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Gain of oncogenic function of p53 mutants regulates E-cadherin expression uncoupled from cell invasion in colon cancer cells

Lauréline Roger, Laurent Jullien, Véronique Gire* and Pierre Roux*‡

Universités Montpellier 2 et 1, CRBM, CNRS UMR 5237, 1919, route de Mende, 34293 Montpellier, France

*These authors contributed equally to this work

‡Author for correspondence (pierre.roux@crbm.cnrs.fr)

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Summary

Mutations in the p53 tumour suppressor gene are associated clinically with tumour progression and metastasis. Downregulation of the E-cadherin cell-cell adhesion molecule is a key event for epithelial to mesenchymal transition (EMT) in tumour progression. Here, we show that wild-type p53 induced to adopt a mutant conformation, and hot-spot p53 mutants, which are both transcriptionally inactive, downregulate E-cadherin expression in the colon carcinoma cell line HCT116. Downregulation of E-cadherin occurred concomitantly with the upregulation of Slug and Zeb-1, transcriptional factors known to repress E-cadherin gene expression. In addition, knockdown of Slug and Zeb-1 expression diminished p53-mediated E-cadherin repression. Knocking down endogenous mutant p53 in MDA-MB-231 and SW620 cancer cell lines lacking E-cadherin protein restored the expression of E-cadherin. Complete loss of E-cadherin expression in HCT116 cells induced morphological alterations along with upregulation of vimentin, a mesenchymal marker. These changes characteristic of the EMT phenotype were, however, not sufficient to confer invasiveness in a three-dimensional matrix. Downregulation of E-cadherin by mutant p53 was not required to promote the invasive phenotype induced by inactivation of p53. These findings indicate that independent control of E-cadherin expression and cell motility could be essential molecular events in p53 mutant-induced invasive phenotypes.

Key words: p53, E-cadherin, Epithelial-to-mesenchymal transition, Invasion, Metastasis

Introduction

Carcinomas are the most frequent type of malignancies in human, and occurrence of metastases due to tumour progression causes the vast majority of cancer-related deaths. Metastatic progression is a multi-step process that includes detachment of cancer cells from the primary tumour mass, migration and invasion, to enable re-establishment of malignant cells at distant sites. In the process of epithelial-mesenchymal transition (EMT), besides loss of adhesive constraints, cancer cells employ developmental process to gain migratory and invasive properties through disruption of cell-cell junctions and extensive reorganization of the actin cytoskeleton (Thiery and Sleeman, 2006; Yilmaz and Christofori, 2009). However, the molecular bases by which tumour cells acquire malignant properties have not yet been completely elucidated.

Malignancy of carcinoma cells is characterized by the loss of both cell-cell adhesion and cellular differentiation, and this has repeatedly been reported to correlate with E-cadherin downregulation. Loss of E-cadherin could be attributed to somatic mutations in some tumour types (Guilford et al., 1998), promoter hypermethylation (Grady et al., 2000) or the action of transcriptional repressors, such as Slug, Snail, Twist1 and Zeb-1 (Cano et al., 2000; Hajra et al., 2002; Postigo and Dean, 1997; Yang et al., 2004). Downregulation of E-cadherin is associated with the development of invasive carcinoma, metastatic dissemination, and poor clinical prognosis (Perl et al., 1998; Vlemingx et al., 1991). Therefore, deregulation of E-cadherin expression may contribute to tumourigenesis.

It has been proposed that genes that confer metastatic ability might be the same genes that, upon mutation promote cell-cycle deregulation leading to the initiation of epithelial carcinogenesis (Bernards and Weinberg, 2002; Roger et al., 2006). These genes include the well-characterized tumour suppressor, *TP53*. The tumour suppression function of p53 relies on its ability to act as a potent sequence-specific transcriptional activator, regulating a program of gene expression that leads to cell cycle arrest, DNA repair and apoptosis (Vousden and Lu, 2002). Among the genes induced by p53 is the gene for the cyclin-dependent kinase inhibitor, p21WAF1 (p21), which blocks cell division, and many genes encoding pro-apoptotic proteins. In addition, p53 controls its own activity via transactivation of E3 ubiquitin-protein ligase Mdm2 (known as Hdm2 in humans), which mediates the ubiquitylation and proteasomal degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Mutation of *TP53* occur in over 50% of human cancers, and epidemiology data show that mutant p53-expressing tumours are aggressive and associated with poor prognosis (Soussi and Beroud, 2001). Most *TP53* alterations are missense mutations within the DNA-binding domain that lead to the synthesis of a stable but transcriptionally inactive p53 protein (Oren, 2003). *TP53* mutations also affect the conformation equilibrium of p53 to varying extents. Changes in p53 conformation can be monitored by reactivity to conformation-specific antibodies that recognize p53 in either a wild-type (pAb1620) or mutant (pAb240) conformation (Milner et al., 1987; Ory et al., 1994). p53 inactivation renders cells non-responsive to signals that challenge genomic integrity, thereby promoting the acquisition of novel phenotypes that are characteristic for cancer

cells such as resistance to apoptosis, neoangiogenesis (Dameron et al., 1994; Teodoro et al., 2007; Ueba et al., 1994), enhanced proliferative and invasive potential (Gadea et al., 2007; Gadea et al., 2002; Roger et al., 2006). Remarkably, several p53 mutants may acquire novel oncogenic function that actually promote cancer progression by specifically regulating cancer invasion (Kastan and Berkovich, 2007). Indeed, tumours emerging from mutant-p53 knock-in mice display aggressive and metastatic traits that are never detected in tumours developing in a p53-deficient mice (Lang et al., 2004; Olive et al., 2004). In addition, *TP53* mutations and p53 protein accumulation in gastric carcinomas correlated with decreased E-cadherin expression (Fricke et al., 2003).

Since both E-cadherin and p53 modulate malignant progression and metastasis formation, we examined whether disruption of p53 wild-type conformation and expression of p53 mutants could downregulate E-cadherin expression in non-invasive colon cancer cells, and if so, whether this alteration is able to enhance the aggressive phenotypes of these cells. Our results indicate that p53 plays an important role in the maintenance of the epithelial phenotype by regulating independently the expression of E-cadherin and cell invasion.

Results

Accumulation of p53 in the absence of p21 leads to EMT-like changes

To assess the contribution of endogenous p53 in the regulation of E-cadherin expression, we used HCT116 colon carcinoma cell lines with different p53 levels. In HCT116-p21^{-/-} cells, which display high p53 levels owing to the deletion of *p21WAF1* (also known as *CDKN1A*) (Javelaud and Besancon, 2002), accumulation of p53 coincided with a dramatic reduction of E-cadherin levels (Fig. 1A). Accumulation of p53 in wild-type HCT116 cells can be achieved by exposure to the DNA damage agent bleomycin that induces p53 stabilization through the activation of kinases that phosphorylate p53 on serine 15, thereby displacing the Mdm2 ubiquitin ligase, a negative regulator of p53 (Vousden and Lu, 2002). Stabilization of p53 resulted in enhanced p53 transcriptional activity, as evidenced by *p21WAF1* induction but did not modify E-cadherin expression (Fig. 1A). Conversely, in HCT116-p21^{-/-} cells, we did not detect changes in p53 phosphorylated at serine 15, suggesting that stimulation of the DNA damage pathway did not account for the enhanced p53 levels and decreased E-cadherin expression observed in these cells (Fig. 1A). Immunostaining analysis clearly confirmed that, in HCT116-p21^{-/-} cells, strong nuclear accumulation of p53 was associated with the disappearance of E-cadherin expression at cell-cell junctions (Fig. 1B). Furthermore, the expression and localization of the cytoplasmic adherens junction protein β -catenin, another epithelial marker, was also affected in HCT116-p21^{-/-} cells (Fig. 1A,B). Loss of epithelial markers, which is characteristic of EMT, is often associated with gain of mesenchymal markers such as vimentin and N-cadherin. Vimentin expression was significantly increased and was detected in the cytoplasm of HCT116-p21^{-/-} cells (Fig. 1A,B). It is important to emphasize that a cadherin switch, i.e. the change from E-cadherin to N-cadherin expression, did not occur in these cells (Fig. 1A). In line with the immunoblotting data (Fig. 1A), HCT116-p21^{-/-} cells showed loss of cell-cell contacts and of the typical epithelial cobblestone-like phenotype, failure to form a confluent monolayer with gaps between the cells and an elongated, spindle shape (Fig. 1B). These phenotypes are reminiscent of the fibroblastoid cells formed during EMT (Thiery and Sleeman, 2006).

