-MB-231 cells. Left: histological analysis of lung colonization. Upper images: hematoxylin and eosin staining of representative sections of entire pulmonary lobes from mice inoculated with shLacZ-infected (control) or shPin1-infected cells (six mice per each group), arrows indicate representative metastases. Lower images: immunohistochemical detection of Pin1 expression and inserts showing a detailed view of the area indicated by the arrows (scale bars are indicated). Right: computer-aided assessment of percentage of lung tissue area occupied by metastases. Data are reported in histograms as mean percentages ± standard error of the mean (Mann-Whitney rank sum test, *p = 0.009).

(D) Transwell migration assays of MDA-MB-231 cells transfected with p53 or control siRNA (C) on transduction with pMSCV (–) or pMSCV HA-Pin1 retroviral vectors. Error bars indicate SD (n = 3, *p < 0.001, two-tailed t test). See Figure S2H for western blot analysis of cell lysates.

(E) Transwell migration assays of MDA-MB-231 cells cotransduced with pMSCV or pMSCV HA-Pin1 along with empty vector (–), HA-p53K280, or HA-p53K280-4M retroviral constructs. Specific depletion of endogenous mutant p53 was achieved transfecting an siRNA targeting the 3′UTR (p53u). Error bars indicate SD (n = 3, *p < 0.001, two-tailed t test). See Figure S2M for western blot analysis of cell lysates.

(F) Transwell migration assays of MDA-MB-231 cells cotransduced with pMSCV or pMSCV HA-Pin1 along with retroviral constructs expressing the indicated mutant p53 constructs or empty vector (–). Endogenous mutant p53 was knocked down as in (E). Error bars indicate SD (n = 3). *p value relative to HA-p53K280-4M (two-tailed t test). pMSCV transduced cells: $p < 0.001$ for p53K280, $p < 0.01$ for p53K280-3MS46, $p < 0.05$ for p53K280-3MS315. pMSCV HA-Pin1 transduced cells: $p < 0.001$ for p53K280 and p53K280-3MS46, $p < 0.05$ for p53K280-3MS315. See Figure S2N for western blot analysis of cell lysates.

See also Figure S2.

could affect mutant p53 phosphorylation, as well as interaction with Pin1 also in human cells, we cotransfected H-Ras^{V12} along with p53K280 in H1299 p53 null cells. H-Ras^{V12} enhanced phosphorylation on two Pin1 binding sites on mutant p53 (S33 and S46) (Figure S2D) and its interaction with Pin1 (Figure S2E), in agreement with the results obtained in H-Ras^{V12} transduced MEFs.

Because induction of migration and invasion are critical aspects of mutant p53 dependent metastatic phenotype (Adorno et al., 2009; Muller et al., 2009), we evaluated whether Pin1 could play a role in these processes. RNAi-mediated knockdown of either mutant p53 or Pin1 significantly reduced cell migration and invasion of MDA-MB-231 cells (Figure 2A; Figures S2F and S2G). The effect of Pin1 on migration was specific and

required its enzymatic activity, because reintroduction of an siRNA-resistant Pin1 construct (Pin1r), but not a catalytically inactive Pin1 mutant (Pin1r S67E), restored migration (Figure 2B; Figure S2H). The impact of Pin1 on promoting invasion and metastasis was further evaluated in vivo, by injecting control- or Pin1-depleted MDA-MB-231 cells intravenously into immunocompromised mice. The ability of Pin1 depleted cells to colonize the lungs was drastically reduced (Figure 2C; Figure S2I).

We next asked whether Pin1 exerts these effects epistatically by modulating mutant p53 functions. As shown in Figure 2D and Figure S2J, Pin1 overexpression in MDA-MB-231 cells causes a significant increase in cell migration and invasion, however, simultaneous depletion of mutant p53 almost completely abolished these effects. In order to test whether direct interaction between Pin1 and mutant p53 is necessary, we generated a modified form unable to bind to Pin1 (p53K280-4M) of the endogenous mutant p53 (p53K280). In this construct alanines replaced serine or threonine residues in the four S/T-P phosphorylation and Pin1 binding sites (S33, S46, T81, and S315) (Mantovani et al., 2007), without perturbing the subcellular localization (Figures S2K and S2L). We then compared the effect of p53K280 and p53K280-4M on migration of MDA-MB-231 cells in which endogenous mutant p53 was selectively depleted using an siRNA targeting the 3′UTR of its mRNA (p53u). Reintroduction of an siRNA-resistant p53K280 resulted in complete rescue of migration, whereas almost no effect was observed in cells expressing p53K280-4M. Notably, on Pin1 overexpression, we observed an increased migration in p53K280 but not in p53K280-4M expressing cells (Figure 2E; Figure S2M).

To evaluate which of these four phosphorylation sites in mutant p53 is important in promoting this phenotype, wild-type amino acids were separately reintroduced into p53K280-4M on each of the alanine substitutions and the resulting four different p53K280-3M constructs were tested for the ability to rescue cell migration on depletion of endogenous mutant p53. Only constructs expressing mutant p53 with wild-type S46 or S315 phosphorylation sites allowed recovery of cell migration, with p53K280-3M-S46 showing the stronger impact, and their effect was even more evident when Pin1 was coexpressed (Figure 2F; Figure S2N). These constructs were able to recover Pin1 binding in CoIP experiments (Figure S2O) further confirming the importance of Pin1 interaction with these phosphorylation sites on mutant p53 to promote cell migration.

Next, we wanted to test which kinases could be involved in phosphorylating mutant p53 on S46. As shown in Figure S2P, treatment of MDA-MB-231 cells with inhibitors of kinases, some of which act downstream of Ras signaling, reduced phosphorylation of endogenous mutant p53 on S46.

Overall, these results strongly suggest that phosphorylation of critical S/T-P sites in mutant p53, triggered by oncogenic signaling, promotes Pin1 binding thus unleashing mutant p53 pro-migration activity.