A recent report demonstrating that p53 induces Mdm2-mediated Slug degradation, a known repressor of E-cadherin gene expression (Wang et al., 2009); prompted us to test whether inactivation of p53 had an effect on the epithelial phenotype. To this end, we used HCT116-p53^{-/-} cells, in which *TP53* has been deleted by homologous recombination [described by Bunz et al. (Bunz et al., 1998)], and HCT116-shp53 cells, in which p53 expression was knocked-down with a retroviral vector expressing a p53-directed short hairpin RNA (shp53). Shp53 induced a total abolition of p53 and p21 expression (Fig. 1C). We did not observe changes in E-cadherin and β -catenin expression in either HCT116-p53^{-/-} or HCT116-shp53 cells compared with their wild-type or control counterparts (Fig. 1C). Furthermore, vimentin and N-cadherin were undetectable in these cells (Fig. 1C). As expected, HCT116-p53^{-/-} cells exhibited intense E-cadherin and β -catenin expression at cell-cell contacts (Fig. 1B). HCT116-p53^{-/-} cells retained a coherent epithelial morphology similar to the parental HCT116 cells (Fig. 1B). Similar observations were made when using HCT116-shp53 cells (data not shown). Unfortunately, we were unable to test the effect of p21 inactivation alone on E-cadherin expression as we failed to completely knock-down p21 expression in HCT116 cells using the shRNA technology.

These data indicate that p53 inactivation alone is insufficient to modulate expression of E-cadherin in colon carcinoma cells and that there are other mechanisms underlying the silencing of E-cadherin in HCT116 cells. Conversely, accumulation of p53 due to the absence of p21, in epithelial cells causes downregulation of E-cadherin and induces some of the molecular and morphological changes that are characteristic of EMT.

p53-mediated repression of E-cadherin expression is bypassed by p21

The loss of E-cadherin expression we observed in HCT116-p21^{-/-} cells could be due to the concomitant higher p53 proteins levels and absence of p21 (as suggested also by the results obtained with bleomycin). To identify the respective role of p21 and p53 in the regulation of E-cadherin expression and in the mesenchymal phenotype of HCT116-p21^{-/-} cells, we knocked-down p53 or stably re-expressed p21 in these cells. To this aim, we generated, by retroviral infection, stable HCT116-p21^{-/-} clones that express shp53, p21WAF1 or empty vector (pBabe). Western blot analysis indicated that silencing p53 expression in these cells restored E-cadherin and β -catenin expression, with a concomitant robust decrease in vimentin expression (Fig. 2A). Immunofluorescence studies showed that E-cadherin properly localized at cell-cell junctions in HCT116-p21^{-/-} cells expressing shp53 (Fig. 2B). Cells were fractionated to document further the E-cadherin localization. The resulting cytoplasmic and membrane-enriched fractions were subjected to western blot analysis for E-cadherin. Subcellular fractionation revealed that E-cadherin is present mainly at the membrane in HCT116-p21^{-/-} cells expressing shp53 (Fig. 2C). The E-cadherin-expressing cells had often lost their fibroblastoid morphology and spread, with an epithelial appearance characteristic of HCT116 cells (Fig. 2B).

Although we selected stable HCT116-p21^{-/-} clones that expressed the highest levels of p21, the levels of restored p21 in HCT116-p21^{-/-} cells did not exceed those observed in parental cycling HCT116 cells (Fig. 2A). First, we verified that exogenous p21 was functional by confirming that it could bind to cyclin A and Cdk2 (supplementary material Fig. S1A) and that its nuclear distribution was cell-cycle dependent, by dual immunofluorescence using

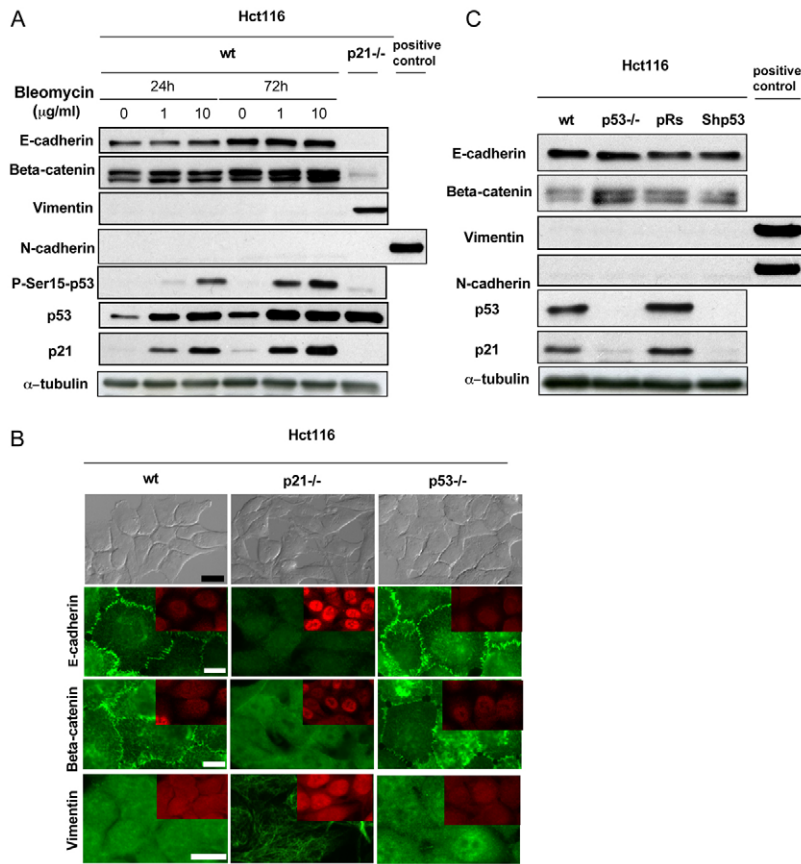


Fig. 1. Accumulation of p53 in the absence of p21 leads to EMT-like changes. (A) Immunoblots of HCT116 cells (wt) treated with bleomycin for the indicated times, and of HCT116-p21^{-/-} (p21^{-/-}) cells. Whole-cell lysates were analysed for expression of E-cadherin, β -catenin, N-cadherin, vimentin, phospho-Ser15 (pSer15)-p53, p53, p21 and α -tubulin (loading control). Film exposure time for p53 was 1 minute. As a positive control for N-cadherin detection, extracts from a rhabdomyosarcoma cell line was used. (B) The upper panels are phase-contrast images to assess the cell morphology of HCT116 (wt), HCT116-p21^{-/-} (p21^{-/-}) and HCT116-p53^{-/-} (p53^{-/-}) cells plated at low density. Scale bar: 100 μ m. The lower panels show immunofluorescence of E-cadherin, β -catenin and vimentin. Insets show immunofluorescence for p53. Scale bars: 20 μ m. (C) Immunoblots of HCT116 (wt), HCT116-p53^{-/-} (p53^{-/-}), and HCT116-shp53 (Shp53) and HCT116 with pRetrosuper vector (pRs) cells. Whole-cell lysates were analysed for expression of E-cadherin, β -catenin, N-cadherin, vimentin, p53, p21 and α -tubulin. Film exposure time for p53 was 5 minutes.

antibodies against cyclin D1 and cyclin A. As expected, exogenous p21 was predominantly localized in the nucleus at G1, as shown by colocalization with cyclin D1, whereas it was virtually absent in S phase (cyclin A-positive cells; supplementary material Fig. S1B). These findings indicate that exogenous p21 expression is cell-cycle regulated and has no effect on HCT116-p21^{-/-} cells proliferation. Re-expression of p21 in HCT116-p21^{-/-} cells was also accompanied by a strong recovery of E-cadherin and β -catenin expression and concomitant loss of vimentin expression (Fig. 2A). In addition, subcellular fractionation and immunofluorescence analyses revealed that E-cadherin accumulated at the plasma membrane and was strongly enriched at sites of cell-cell contact (Fig. 2B,C). Of note, p53 protein remained nuclear in these cells (Fig. 2B).

Loss of E-cadherin expression in cancer cells frequently involves transcriptional repression (Peinado et al., 2004). Analysis of E-cadherin mRNA levels by Q-PCR showed a complete absence of endogenous E-cadherin transcripts in HCT116-p21^{-/-} cells (Fig. 3A), suggesting that E-cadherin gene expression was repressed at the transcriptional level. By contrast, HCT116-p21^{-/-} cells expressing p21 and shp53 strongly expressed E-cadherin transcripts (Fig. 3A). The upregulation (derepression) of E-cadherin gene expression in HCT116-p21^{-/-} cells expressing p21 and shp53 was further analyzed at the promoter level. HCT116-p21^{-/-} cells were transiently transfected with Ecad5-luc (a plasmid in which the luciferase gene is under the control of the E-cadherin proximal regulatory promoter) and increasing amounts of a retroviral vector encoding shp53. Gradual silencing of p53 led to a modest but significant increase of E-cadherin proximal promoter activity compared with control-transfected cells (Fig. 3B). Similarly, transient transfection of

HCT116-p21^{-/-} cells with Ecad5-luc and increasing amounts of pcDNA3-p21WAF1 strongly activated the E-cadherin proximal promoter in a dose-dependent manner (Fig. 3C).

These findings indicate that concomitant accumulation of p53 and absence of p21 are both needed for repression of E-cadherin expression and acquisition of the morphological changes characteristics of EMT observed in HCT116-p21^{-/-} cells.

p53 accumulates in a mutant conformation in the absence of p21

We then wanted to investigate whether the mechanisms by which HCT116-p21^{-/-} cells, with a wild-type TP53, downregulate E-cadherin protein operate at the level of the p53 protein itself. Since the conformation of p53 appears to be important for its biological functions, we hypothesized that changes in the conformational status of p53 could account for its distinct biological activities in HCT116-p21^{-/-} and in HCT116 cells treated with bleomycin.

To this aim, we assessed the conformational status of p53 in HCT116 and HCT116-p21^{-/-} cells by immunoprecipitation using PAb1620 and PAb240, two conformation-specific monoclonal antibodies against p53, which discriminate between the folded and unfolded tertiary structure of p53 (Meplan et al., 2000). Specifically, PAb1620 recognizes an exposed epitope in the wild-type p53 that is competent for DNA binding. By contrast, PAb240 recognizes a cryptic epitope that is competent for DNA binding only when exposed by denaturation. Many p53 mutants are recognized by PAb240 but not by PAb1620, leading to the identification of 'wild-type' (PAb1620+; PAb240-) and 'mutant' (PAb1620-; PAb240+) protein conformation (Meplan et al., 2000). In addition, DO1, an antibody which recognizes an epitope localized in the N-terminal

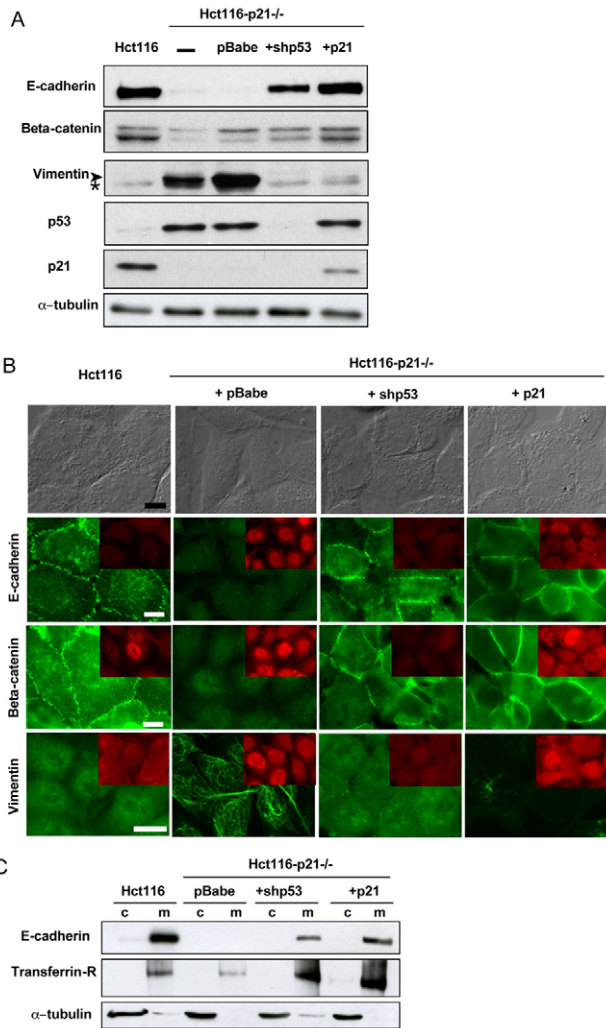


Fig. 2. p21 hinders p53-dependent repression of E-cadherin in HCT116-p21^{-/-} cells. (A) Immunoblots of HCT116 and HCT116-p21^{-/-} cells, HCT116-p21^{-/-} clones stably expressing pRetrosuper-shp53 (+shp53), pBabepuro-p21WAF1 (+p21), and pBabepuro (pBabe) vector alone. These stable clones were generated by retroviral infection. Whole-cell lysates were analysed for their content in E-cadherin, β -catenin, vimentin, p53, p21 and α -tubulin. The asterisk indicates a non-specific band. Film exposure time for p53 was 30 seconds. (B) The upper panels are phase-contrast images of the cell lines described in A. The lower panels are indirect immunofluorescence images showing E-cadherin, β -catenin and vimentin localization. Insets show immunofluorescence for p53. Scale bars: 20 μ m. (C) Localization of E-cadherin protein by subcellular fractionation. Equal amounts of proteins from cytoplasmic (c) and cell membrane (m) fractions of the cell lines described in B were subjected to western blot analysis using E-cadherin antibody. Tubulin was used as a loading control for the cytoplasmic fraction and transferrin receptor (Transferrin-R) was used for the cell membrane fraction.

domain of p53, was used to immunoprecipitate both forms of p53. The relative amount of p53 immunoprecipitated by each antibody was considered as an indication of the conformation of p53.

As expected, DO1 immunoprecipitated p53 in all tested cells lines, whereas PAb1620 and PAb240 clearly did not (Fig. 4A). PAb1620 (wild-type conformation) immunoprecipitated p53 in control HCT116 and bleomycin-treated HCT116 cells and PAb

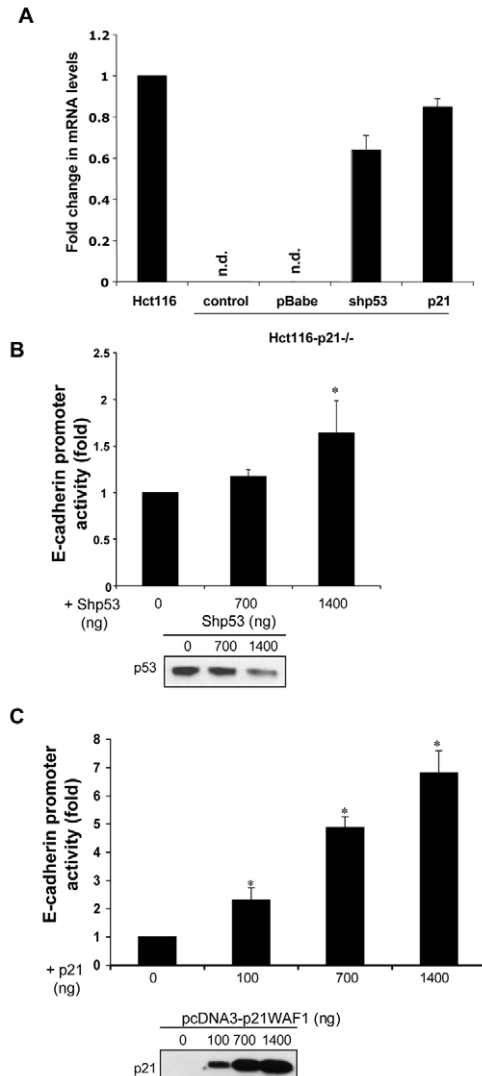


Fig. 3. E-cadherin downregulation in HCT116-p21^{-/-} cells is associated with transcriptional repression. (A) Expression of E-cadherin in HCT116, HCT116-p21^{-/-} cells (control) and HCT116-p21^{-/-} clones stably expressing pRetrosuper-shp53 (shp53), pBabepuro-p21WAF1 (p21) and pBabepuro (pBabe) vector alone. Transcript levels of E-cadherin were determined by Q-PCR and standardized for tubulin mRNA. The mRNA levels in HCT116 cells were arbitrarily set to 1. Mean values from two independent experiments are presented with the s.d.; n.d., not detectable. (B) Luciferase reporter gene assay in HCT116-p21^{-/-} cells co-transfected with the Ecad-luc plasmid and the indicated quantities of pRetrosuper-shp53 or scramble shRNA control vector. The level of luciferase activity was normalized to the expression of the co-transfected *Renilla* luciferase gene and the fold induction relative to the vector only is shown. Inhibition of p53 protein expression was evaluated by western blot analysis (lower panels). (C) Luciferase reporter gene assay as in B, except that the indicated quantity of pcDNA3-p21WAF1 were used. Expression of p21 was evaluated by western blot analysis (lower panels).