Pin1 Impacts Mutant p53 Transcriptional Regulation

Mutant p53 was reported to enhance cell migration and invasion by sequestering and inactivating the antimetastatic factor p63 (Adorno et al., 2009; Muller et al., 2009). Therefore, we asked whether Pin1 could promote the mutant p53-p63 interaction. CoIP assays in MDA-MB-231 cells showed that Pin1 overexpression increased the association between endogenous p63 and mutant p53, whereas Pin1 downregulation reduced their interaction (Figure 3A). Likewise, p53K280-4M did not efficiently associate with p63 (Figure 3B), indicating that phosphorylation on these sites and Pin1 binding are required for the interaction with p63. Pin1 downregulation also reduced the interaction between Smad2 and p63, which was shown to be required for mutant p53 binding to p63 on TGF-β treatment (Adorno et al., 2009) (Figure S3A).

Accordingly, on Pin1 overexpression, the transcriptional function of p63 was curbed, as judged from reduced expression of p63 targets *CCNG2* and *Sharp-1* (Adorno et al., 2009) (Figure 3C); an effect that was mutant p53-dependent because concomitant downregulation of mutant p53 caused their induction (Figure 3D). Moreover, silencing of Pin1 caused upregulation of these and other p63 target genes such as *Dicer* (Su et al., 2010). This effect required the presence of p63, because their expression levels decreased when Pin1 and p63 were simultaneously downregulated (Figures S3B and S3C).

In vitro GST-Pin1 pull down and CoIP assays in MDA-MB-231 cells demonstrated that p63 binds Pin1 (Figure S3D), thus suggesting that Pin1 could also affect directly p63 functions. Indeed, it enhanced TAp63α transcriptional activity when coexpressed in H1299 p53 null cells (Figure S3E). This demonstrates that, in the absence of mutant p53, Pin1 does not inhibit p63 but rather it increases p63 transcriptional activity.

Interestingly, in experiments where Pin1 was overexpressed, we also observed a consistent increase in transcription of *CCNA2*, a known direct target gene of the mutant p53-NF-Y complex (Di Agostino et al., 2006) (Figure 3E). Overall, these results show that Pin1 contributes to mutant p53 oncogenic properties both by potentiating its ability to block p63 transcriptional activity and by influencing other mutant p53 transcriptional functions.

Pin1/Mutant p53 Axis Activates a Pro-Aggressiveness Transcriptional Program

To investigate how Pin1 and mutant p53 may globally alter gene expression, we analyzed the transcription profiles of MDA-MB-231 cells on depletion of either Pin1 or mutant p53. Unbiased analysis of functional annotation (AFA) showed that the same categories of genes were affected under both conditions (Figure S4A), implying that Pin1 and mutant p53 are involved in the regulation of similar cellular processes, including proliferation, motility, cytoskeleton dynamics, metabolism and signal transduction (Tables S3 and S4). In particular, we identified 386 genes that were upregulated and 303 genes that were downregulated by depletion of either Pin1 or mutant p53, suggesting that a group of genes may be regulated by the concerted action of these two proteins (Figure 4A). The effect of Pin1 and mutant p53 depletion on the expression of selected genes was confirmed by qRT-PCR (Figure S4B).

We hypothesized that if the transcriptional program induced by mutant p53 and Pin1 contained genes relevant for tumor aggressiveness, it could influence breast cancer prognosis. To test this idea, we analyzed primary tumors for associations between the expression of genes induced by Pin1 and mutant p53 (i.e., genes downregulated in our profiling experiments) and disease prognosis, using four independent data sets of breast cancer (Desmedt et al., 2007; Miller et al., 2005; Pawitan et al., 2005; Sotiriou et al., 2006), which collectively consist of >800 patients. Among

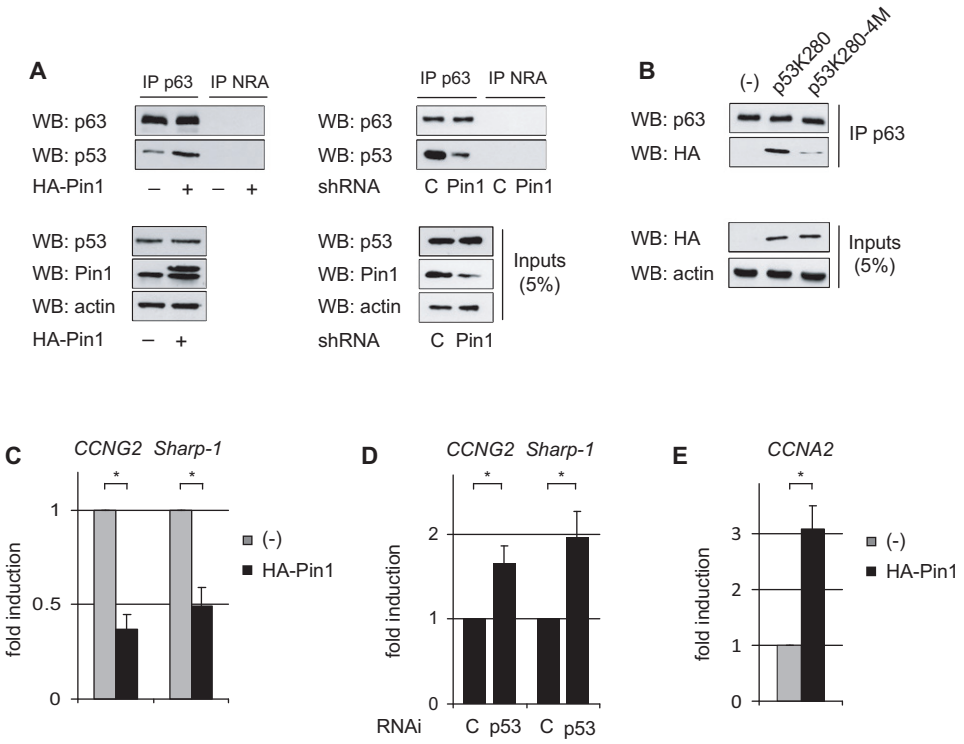


Figure 3. Pin1 Impacts Mutant p53 Transcriptional Regulation

(A) Left: CoIP of endogenous p63 and mutant p53 from MDA-MB-231 cells transduced with HA-Pin1 (+) retroviral construct or empty vector (–). Right: CoIP of endogenous p63 and mutant p53 from MDA-MB-231 cells transduced with either a retroviral construct expressing an shRNA targeting Pin1 or a control sequence (C). NRA, not related antibody. Protein inputs (5% of lysate) are shown below.