240 (mutant conformation) recognized the p53-A143 mutant (Fig. 4A) (Ory et al., 1994). Strikingly, in HCT116-p21^{-/-} cells, a fraction of p53 was immunoprecipitated by PAb240, indicating that it had the 'mutant-like' conformation. Following p21 re-expression the fraction of immunoprecipitated p53 with mutant

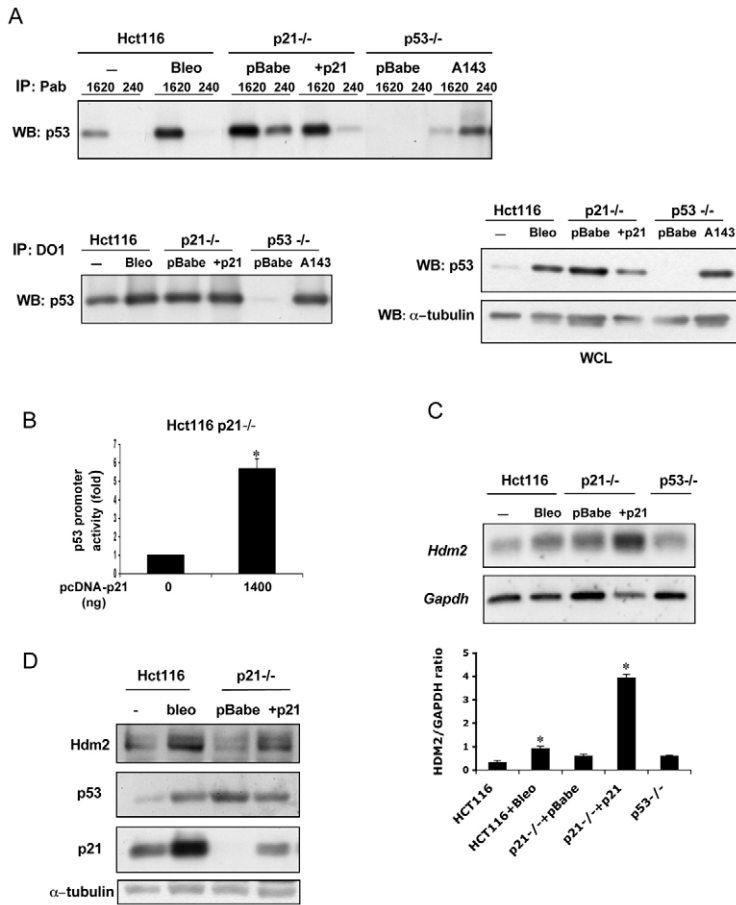


Fig. 4. Conformation analysis and transactivation properties of wild-type p53 in HCT116-p21^{-/-} cells. (A) Wild-type p53 accumulates in a mutant conformation in HCT116-p21^{-/-} cells. Immunoprecipitation (IP) and western blot (WB) analysis of the indicated cell extracts with wild-type conformation-specific (Pab1620) or mutant conformation-specific (Pab240) antibodies followed by western analysis for p53 (top panel). Immunoprecipitates were revealed using CM1, a rabbit polyclonal anti-p53 antibody, which detect the total amount of immunoprecipitated p53. Immunoprecipitation with DO1, which recognizes both forms, was used as a positive control (bottom panel). Whole cell lysates (WCL) were subjected to western blot analysis with antibodies to p53 and α-tubulin (right bottom panels). Bleo, bleomycin; p21^{-/-}, HCT116-p21^{-/-} cells transfected with vector alone (pBabe) or with pBabe-p21WAF1 (+p21); HCT116-p53^{-/-} cells transfected with vector alone (pBabe) or with the p53-A143 mutant (A143). (B) p21 re-expression in HCT116-p21^{-/-} cells leads to upregulation of p53-dependent transactivation activity. Luciferase reporter gene assay of HCT116-p21^{-/-} cells co-transfected with the PG-13-luc plasmid and the indicated quantity of pcDNA3-p21WAF1 or empty vector. The level of luciferase activity was normalized to the expression of the co-transfected *Renilla* luciferase gene and the fold induction relative to the vector only is shown. (C) Restoration of p53 transactivation activity in HCT116-p21^{-/-} cells by p21 expression. RT-PCR analysis shows *Hdm2* (p53-induced gene) mRNA levels in HCT116-p21^{-/-} cells stably expressing empty vector (pBabe) or pBabe-p21WAF1 (+p21). As a positive control for p53 transactivation activity, HCT116 cells treated with bleomycin (Bleo) were included. The level of the housekeeping gene *GAPDH* is shown as loading control. Graph below the RT-PCR gives the densitometric values normalized to the relative *GAPDH* value. (D) Western blot analysis showing *Hdm2*, p53, p21 and α-tubulin protein levels. Expression of *Hdm2* protein was induced in HCT116-p21^{-/-} stably re-expressing p21.

conformation decreased (Fig. 4A). It should be underlined that wild-type p53, as recognized by the Pab1620 antibody, was still present in HCT116-p21^{-/-} cells and only a fraction of the total p53 showed immunoreactivity to the Pab240 antibody. These results suggest that absence of p21 favours a p53 conformational change.

To explore whether this conformational change was linked to a modification of the biological activity of p53, HCT116-p21^{-/-} and HCT116-p21^{-/-} cells expressing p21 were transiently transfected with a plasmid containing the luciferase reporter gene under the control of p53-binding elements. The luciferase activity measured in HCT116-p21^{-/-} cells expressing p21 was fivefold higher than that measured in HCT116-p21^{-/-} cells (Fig. 4B). Therefore, it appears that p21 re-expression in HCT116-p21^{-/-} cells enhances the function of p53 as a transcriptional activator. These findings indicate that HCT116-p21^{-/-} cells accumulate p53 protein with a mutant conformation that has reduced transcriptional activity compared with wild-type p53.

To complement these results based on a reporter gene assay, we then examined the expression of *Hdm2* (*MDM2*), a p53-responsive gene in the different HCT116 cell lines. *Hdm2* mRNA was detected in control HCT116 cells (Fig. 4C) and treatment of these cells with bleomycin increased its expression, as a result of activation of p53-dependent transcription (Fig. 4C). By contrast, in HCT116-p21^{-/-} cells, no significant increase in *Hdm2* mRNA expression was observed in comparison to control HCT116 cells (Fig. 4C). However, p21 re-expression in HCT116-p21^{-/-} cells resulted in *Hdm2* induction (Fig. 4C). These results were confirmed also by

western blot analysis (Fig. 4D) and are consistent with the observation that p53 in HCT116-p21^{-/-} cells failed to activate p53-dependent gene expression (Fig. 4B,C). These findings indicate that absence of p21 in HCT116 cells promotes a shift from wild-type to conformation mutant p53 that is transcriptionally inactive. This change allows p53 accumulation by antagonizing the *Hdm2* auto-regulatory feedback loop.

E-cadherin is negatively regulated in a p53-dependent manner

Next, we sought to examine whether the effects on E-cadherin expression due to the expression of p53 with mutant conformation were also recapitulated by human tumour-derived p53 mutants. For these studies, HCT116-p53^{-/-} clones expressing either a temperature-sensitive p53 allele encoding valine at codon 143 (p53-A143) or empty vector were generated by retroviral infection. At 32°C, p53 was expressed in wild-type conformation and shifting the temperature to 37.5°C induced expression of the p53-A143 protein in a mutant conformation (Zhang et al., 1994). The temperature shift to 37.5°C had no effect on E-cadherin protein levels in parental HCT116-p53^{-/-} cells whereas it resulted in a significant decrease of E-cadherin expression in HCT116-p53^{-/-} cells expressing p53-A143 (Fig. 5A). To investigate the involvement of p53-A143 in E-cadherin transcription, we assessed changes in E-cadherin mRNA levels. Q-PCR analyses showed that the temperature shift to 37.5°C and the mutant p53 induction in HCT116-p53^{-/-} cells expressing p53-A143 resulted in a decrease in E-cadherin mRNA expression (Fig. 5B).

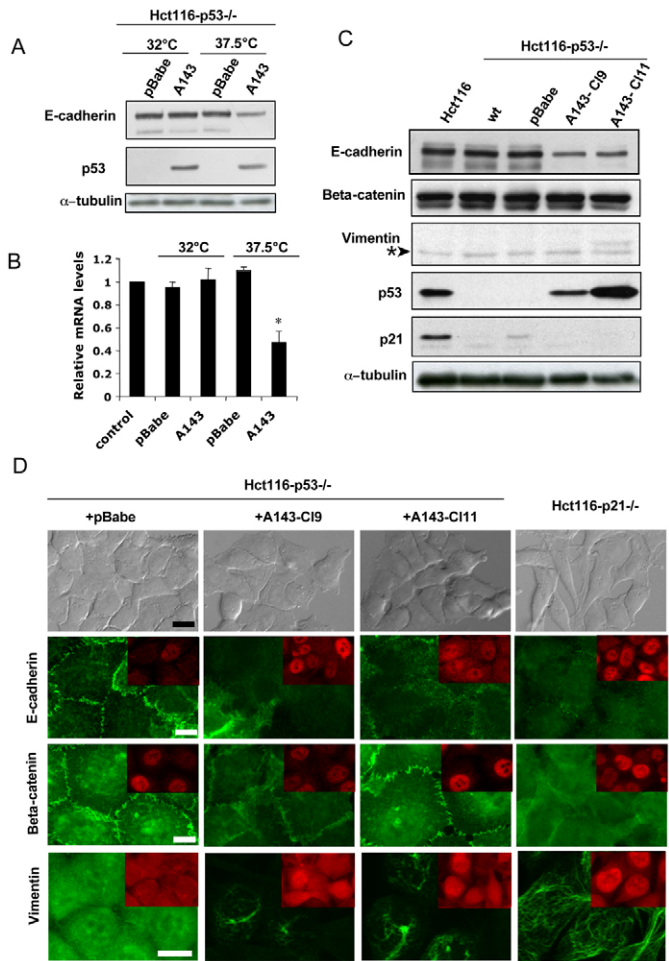


Fig. 5. Downregulation of E-cadherin and upregulation of vimentin in HCT116 p53^{-/-} cells expressing p53-A143. (A) E-cadherin is downregulated by induction of mutant p53. Immunoblots showing E-cadherin and p53 expression in HCT116-p53^{-/-} cells infected with vector alone (pBabe) or temperature-sensitive p53-A143 (A143). Cells were shifted to 32°C (wild-type p53 conformation) or to 37.5°C (mutant p53 conformation) for 72 hours. α -tubulin was used as a loading control. (B) Expression of E-cadherin in the cell lines described in A. Transcript levels of E-cadherin were determined by Q-PCR and standardized for *tubulin* mRNA. The mRNA levels in HCT116-p53^{-/-} cells (control) were arbitrarily set to 1. Mean values from two independent experiments are presented with the s.d. (C) Representative immunoblots of HCT116, HCT116-p53^{-/-} and HCT116-p53^{-/-} clones, stably expressing pBabe-p53-A143 [clones 9 (A143-C19) and 11 (A143-C11)] or pBabe alone. Cells were grown at 37°C (mutant p53 conformation for clones 9 and 11) for 72 hours. Whole-cell lysates were analysed for E-cadherin, β -catenin, vimentin, p53, p21 and α -tubulin (loading control). The asterisk indicates a nonspecific band. (D) Upper panels: phase-contrast images of the indicated cell lines plated at low density. Scale bar: 100 μ m. Lower panels: localization of E-cadherin, β -catenin and vimentin. Insets show immunofluorescence for p53. Scale bars: 20 μ m.

We then evaluated whether p53-A143 led to the acquisition of EMT-like properties in HCT116-p53^{-/-} cells. In two independently derived clones that stably expressed high levels of p53-A143, both E-cadherin expression and the number of cells with strong E-cadherin expression at cell-cell boundaries were markedly reduced (Fig. 5C,D). Conversely, in control cells (pBabe), E-cadherin

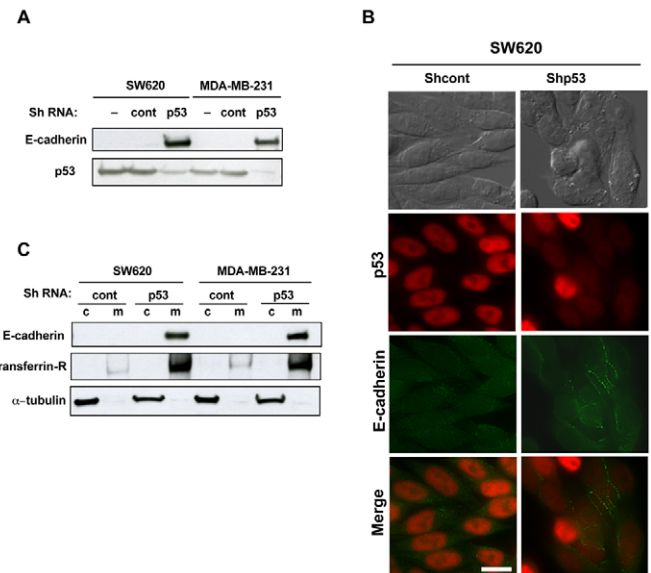


Fig. 6. Phenotypic effects induced by p53 mutant inhibition in SW620 and MDA-MB-231 cells. (A) ShRNA knockdown of mutant p53 reverses p53-mediated E-cadherin downregulation. SW620 and MDA-MB-231 cells were transfected with pRetrosuper-shp53 (p53) or control shRNA vector (cont) and the efficiency of mutant p53 protein knockdown and expression of E-cadherin was evaluated by immunoblotting 72 hours post-transfection. (B) ShRNA knockdown of mutant p53 has no obvious effect on cell morphology of SW620 cells. The upper panels are phase-contrast images of SW620 cells transfected with pRetrosuper-shp53 (shp53) or control shRNA vector (shcont). Only control shRNA vector-transfected SW620 cells are shown, as there were no visible differences between control SW620 and parental SW620 cells. The lower panels show immunofluorescence localization of E-cadherin and expression of p53 in SW620 cells transfected with pRetrosuper-shp53 (shp53) or control shRNA vector (shcont). Merged images show E-cadherin (green) expression only in cells that have lost p53 staining (red) in shp53 transfectants. Scale bar: 10 μ m. (C) Immunoblot analysis of the expression of E-cadherin, transferrin receptor (transferrin-R) and α -tubulin in the cytosolic (c) and membrane (m) fractions of SW620 and MDA-MB-231 cells transfected with pRetrosuper-shp53 (p53) or control shRNA vector (cont).

was still localized along cell-cell contact sites (Fig. 5D). Immunofluorescence analysis of vimentin expression showed that the protein was detectable in both clones (Fig. 5D), whereas β -catenin expression remained unchanged (Fig. 5C,D). Despite the switch from epithelial to mesenchymal markers observed in HCT116-p53^{-/-} cells expressing p53-A143, their overall epithelial morphology was not affected (Fig. 5C, upper panels). These data indicate that expression of p53-143A induces some molecular markers associated with EMT; however, this is not enough to induce a complete morphological EMT.

To further confirm the negative regulation of E-cadherin expression by mutant p53, we knocked-down endogenous mutant p53 by using p53-shRNA in colon and breast cancer cell lines, SW620 (p53R273H) and MDA-MB-231 (p53R280K), respectively. Both cell lines expressed high levels of mutant p53 proteins and exhibited very low or undetectable levels of E-cadherin protein (Fig. 6A). Immunoblotting of total cell lysates revealed a significant decrease of mutant p53 protein levels upon expression of shp53 (Fig. 6A). Remarkably, mutant p53 inactivation substantially restored E-cadherin expression (Fig. 6B). In addition, as indicated

by immunofluorescence analysis, p53 knockdown leads to a partial restitution of E-cadherin staining at the cellular boundaries of SW620 cells (Fig. 6B). Subcellular fractionation confirmed that E-cadherin is integrated into the membrane (Fig. 6C). However, expression of shp53 did not significantly alter cell morphology of SW620 cells compared with control-transfected cells (Fig. 6B). Similar results were also obtained in MDA-MB-231 cells (Fig. 6A,C), supporting a general function of mutant p53 on E-cadherin gene silencing in cancer cells.

p53 mutants regulate E-cadherin transcription

To assess whether the downregulation of E-cadherin transcripts is due to repression of the E-cadherin proximal regulatory promoter, we tested whether the activity of a luciferase reporter gene under the control of the E-cadherin promoter could be regulated by p53 mutants in HCT116-p53^{-/-} cells. Increased amounts of co-expressed p53-A143 repressed the E-cadherin promoter by up to twofold in HCT116-p53^{-/-} cells (Fig. 7A). To verify whether the transcriptional effects exerted by p53-A143 on the E-cadherin promoter were also recapitulated by other human tumour-derived p53 mutants, we also used p53-H175, which, like p53-A143, adopts an unfolded conformation, preventing its activity (Gannon et al., 1990; Ory et

al., 1994), and p53-H273, which contains a DNA-contact mutation inactivating its ability to form contact with DNA response elements. Both p53-H175 and p53-H273 showed dose-dependent repression of the E-cadherin promoter construct (Fig. 7A). However, none of these p53 mutants could repress a construct in which all three of the E-box elements were mutated (Fig. 7B). These data show that mutant p53 are capable of repressing E-cadherin transcription in vitro, and this repression is mediated via the E-box elements in the proximal E-cadherin promoter.