(B) CoIP of endogenous p63 and ectopically expressed HA-p53K280 or HA-p53K280-4M in MDA-MB-231 cells by transducing the indicated retroviral constructs. As a negative control, empty vector (–) was used. Protein inputs (5% of lysate) are shown below.

(C) qRT-PCR of *CCNG2* and *Sharp-1* mRNA levels in MDA-MB-231 cells on transduction with empty vector (–) or HA-Pin1 retroviral construct.

(D) qRT-PCR of *CCNG2* and *Sharp-1* mRNA levels in MDA-MB-231 cells transduced with HA-Pin1 retroviral construct on transfection of either p53 or control (C) siRNAs.

(E) qRT-PCR of *CCNA2* mRNA levels in MDA-MB-231 cells on transduction with either empty vector (–) or HA-Pin1 retroviral construct. (C–E) Error bars indicate SD (n = 3, *p < 0.001, two-tailed t test).

See also Figure S3.

the 303 downregulated genes, we selected the top repressed ones in both conditions of silencing (31 genes). The Desmedt data set (Desmedt et al., 2007) was used as a training set to build a gene rank based on clinical data correlation (Table S5) and we selected the 10 top scoring genes to define a Pin1/mutant p53 signature (Table S6). Kaplan-Meier survival analysis showed that expression of these genes significantly correlated with clinical outcome: patients expressing high levels displayed a shorter time to distant metastasis (TDM) and a reduced overall survival (OS) (Figure 4B; Figures S4C–S4F).

We next investigated whether these ten genes are regulated by the concerted action of Pin1 and mutant p53. As shown in Figure 4C, in MDA-MB-231 cells depleted of endogenous mutant p53, reintroduction of p53K280, but not of p53K280-4M, induced the expression of all these genes. More important, ectopic expression of Pin1 together with p53K280 strongly enhanced their transcription. On the contrary, when Pin1 was overexpressed alone or together with p53K280-4M, the expression of these genes was not affected (Figure 4C).

To dissect the mechanisms by which the concerted action of Pin1 and mutant p53 enhances transcription, chromatin immunoprecipitation experiments (ChIP) were performed in MDA-MB-231 cells. As shown in Figure 4D and Figure S4G, mutant p53 is specifically recruited on the promoters of all the Pin1/mutant p53 signature genes, whereas on Pin1 depletion its recruitment was reduced by ~2-fold. On the other hand, Pin1 is also present on these promoters, but only in a mutant p53 dependent manner (Figure 4E; Figure S4H). Of note, neither mutant p53 nor Pin1 were recruited to nonspecific regions on these promoters. Our data suggest that these genes are direct targets for transcriptional induction by the Pin1/mutant p53 axis and that Pin1 could be required for the proper interaction of mutant p53 with functional sites on chromatin. Inspection of these promoter regions revealed the presence of consensus binding sites for transcription factors known to interact with mutant p53, such as Ets-1, NF-Y, Sp1, and VDR (Brosh and Rotter, 2009) (data not shown). The majority of these genes appeared to be regulated by Pin1 and mutant p53 also in MDA-MB-468 cells that express endogenous p53H273 (Figure S4I), indicating that Pin1 could also affect the transcriptional activity of other p53 mutants. Importantly, induction of these genes by Pin1 and mutant p53 did not rely on p63, because their

expression was unaffected by p63 knockdown. An exception to this behavior was found for *DEPDC1*, whose expression was reduced on p63 downregulation (Figure S4J).

To investigate the functional roles of these 10 genes as mediators of Pin1/mutant p53-dependent cell migration, we analyzed the effect of their silencing in MDA-MB-231 cells. We identified six genes that, when depleted, reduced migration without affecting cell viability (Figure 5A; Figure S5A and Table S7). Interestingly *DEPDC1*, which was reported to be involved in proliferation of bladder cancer cells and to be overexpressed in breast cancer (Kanehira et al., 2007; Sparano et al., 2009), showed the strongest effect and had an impact also on invasiveness (Figure 5B; Figure S5B). Moreover, when overexpressed in MDA-MB-231 cells, DEPDC1 enhanced migration and, importantly, it was able to partially rescue the phenotype caused by mutant p53 depletion (Figure 5C; Figure S5C). We also analyzed whether genes downregulated by the Pin1/mutant p53 axis (Figure S4B) may interfere with this pro-migration phenotype. Indeed, when Dicer was overexpressed in MDA-MB-231 cells, it was able to overcome Pin1 induced cell migration (Figure 5D; Figure S5D).

Collectively, these results show that Pin1 acts in concert with mutant p53 to reprogram gene expression in tumor cells, regulating a transcriptional program that entails on one hand the activation of a previously unknown set of mutant p53 direct target genes relevant for migration and invasion and on the other the repression of negative regulators of these processes.

In confirmation of this, simultaneous depletion of either mutant p53 or Pin1 along with *Sharp-1* or *CCNG2* exerted only a slight increase of cell migration in our experimental conditions (data not shown), as compared to previously published results, where depletion of these genes was shown to control this phenotype in a TGF-β dependent way (Adorno et al., 2009).