To further explore the mechanism of mutant p53-mediated E-cadherin silencing, we examined the mRNA expression levels of known E-cadherin repressors that initiate EMT: Snail, Slug, Twist1 and Zeb-1 (Peinado et al., 2007). Q-PCR analyses showed that the temperature shift to 37.5°C and induction of the mutant p53 in HCT116-p53^{-/-} cells expressing p53-A143 led to an induction of Slug and Zeb-1 transcripts (Fig. 7C). These observations were confirmed in HCT116-p21^{-/-} cells, showing also a significant upregulation of *Slug* and *Zeb-1* mRNA (Fig. 7E). Interestingly, *Twist1* and *Snail* mRNA did not differ between HCT116 and HCT116-p21^{-/-} cells. Stable expression of p21 and shp53 in HCT116-p21^{-/-} clones, which upregulated E-cadherin at both mRNA and protein levels (Fig. 2A, Fig. 3A) and gained an

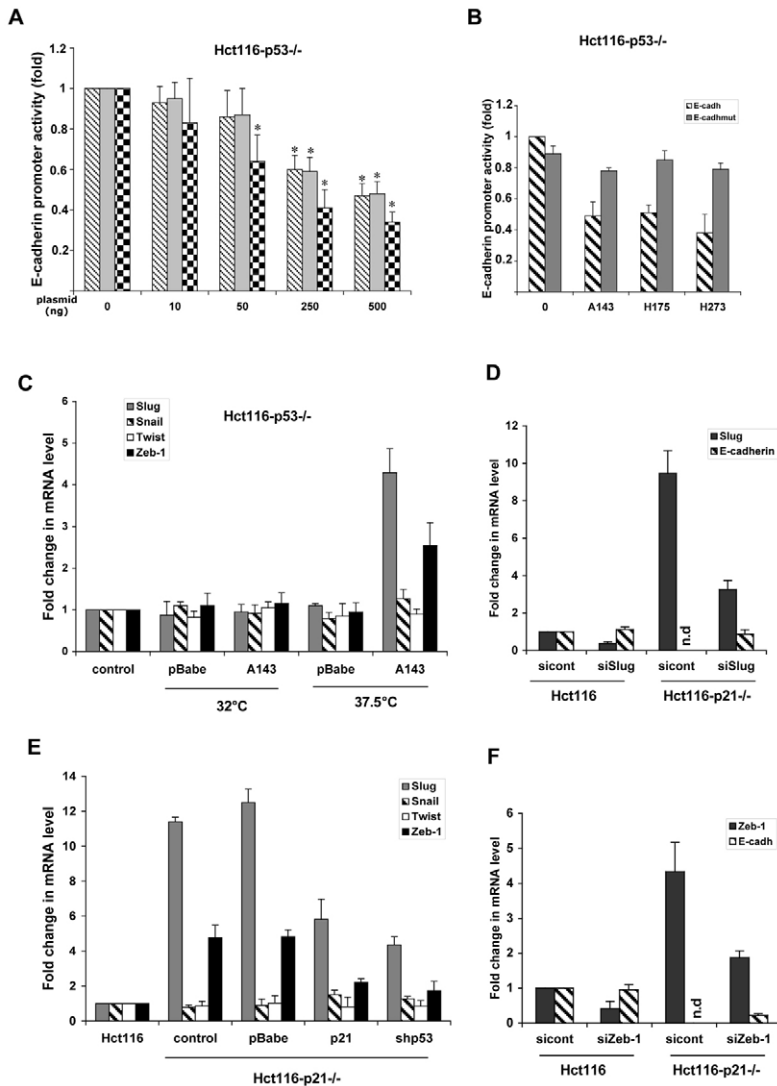


Fig. 7. Mutation of p53 leads to repression of E-cadherin gene expression and upregulation of *Slug* and *Zeb-1* transcripts. (A) p53 mutants repress the activity of the human E-cadherin promoter in transient HCT116-p53^{-/-} transfectants. HCT116-p53^{-/-} cells were co-transfected with wild-type E-cadherin reporter construct in the presence of the indicated quantities of pCMV vectors expressing p53-H175 (hatched bars), p53-A143 (grey bars) or p53-H273 (checked bars). (B) Repression of the E-cadherin promoter by mutant p53 is mediated via the E-box elements in the proximal promoter. HCT116-p53^{-/-} cells were co-transfected with 500 ng of the indicated mutant p53 vectors and wild-type or mutated E-cadherin reporter constructs. The wild-type promoter construct contained a -108 to +125 fragment of the human E-cadherin proximal promoter (E-cadh), whereas the mutated version contained mutations in all three proximal E-boxes (E-cadmut). The activity of the promoter constructs is represented relative to that of the wild-type construct detected in control-transfected cells. (C) Q-PCR analysis of the abundance of mRNAs of *Slug*, *Snail*, *Twist* and *Zeb-1* in HCT116-p53^{-/-} cells infected with vector alone (pBabe) or temperature-sensitive p53-A143 (A143). Cells were shifted to 32°C (wild-type p53 conformation) or to 37.5°C (mutant p53 conformation) for 72 hours. The mRNA levels in HCT116-p53^{-/-} cells (control) were arbitrarily set to 1. (D) Q-PCR analysis of the expression of *Slug* and E-cadherin mRNA in HCT116 and HCT116-p21^{-/-} cells transiently transfected with sicontrol and siSlug. Data are from two independent experiments. n.d., not detectable. (E) Q-PCR analysis of the expression of *Slug*, *Snail*, *Twist* and *Zeb-1* in HCT116, HCT116-p21^{-/-} cells (control) and HCT116-p21^{-/-} clones stably expressing pRetrosuper-shp53 (shp53), pBabepuro-p21WAF1 (p21) and pBabepuro (pBabe) vector alone. The mRNA levels in HCT116 cells were arbitrarily set to 1. (F) Q-PCR analysis of the expression of *Zeb-1* and E-cadherin mRNA in HCT116 and HCT116-p21^{-/-} cells transiently transfected with sicontrol and siZeb-1. Data are from two independent experiments. n.d., not detectable.

epithelial phenotype (Fig. 2B) resulted in the reduction of both *Slug* and *Zeb-1* mRNA (Fig. 7E).

To prove that *Slug* and *Zeb-1* were responsible for E-cadherin gene repression in HCT116-p21^{-/-} cells, *Slug* and *Zeb-1* were targeted using small interfering RNA (siRNA). Knockdown of *Slug* was confirmed by Q-PCR and was found to be sufficient to restore expression of E-cadherin (Fig. 7D). In addition, transient siRNA-mediated downregulation of *Zeb-1* also led to the reactivation of E-cadherin gene expression in HCT116-p21^{-/-} cells (Fig. 7F).

These data identify *Slug* and *Zeb-1* as key players in the mechanism of mutant p53-mediated E-cadherin gene repression.

p53 mutant does not show gain-of-function properties involved in promotion of cell invasiveness in three-dimensional Matrigel

To evaluate whether repression of E-cadherin by p53 mutants also contributes to cell invasion, we performed Matrigel invasion transwell assays as described previously (Gadea et al., 2007). We observed a significant increase in the invasive capacity of HCT116-p53^{-/-} cells compared with parental HCT116 cells (Fig. 8A). However, the invasiveness of HCT116-p53^{-/-} cells stably expressing p53-A143 was not further enhanced compared to that of parental HCT116-p53^{-/-} cells (Fig. 8A). These data support the notion that the p53 mutant does not show gain-of-function properties involved in promotion of invasion in p53-null cells. In addition, the invasive activity acquired by loss of p53 did not require downregulation of E-cadherin.

In other cell types, cell migration in this assay is influenced by the status of p53 and of the small GTPase, RhoA (Gadea et al., 2007). To evaluate the activation of the RhoA protein, we measured the amount of GTP-loaded active forms of RhoA. Measurement of RhoA activity revealed that the abundance of GTP-bound, active RhoA is greatly increased in HCT116-p53^{-/-} cells compared with parental HCT116 cells, whereas total RhoA protein level remained

constant (Fig. 8B). By contrast, active RhoA and RhoA protein abundance remained unaltered in HCT116-p53^{-/-} cells stably expressing p53-A143 compared with control HCT116-p53^{-/-} cells (Fig. 8B). To test whether the RhoA activation observed in HCT116-p53^{-/-} cells is required to mediate the enhanced cell invasiveness, we used a chimaeric membrane-penetrating Rho inhibitor, Tat-C3 (Coleman et al., 2001). Tat-C3 treatment effectively inhibited cell invasiveness of vector control HCT116-p53^{-/-} and HCT116-p53^{-/-} cells stably expressing p53-A143, in which RhoA activity is high (Fig. 8A,B), suggesting that RhoA activity is required for motility in Matrigel.

These results indicate that p53 inactivation increases cell invasion via RhoA activation independently of the functional status of E-cadherin. We further tested whether HCT116-p21^{-/-} cells show similar effects. In these cells, both RhoA activity and cell invasiveness are low (Fig. 8C,D), although E-cadherin gene expression is silenced. Under these conditions, HCT116-p21^{-/-} cells were not more invasive than parental HCT116 cells, whereas HCT116-p53^{-/-} cells showed the highest invasive potential in vitro (Fig. 8C). By contrast, both activation and abundance of RhoA protein were significantly increased in HCT116-p21^{-/-} cells expressing p21 and shp53 compared with that of vector only HCT116-p21^{-/-} cells (Fig. 8D). However, these cells stably expressing p21 and shp53 had invasive properties similar to those of HCT116-p21^{-/-} cells infected with an empty vector in our experimental assays (Fig. 8D), indicating that restored RhoA expression is not sufficient for induction of cell invasion in HCT116-p21^{-/-} cells.

Discussion

The p53 tumour suppressor gene contributes to cancer invasion by regulating the expression of some metastasis-related genes (Sun et al., 1999). Our data reveal that wild-type p53 induced to adopt a mutant conformation, and the oncogenic p53 mutants negatively

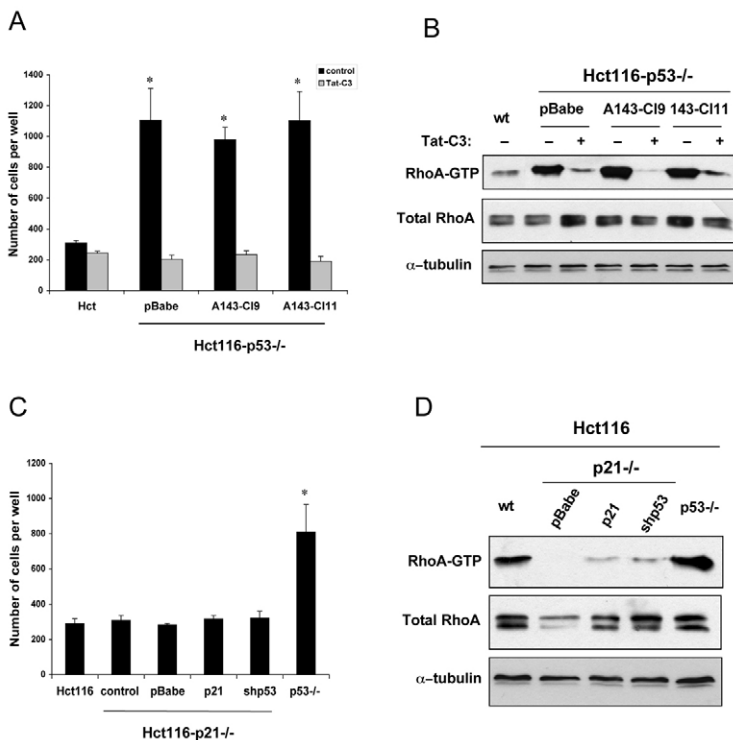


Fig. 8. p53 mutant does not show gain-of-function properties involved in promotion of cell invasiveness. (A) Matrigel invasion assay. HCT, HCT116-p53^{-/-} and HCT116-p53^{-/-} cells expressing vector only (pBabe) and p53-A143 (A143-C19 and A143-C111) in the absence (control) or presence of the Rho inhibitor Tat-C3 (10 µg/ml) were assayed for their ability to migrate through Matrigel. Values are means ± s.d. of two independent experiments. (B) RhoA-GTP loading was measured by a Rho activity pull-down assay. GTP loading of RhoA in HCT (wt) and HCT116-p53^{-/-} cells expressing p53-A143 (A143-C19 and A143-C111) in the absence or presence of Tat-C3 was measured by pull-down assays from cell lysates incubated with Rhotekin protein (see Materials and Methods). RhoA protein levels were determined by western blotting with α-tubulin as a loading control. Experiments were carried out twice and a representative experiment is shown. (C) Matrigel invasion assays were performed using HCT116, HCT116-p53^{-/-} (p53^{-/-}), HCT116-p21^{-/-} (control) cells and HCT116-p21^{-/-} clones stably expressing pBabepuro-p21 WAF1 (p21), pRetrosuper-shp53 (shp53) and pBabepuro (pBabe) vector alone. (D) RhoA-GTP loading of the cells described in C was measured by a Rho activity pull-down assay. RhoA protein abundances were evaluated by western blotting with α-tubulin as a loading control. Experiments were carried out twice and a representative experiment is shown.

regulate E-cadherin gene expression and promote some molecular alterations that are part of the EMT phenotype. In addition, p53 inactivation increases cell invasion via RhoA activation independently of the functional status of E-cadherin. This highlights the importance of p53 in governing the progression from non-invasive to invasive phenotype. Hence, we propose that inactivation of p53 contributes not only to cellular growth but permits tumour progression towards an invasive phenotype and that inhibition of EMT is a novel tumour-suppressor function of p53.

Our work provides evidence for another mechanism that tumour cells may use to promote metastasis, especially in tumour where *TP53* is not mutated but its expression is high. We show that absence of p21 inhibits p53 activity by favouring the expression of an inactive, 'mutant-like' form with impaired trans-activation activity. Moreover, although p53 in HCT116-p21^{-/-} cells is still recognized by PAb1620 (wild-type conformation), this does not equate to retention of a true wild-type conformation (Fig. 4). Indeed, in HCT116-p21^{-/-} cells, p53 does not act as a transcriptional activator any longer. As a consequence, it fails to trans-activate Hdm2, which in turn cannot target p53 for degradation, leading to p53 stabilization. Upon re-expression of p21, the levels of Hdm2 increase and those of p53 decrease concomitantly (Fig. 4D). Importantly, this conformational mutant form of p53 is associated with downregulation of E-cadherin expression and morphological changes (Fig. 1). The silencing effect of p53 on E-cadherin expression can be hindered by re-expression of p21 in HCT116-p21^{-/-}, which is followed by a strong decrease of the PAb240-positive inactive p53 (Fig. 4). These observations suggest that the absence of p21 perturbs the folding of p53 in a direct or indirect manner and therefore p21 may participate in the physiological control of p53 functions by affecting the fine tuning of p53.

Mutations of *TP53* are the most common genetic abnormality described in human cancer. The presence of p53 mutations is coincident with tumour invasion and aggressiveness in colorectal tumours (Tortola et al., 1999), and functional loss of p53 has been implicated in high-grade human breast cancers (Done et al., 2001). In addition, cadherin dysfunction promotes malignant progression of human cancers (Vlemingckx et al., 1991). We show a causal relationship between mutant p53 expression and decreased E-cadherin levels in colon cancer cell lines. Using Q-PCR and E-cadherin reporter assays, we demonstrate that mutant p53 decreased E-cadherin levels through a transcriptionally dependent event (Fig. 5, Fig. 7A).

To determine whether a gain-of-function effect enabled mutant p53 to repress the E-cadherin gene, we used cells devoid of p53 activity and demonstrated that mutant p53 acts in a gain-of-function fashion. Further analyses revealed that downregulation of E-cadherin expression occurred concomitantly with the upregulation of Slug and Zeb-1 (Fig. 7C,E), transcriptional factors known to repress E-cadherin expression (Peinado et al., 2007). In addition, siRNA-mediated downregulation of Slug and Zeb-1 expression diminished p53-mediated E-cadherin repression in HCT116-p21^{-/-} cells, as demonstrated by Q-PCR analysis (Fig. 7D,F). These data demonstrate that E-cadherin gene repression by mutant p53 is mediated by Slug and Zeb-1 and results in decreased E-cadherin protein.

Our data reveal that E-cadherin downregulation by p53 in colon cancer cells is not likely to result in complete EMT. Indeed, we showed that complete disappearance of the E-cadherin- β -catenin adhesion complex from the cell membrane and the acquisition of the mesenchymal marker vimentin were accompanied by a switch

to a fibroblast-like morphology but not by expression of N-cadherin (Fig. 1). Moreover, these morphological and molecular changes were not sufficient to confer invasive properties on HCT116-p21^{-/-} cells (Fig. 8). Importantly, expression of p53-A143 in HCT166-p53^{-/-} cells did not significantly affect cell invasiveness, although it modulated the expression of some epithelial and mesenchymal markers (Fig. 5). This is intriguing since loss of E-cadherin has been shown to be the rate-limiting step in the transition from adenoma to invasive carcinoma (Perl et al., 1998). Nevertheless, loss of E-cadherin-mediated cell adhesion and acquisition of the fibroblast-like morphology in HCT116-p21^{-/-} cells could be regarded as a partial EMT. It will be important to determine whether metastasis formation induced by expression of EMT regulators, such as SIP1, Snail, Slug and Twist, is solely mediated through repression of E-cadherin, or whether other transcriptional targets contribute also to this process. Indeed, since loss of E-cadherin alone in normal epithelial cells results in cell death rather than EMT, it is likely that the core EMT program includes more than just repression of E-cadherin.