Pin1 Overexpression and p53 Mutation Modulate Clinical Outcome in Breast Cancer

We next investigated the association of Pin1 expression and p53 mutation status with clinical outcome in breast cancer. Quantitative immunohistochemistry (IHC) was used to analyze Pin1 and p53 protein expression in primary breast carcinomas (data not shown). The status of p53 was analyzed by direct sequencing, and only tumors bearing either wild-type p53 or missense p53 mutations were included in this study, resulting in a series of 212 cases. Pin1 was overexpressed in 144/212 (68%) cases whereas missense mutations in *TP53* were detected in 46/212 (22%) cases (Table S8). Overall survival (OS) was not associated with Pin1 overexpression (Figure S6A). However, consistent with previous observations (Langerød et al., 2007; Olivier et al., 2006), OS was decreased in cases with missense p53 mutations (Figure S6B). Remarkably, when we determined OS as a function of the combination between Pin1 expression and presence of p53 missense mutations, we found that it was significantly decreased in patients with tumors expressing high levels of Pin1 (high Pin1) and mutant p53, compared to low Pin1 expression (low Pin1) and mutant p53, or wild-type p53 cases (Figure 6A).

Moreover, multivariate Cox proportional hazards analysis showed that the combination of Pin1 overexpression and p53 mutation behaved as an independent predictor of poor clinical outcome (Figure 6B). The dependence of the prognostic value of p53 mutation on Pin1 expression levels is further highlighted by the observation that p53 mutation correlates with shorter OS only in cases with high Pin1 expression levels (Figures 6C and 6D). Of note, high Pin1 expression levels and p53 missense mutation were associated with poor outcome in patients treated with anthracycline-based adjuvant chemotherapy. Also in this subgroup the combination of Pin1 overexpression and p53 mutation behaved as an independent prognostic factor (Figure S6C).

DISCUSSION

Increasing evidence has demonstrated that missense mutations in the *TP53* tumor suppressor gene are associated with tumor progression and metastasis. It is only recently that the mechanistic aspects of the acquisition of malignant phenotypes by mutant p53 are being unveiled. Indeed, studies performed in mutant p53 knockin mice have underscored the requirement of an oncogenic context to activate mutant p53 function (Lang et al., 2004; Olive et al., 2004; Terzian et al., 2008). Here, we provide molecular insights into the causal link between oncogenic signaling and the acquisition of a tumor promoting behavior by mutant p53. Indeed, we clearly demonstrated both in mice and in human cancer cells, that oncogenic signaling, as mimicked by H-Ras^{V12} overexpression, causes phosphorylation of mutant p53 on critical sites that are recognized by the phosphorylation-dependent prolyl isomerase Pin1. Only on Pin1 mediated isomerization mutant p53 is enabled to fully exert its malignant activities. Indeed, lack of Pin1 hampers mutant p53 dependent tumorigenesis, strongly suggesting a critical role for this enzyme in transducing oncogenic signaling to activate mutant p53 gain of function.

The contribution of Pin1 to mutant p53 activities is further emphasized by our data from H-Ras^{V12} transduced *p53$^{M/M}$* MEFs, where enhanced tumorigenicity was strictly dependent on Pin1. Our results suggest that the in vivo cooperation between Ras and mutant p53 described in mouse models of malignant skin and pancreatic carcinomas (Caulin et al., 2007; Hingorani et al., 2005), that leads to increased genomic instability and metastasis, could also depend on Pin1.

As observed in H-Ras^{V12} transformed MEFs, also in human breast cancer cells we have shown that aberrant signaling causes phosphorylation of mutant p53 on critical S/T-P sites, which promotes its recognition by Pin1 as a necessary step to unleash mutant p53 dependent migration and invasion. These phenotypes rely in part on inhibition of the metastasis suppressor p63 by mutant p53 that has been shown to act as a molecular switch for TGF-β triggered metastasis (Adorno et al., 2009). We have found that Pin1 potentiates the complex formed by Smad2, p63, and mutant p53 suggesting that this isomerase could function downstream of TGF-β signaling, contributing to downregulate p63 antimetastatic target genes. Notably, Pin1 also interacts directly with p63 and enhances p63 transcriptional activity in the absence of mutant p53. Thus Pin1 may promote p63 tumor suppressive functions, similarly to what occurs with the other members of the p53 family (Zacchi et al., 2002; Mantovani et al., 2004). However, in the presence of mutant p53 Pin1 contributes to the inhibition of p63 functions by enhancing p63 interaction with mutant p53. In this condition,

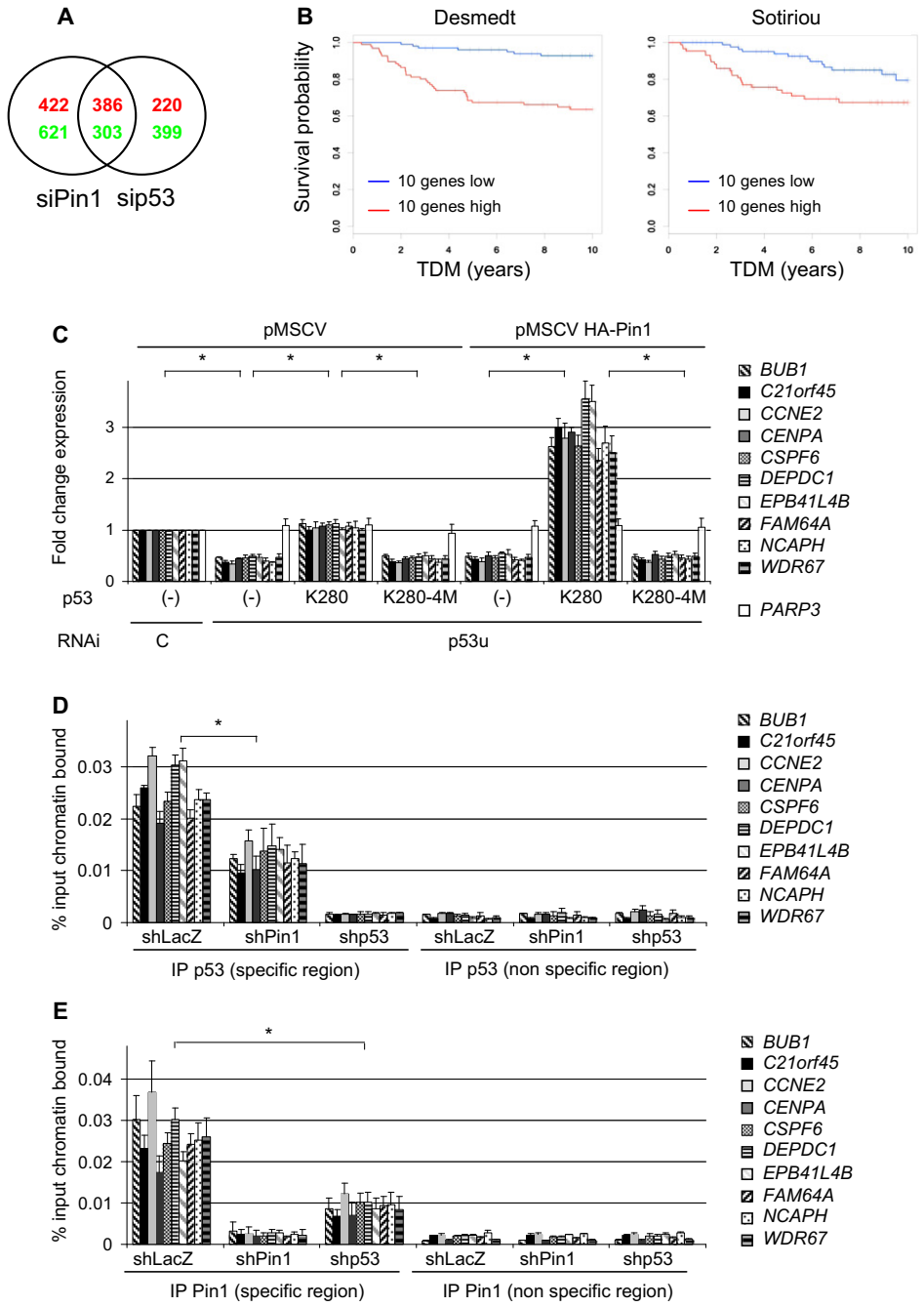


Figure 4. Pin1 and Mutant p53 Reprogram Gene Expression

(A) Venn diagram showing the overlap between genes perturbed by depletion of either Pin1 (siPin1) or mutant p53 (sip53) in MDA-MB-231 cells, red: upregulated genes, green: downregulated genes.

(B) Kaplan-Meier survival curves of time to distant metastasis (TDM) of breast cancer patients classified according to the expression of the Pin1/mutant p53 signature. Red line: cases with high expression of the 10 genes, blue line: cases with low expression of the 10 genes. Left: Desmedt data set (Desmedt et al., 2007) (χ^2 = 25.6, p = 4.1 × 10^{-7}, n = 198, log rank test). Right: Sotiriou data set (Sotiriou et al., 2006) (χ^2 = 7.2, p = 0.0074, n = 179, log rank test).

(C) Bar graph showing fold change in expression of the Pin1/mutant p53 signature genes or an unaffected control gene (*PARP3*) analyzed by qRT-PCR in MDA-MB-231 cells on cotransduction of pMSCV or pMSCV HA-Pin1 with the indicated p53 mutants or empty vector (–) as a negative control. Endogenous mutant p53 was depleted as in Figure 2E. (n = 3, *p < 0.001 comparing the expression of each gene from the Pin1/mutant p53 signature between the indicated conditions, two-tailed t test).

(D) ChIP analysis of mutant p53-bound chromatin (IP p53) from MDA-MB-231 cells on transduction with shRNA vectors targeting endogenous Pin1 (shPin1), mutant p53 (shp53), or LacZ as a negative control (shLacZ). Chromatin was immunoprecipitated with DO1 antibody or mouse purified IgGs as negative control (see Figure S4G). Promoter occupancy was analyzed by qRT-PCR and calculated as percentage of input chromatin. (n = 3, *p < 0.013 comparing promoter occupancy for each gene from the Pin1/mutant p53 signature between the indicated conditions, two-tailed t test).

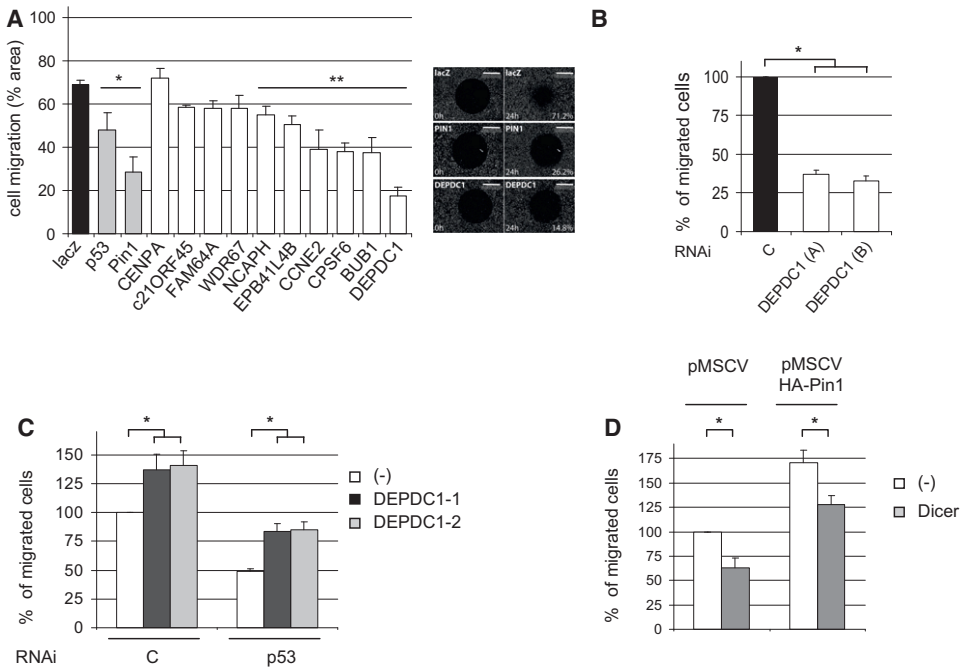


Figure 5. Pin1/Mutant p53 Target Genes Affect Cell Migration and Invasion

(A) Wound healing-based siRNA analysis. Left: bar graph showing the percentage of well surface covered by migrating cells 24 hr on silencing of the indicated genes. Error bars indicate SD (n = 4, ***p < 0.001 relative to LacZ siRNA, two-tailed t test). Right: representative images of the assay. Scale bar represents 1 mm.

(B) Transwell migration assays of MDA-MB-231 cells on *DEPDC1* depletion using two different siRNA sequences (A and B). Error bars indicate SD (n = 3, *p < 0.001). See Figure S5B for qRT-PCR analysis of *DEPDC1* depletion.

(C) Transwell migration assays performed in MDA-MB-231 cells transfected with vectors expressing two DEPDC1 isoforms (DEPDC1-1 and DEPDC1-2) on control (C) or mutant p53 siRNA transfection. As a negative control empty vector was used (−). Error bars indicate SD (n = 3, *p < 0.01). See Figure S5C for a western blot analysis of cell lysates.

(D) Transwell migration assays performed in MDA-MB-231 cells transfected with pcDNA3 Dicer or empty vector (−) as a control, on transduction with retroviral constructs pMSCV HA-Pin1 or empty vector. Error bars indicate SD (n = 3, *p < 0.005). See Figure S5D for western blot analysis of cell lysates.

See also Figure S5 and Table S7.

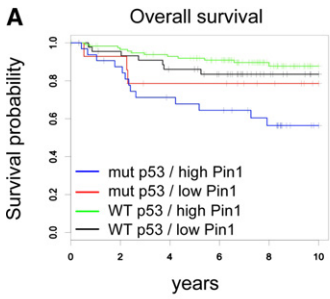
besides inhibiting p63 target genes such as *CCNG2*, *Sharp-1*, and *Dicer*, we have shown that Pin1 and mutant p53 also repress the expression of several other genes known to counteract tumor formation and progression like *TIMP3* (Anania et al., 2011; Song et al., 2010), *CAV1* (Bouras et al., 2004), and *HPGD* (Wolf et al., 2006).

In addition to the repression of the above mentioned genes, we have demonstrated that the Pin1/mutant p53 axis must also induce a specific set of genes to fully establish malignant phenotypes (Figure 7). Among this group we identified 10 mutant p53 direct target genes, as evidenced by our ChIP experiments. The regions analyzed on these promoters contain consensus binding sites for transcription factors previously shown to tether mutant p53 on cognate promoters and to contribute to mutant p53 gain of function, such as Ets-1, NF-Y, Sp1, and VDR (Brosh and Rotter, 2009). The evidence that Pin1 depletion hampers mutant p53 recruitment on chromatin suggests that Pin1 could be required for the proper interaction of mutant p53 with these transcriptional partners on specific gene promoters. Nine of these ten genes were unaffected by p63 downregulation in our cellular system, indicating that the Pin1/mutant p53 axis promotes aggressiveness by at least two independent but complementary mechanisms. A notable exception was *DEPDC1*, whose expression was partially reduced in this condition. *In silico* inspection revealed the presence of a p63 binding site at −27 kb from *DEPDC1* transcription start site. We speculate that p63 may positively regulate *DEPDC1* expression, possibly through still uncharacterized mechanisms that could involve its interaction with mutant p53.

The relevance of these 10 genes (Pin1/mutant p53 signature) for the metastatic phenotype was supported by their activity as key effectors of mutant p53-dependent migration and invasion and by their significant correlation with clinical outcome found in four independent breast cancer data sets. Interestingly, three of these genes, *CCNE2*, *BUB1*, and *CENPA* are present in a gene signature predictive of early metastasis in breast cancer (van't

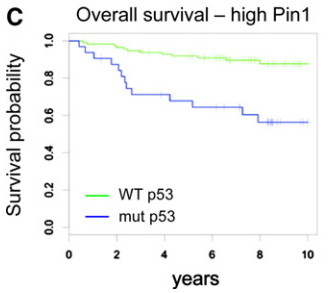
(E) ChIP analysis of Pin1-bound chromatin (IP Pin1) from MDA-MB-231 cells on transduction with shRNA vectors targeting endogenous Pin1 (shPin1), mutant p53 (shp53), or lacZ as a negative control (shLacZ). Chromatin was immunoprecipitated with Pin1 polyclonal antibody or rabbit purified IgGs as negative control (see Figure S4H). Promoter occupancy was analyzed as in (D). (D and E) As a control of specificity, a promoter region not bound by mutant p53 nor Pin1 was analyzed (nonspecific region). (C–E) Error bars indicate SD (n = 3, two-tailed t test).

See also Figure S4 and Tables S3–S6.



B Cox regression analysis

	Hazard Ratio	p-Value	lower .95	upper .95
Tumor size	1.022	0.030	1.002	1.042
Tumor grade	1.498	0.001	1.201	1.869
Node	1.085	0.003	1.027	1.146
ER negative status	1.905	0.248	0.639	5.680
HER2 negative status	0.910	0.833	0.379	2.187
PGR negative status	1.985	0.210	0.679	5.804
Age	1.000	0.981	0.975	1.025
High Pin1	0.620	0.327	0.238	1.613
p53 mutation	0.568	0.451	0.130	2.474
High Pin1 and p53 mutation	5.679	0.038	1.104	29.200



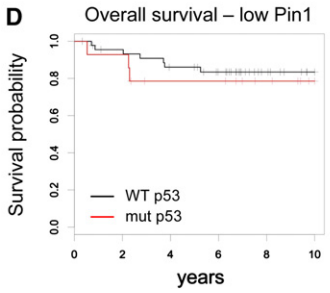


Figure 6. High Pin1 Levels Influence the Prognostic Value of p53 Missense Mutation in Breast Cancer

(A) Kaplan-Meier survival curves of breast cancer patients (observation threshold 10 years, clinical variable = OS) as a function of the combination of Pin1 overexpression and presence of p53 missense mutations. Low Pin1, tumors not overexpressing Pin1; high Pin1, tumors overexpressing Pin1 ($\chi^2 = 17.2$, $p < 10^{-4}$, n = 212, log rank test).

(B) Multivariate analysis of risk of death. Multivariate analysis was performed on the same cohort of patients analyzed in (A) using Cox proportional hazards regression modeling. The relationships between survival and high Pin1 levels and *TP53* missense mutation or other clinical variables used in the clinical practice are shown.

(C) Kaplan-Meier survival curves of breast cancer patients with high expression of Pin1 (observation threshold 10 years, clinical variable = OS) as a function of the presence of missense mutant or wild-type p53 ($\chi^2 = 14.1$, $p < 10^{-4}$, n = 143, log rank test).

(D) Kaplan-Meier survival curves of breast cancer patients with low expression of Pin1 (observation threshold 10 years, clinical variable = OS) as a function of the presence of missense mutant or wild-type p53 ($\chi^2 = 0.4$, p = 0.548, n = 69, log rank test).

See also Figure S6 and Table S8.

Veer et al., 2002). In addition, *C21orf45* was shown to sustain tumor cell addiction to oncogenic K-Ras (Luo et al., 2009), whereas *CPSF6* was found both upregulated by Snail in human colon cancer and fused to *FGFR1* in hematological malignancies (Hidalgo-Curtis et al., 2008; Larriba et al., 2010).

A further demonstration of the clinical relevance of the Pin1/mutant p53 axis was provided by our results in a cohort of breast cancer patients. This analysis revealed that the prognostic value of p53 mutation was strongly improved by combining it with quantification of Pin1 protein levels. Studies including larger numbers of cases are required to validate this combination as a prognostic and potentially predictive tool. Nevertheless, multivariate analysis showed that concomitant high Pin1 expression and p53 mutation behaved as an independent and strong predictor of clinical outcome. Indeed, in tumors overexpressing Pin1, p53 status allowed a better stratification of patients into groups of long- or short-term overall survival and this was also observed among patients who received anthracycline-based adjuvant chemotherapy.

Our data suggest that patients bearing high Pin1 levels and mutant p53 could benefit from inhibition of the Pin1/mutant p53 axis. Proposed strategies based on inactivation of mutant p53 are represented by small molecules that reactivate wild-type like functions (Selivanova and Wiman, 2007), or peptide aptamers that kill cancer cells expressing mutant p53 (Guida et al., 2008). By dissecting upstream and downstream molecular mechanisms that impinge on mutant p53 function we identified additional potential targets for clinical intervention such as Pin1 or the kinases acting on mutant p53, as well as downstream effectors of the Pin1/mutant p53 axis.

Mutations in *TP53* are frequent in basal-like breast cancer (Langerød et al., 2007), a subgroup of tumors consisting mostly of estrogen receptor-, progesterone receptor-, and Her2-negative (triple-negative) immunohistochemical phenotype, that have a higher risk of recurrence and whose management still represents a clinical challenge. Some of the Pin1/mutant p53 signature genes (e.g., *BUB1*, *CENPA*, and *DEPDC1*), that we found modulated in two triple negative breast cancer cell lines, are also overexpressed in triple negative breast cancers (Sparano et al., 2009). Therefore our findings carry therapeutic implications for this subtype of breast cancer and potentially also for other epithelial cancers bearing mutant p53 and high levels of Pin1.

EXPERIMENTAL PROCEDURES

Additional methods can be found in the Supplemental Experimental Procedures.

Mice Strains and Analysis

Mouse cohorts were generated by mating $p53^{M/+}$ or $p53^{+/-}$ with $Pin1^{+/-}$ mice maintained on a C57BL/6 background and genotyping was performed by

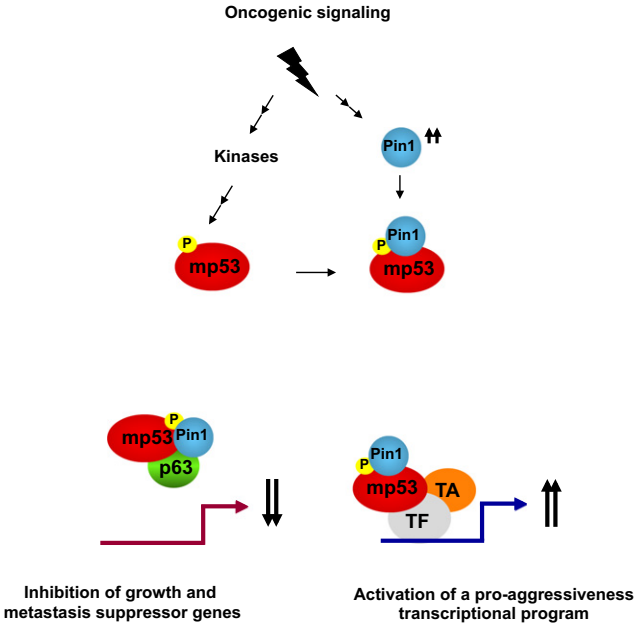


Figure 7. Schematic Representation Summarizing the Proposed Mechanism of the Concerted Action of Pin1 and Mutant p53 in the Promotion of an Aggressive Tumor Phenotype

P, phosphate group; TA, transcriptional activator; TF, transcription factor.

polymerase chain reaction (PCR) analysis as described (Atchison et al., 2003; Lang et al., 2004). Animals showing signs of illness or evident tumor burden were sacrificed and organs were fixed in 10% formalin/PBS for 24 hr. Tissues were embedded in paraffin, sectioned at 5 μm, dewaxed, and stained with hematoxylin and eosin (H&E) before pathological analysis. Procedures involving animals conformed to institutional guidelines that comply with international laws and policies (UKCCCR, 1989). All experimental protocols were approved by the Animal Ethics Committee of the athenaeum of the University of Trieste.

Transformation Assays and In Vivo Tumorigenicity

Low-passage MEFs infected with pLPC H-Ras^{V12} or empty vector were resuspended in DMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 IU/ml), containing 0.3% agarose and plated on top of 1% agarose in the same medium, at a cell density of 1×10^5 for each 60-mm plate. After 14 days in culture, colonies reaching at least 100 μm were scored. Experiments were performed in triplicate using at least two different clones for each genotype. To assess tumorigenicity of H-Ras^{V12}, infected MEFs 1×10^6 cells were injected subcutaneously on both sides in NOD SCID mice and after 2 weeks tumor volume was determined by caliper measurements as described (Rustighi et al., 2009). Procedures involving animals and their care were in conformity with institutional guidelines (D.L. 116/92 and subsequent complementing circulars) and all experimental protocols were approved by the Animal Ethics Committee of the athenaeum of the University of Trieste.

Migration and Invasion Assays

For wound healing assays, transfected cells were plated on 35-mm plates and cultured to 90% confluence. Cells were scraped with a 200 μl tip and wound closure was followed for 36 hr. For transwell migration assays 24-well PET inserts were used (8.0 μm pore size, Falcon). Invasion assays were performed in 24-well PET inserts (8.0 μm pore size, Falcon) with matrigel-coated filters.

In Vivo Metastasis Assays

For lung colonization assays MDA-MB-231cells were cotransduced with a lentiviral vector coding for the Firefly Luciferase reporter gene and pSR shLacZ or pSR shPin1 (six animals per group). 1×10^6 cells were resuspended in 100 μl of PBS and inoculated in the tail vein of SCID mice. For in vivo imaging bioluminescence images were acquired at several time points after cell injection using a cooled charge-coupled device camera mounted on a light-tight specimen box (IVIS Lumina II Imaging System; Caliper Life Sciences, Alameda, CA). Ten minutes before imaging, anesthetized animals were administered intraperitoneally with 150 mg/kg D-luciferin (Caliper Life Sciences) in PBS. Histological analysis was carried out 1 month after MDA-MB-231 cells injection on paraffin embedded lungs by H&E staining. For computer-aided assessment of lung tissue, the area occupied by metastatic foci, identifiable in sections of lung lobes, were calculated using a Leica DM200 microscope equipped with a Leica DFC295 digital color camera and the software Leica Application Suite (LAS) V3, summed, and total value was finally compared to the whole area of the lung lobe. IHC detection of Pin1 expression was carried out using Pin1 monoclonal antibody (see Supplemental Experimental Procedures). Procedures involving animals and their care were in conformity with institutional guidelines (D.L. 116/92 and subsequent complementing circulars) and all experimental protocols were approved by the ethical Committee of the University of Padua (CEASA).

Gene Expression Analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and subjected to DNase-I (Ambion) treatment. For microarray analysis three biological mRNA replicates for each group (siPin1 or sip53) were hybridized on Affymetrix GeneChip Human Genome U133A 2.0 array. For quantitative RT-PCR mRNA was transcribed using Superscript III (Invitrogen). Real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) and StepOne Plus cycler (Applied Biosystems). Primer sequences are shown in the Supplemental Experimental Procedures.

Survival Analysis for Gene Rankings

Cox proportional hazards model was fitted to test the association between gene expression of 31 common top downregulated genes and survival (Desmedt data set GSE7390). From the clinical associated rank, we selected genes with score >3, obtaining in this way the 10-gene signature. To verify the correlation of the 10-gene signature and breast cancer clinical data, a Mantel-Haenszel test was applied to the other normalized data sets we used (GSE2990, GSE3494, GSE1456) (survival R package).

Chromatin Immunoprecipitation

Chromatin was immunoprecipitated with p53 DO1 (Santa Cruz) or polyclonal Pin1 antibody (Zacchi et al., 2002). As negative controls IgGs purified from rabbit or mouse serum were used. For a detailed protocol see Supplemental Experimental Procedures. Coimmunoprecipitated DNA was analyzed by real-time PCR on a StepOne Plus cycler (Applied Biosystems), using SYBR Green Universal PCR Master Mix (Applied Biosystems). Promoter occupancy was calculated as percent of input chromatin immunoprecipitated using the $2^{-\Delta Ct}$ method. Primer sequences are shown in the Supplemental Experimental Procedures.

Tissue Microarray Survival Data Analysis

The tissue microarray (TMA) used in this study consisted only of historical breast cancer samples, donated from each of 212 primary previously untreated and otherwise unselected tumors. The results generated were exported via Aperio Web Services. Overall survival was calculated from date of treatment start to date of death, or date of censor if alive. Survival curves were constructed using Kaplan-Meier methodology. Log rank tests assessed differences in tumor characteristics. Adjuvant chemotherapy typically consisted on anthracycline-based regimens. Breast cancer samples were collected and formed into a TMA by the Tayside Tissue Bank (http://www.tissuebank.dundee.ac.uk) under delegated ethical authority of the Tayside Local Research Ethics Committee with written informed consent from contributing patients.

Multivariate Analysis Using a Cox Proportional Hazards Model

The analysis of the death risk for the 212 tumors from the TMA was conducted using Cox proportional hazards regression modeling. We examined the relationship between overall survival and the presence of *TP53* missense mutation and Pin1 levels and other predictors commonly used in the clinical practice,

including tumor size, estrogen receptor status (ER status), progesterone receptor status (PGR status), nodal status, HER2 status, node infiltration, and tumor grade. We fitted Cox proportional hazards regression model first by using clinical variables only and then adding *TP53* missense mutation and Pin1 level variables. Clinical parameters were treated as follows:

Tumor size: continuous variable;
Tumor grade: grade 1, grade 2, grade 3;
Node infiltration: negative if $\leq$4 (0), positive >4 (1);
ER status: negative if $\leq$4 (1), positive >4 (0);
PGR status: negative if $\leq$4 (1), positive > 4 (0)
HER2 status: positive staining or negative staining (0, 1); and
Pin1 status: positive nuclear staining of Pin1 >5 (1), negative if $\leq$5 (0).

ACCESSION NUMBERS

The accession number in the Gene Expression Omnibus public database for the MDA-MB-231 expression array experiment is GSE26262.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, eight tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.06.004.

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