Our findings also reveal that whereas loss of E-cadherin alone does not suffice to induce a cadherin switch or an obvious EMT phenotype, p53-deficiency enables cell invasion through a pathway that is independent of downregulation of E-cadherin expression (Figs 1 and 8). This effect as reported previously, and further confirmed in this work, is largely due to p53 regulation of signalling of Rho proteins that are important regulators of cell migration (Gadea et al., 2007; Guo and Zheng, 2004; Xia and Land, 2007) (Fig. 8). Therefore, cell migration events controlled by p53 do not necessarily require a complete EMT phenotype. This result is consistent with clinical observations showing that the majority of human breast carcinoma metastases still express E-cadherin and maintain their epithelial morphology, suggesting that they have disseminated without switching to a mesenchymal phenotype (Thompson et al., 1995). Studies in mouse tumour models have also suggested that tumour invasion and metastasis can be achieved without an obvious EMT phenotype (Han et al., 2005). Nevertheless, loss of both E-cadherin and p53 resulted in the accelerated development of invasive and metastatic mammary carcinomas in mice (Derksen et al., 2006).

Further work is necessary to understand whether the role of p53 in the regulation of E-cadherin expression and cell invasiveness influences metastatic development in human cancers. Research addressing this aspect will also need to ascertain the relative contribution of each functional property of p53, i.e. control of the cell cycle, apoptosis, cell-cell adhesion and migration, in tumour aggressiveness.

Materials and Methods

Cell culture, small interfering RNA transfection and retroviral infection

Human colon carcinoma cell lines, HCT116-p21^{-/-} (which expresses wild-type p53 as determined by sequencing of PCR-amplified cDNA), HCT116-p53^{-/-} and parental HCT116 have been described previously (Bunz et al., 1998) and were gifts from B. Vogelstein (Johns Hopkins University, Baltimore, MD). HCT116 and derivative cell lines were cultured in McCoy's 5A medium (Sigma) supplemented with 10% foetal bovine serum (HyClone Laboratory Inc.), 2 μ g/ml penicillin-streptomycin, and 2 mM L-glutamine. SW620 and MDA-MB-231 cell lines were cultured in RPMI 1640 (Gibco) and 10% foetal bovine serum.

pRetrosuper retroviral vector encoding short RNA hairpins directed against *TP53* (pRetrosuper-shp53) was a gift from W. Hahn (Dana-Farber Cancer Institute, Boston, MA). A cDNA encoding human *p21WAF1* and the A143 mutant of p53 were subcloned into the pBabe retroviral vector that contains a puromycin resistance gene (pBabe-puro). Methods used for retroviral production and infection have been described previously (Gire et al., 2004).

siRNAs targeting the following sequences were used: 5'-GCATTGCGACAG-GTCAAAT-3' (Slug siRNA), 5'-AATGCAGATTGAGGCTGATCACCTGTCTC-3'

(Zeb-1 siRNA) and 5'-GTTGCTTGCACGTCCTAGAT-3' (sicont). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) and were harvested 4 days after transfection.

Luciferase reporter assays

The following plasmids were used in transfection experiments: pCMV-p53-A143, pCMV-p53-H175, pCMV-p53-H273 (gifts from B. Vogelstein), pcDNA3-p21WAF1 (gift from B. Ducommun, Université Paul Sabatier, Toulouse, France), pRetrosuper-shp53, and pcDNA3. The human E-cadherin gene promoter-firefly luciferase construct in pGL2-Ecad5-Luc (-362/+125), pGL2-Ecad1-Luc (-108/+125) and the mutated version contained mutations in all three proximal E-boxes were obtained from E. Fearon (University of Michigan, Ann Arbor, MI). The PG-13-luc consists of the polyomavirus early promoter and 13 copies of a synthetic consensus followed by the luciferase gene (el-Deiry et al., 1994). HCT116 cells were seeded in duplicate in 6-well plates, at a density of 1.5×10^5 and were transfected with 0.5 μ g of the E-cadherin gene-luciferase construct or of the PG-13-luciferase construct and 0.1 μ g of an internal control vector encoding *Renilla* luciferase using the JetPei reagent (Qbiogene). The constructs, at the indicated amounts, were co-transfected with the luciferase plasmids. After 24 hours, cells were lysed, and 20 μ l of cell lysates were assayed using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Expression of proteins was confirmed by western blotting. Firefly luciferase activity under the control of the E-cadherin gene promoter was normalized to that of *Renilla* luciferase.

RT-PCR and real-time PCR

Total RNA was purified from the indicated cell lines using the RNEasy mini kit (Qiagen) according to the manufacturer's recommended protocol. First strand cDNA was synthesized with the SuperScript II First-strand Synthesis system for RT-PCR (Invitrogen) and oligo(dT) primers. PCR amplification was performed using *Taq* DNA polymerase (Roche) for 19 (GAPDH) and 30 (Hdm2) cycles with a denaturing step at 95°C for 1 minute, annealing for 1 minute (at 60°C for Hdm2 and 55°C for GAPDH), and extension at 72°C for 1 minute. PCR products were run on 2% agarose gels.

mRNA expression was measured by quantitative real-time PCR (Q-PCR) with SYBR Green master mix (Roche) using a Mx3000P Q-PCR system (Stratagene). PCR conditions consisted of an 8-minute hot start at 95°C, followed by 40 cycles of 30 seconds at 95°C and 30 seconds at 65°C. Relative gene expression was determined by normalizing to tubulin using the ΔC_t method. Primer sequences are listed in supplementary material Table S1.

Subcellular fractionation and western blot analysis

Subcellular fractionation was performed using the subcellular protein fractionation kit (Thermo Scientific Pierce) according to the manufacturer's instructions. Whole lysates of cells cultured to confluence were prepared by scraping cells with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and 0.5% deoxycholate) containing protease inhibitors (mixture from Sigma). Protein concentration was determined by the bicinchoninic acid (BCA) method. Proteins were separated on 8% or 15% gels by SDS polyacrylamide gel-electrophoresis (PAGE) and transferred onto Immobilon-P membranes (Millipore). Primary antibodies used were anti-p21WAF1 (sc-397), -p53 (clone DO1), -Mdm2 (SMP14), -RhoA (26C4), - α -tubulin (all from Santa Cruz Biotechnology), -E-cadherin (clone 3), -N-cadherin (clone 32), - β -catenin (clone 14) (all three from Transduction Laboratory), -phosphorylated p53 on serine 15 (Cell Signaling Technology), -vimentin (1:250; clone V9, Sigma), -transferrin receptor (clone H68.4, Invitrogen). Bound primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies (Amersham Biosciences), followed by enhanced chemiluminescence (Amersham Biosciences).

Immunoprecipitation analysis

To analyse p53 conformation, cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, and protease inhibitors) on ice for 30 minutes and cell debris were cleared by centrifugation at 12,000 g at 4°C. To prevent non-specific binding, 100 μ g of protein extracts were pre-cleared with 50 μ l protein-A/G-Sepharose beads (Amersham) on ice for 1 hour followed by centrifugation. The resulting supernatant was immunoprecipitated with 1 μ g of the conformation-specific PAb1620 (wild-type p53), PAb240 (mutant p53) and DO1 (which recognizes both wild-type and mutant p53) antibodies at 4°C overnight. Immuno-complexes were collected with protein-A/G-Sepharose beads at 4°C for 40 minutes, centrifuged and washed with ice-cold lysis buffer three times. Immunoprecipitated p53 was analyzed by western blotting using the rabbit polyclonal anti-p53 antibody CM1 (a gift of J.-C. Bourdon).

Immunofluorescence

Cells grown on glass coverslips were fixed in 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. Cells were subsequently incubated with primary antibodies at room temperature for 1 hour: anti-cyclin A (clone 6E6, Novocastra), -cyclin D1 (clone DCS-6, Pharmingen) and anti-E-cadherin (clone HECD-1, Zymed). Alexa-Fluor-conjugated antisera (Molecular Probes) were used as secondary antibodies, and DNA

was visualized with Hoechst dye. Images were captured with a DMRA Leica epifluorescence microscope with a $\times 40$, NA 1.4 oil immersion objective (Zeiss, Inc.) using a CoolSnap HQ2 camera (Photometrics, Tucson, AZ) driven by Metamorph software 7.1 (Universal Imaging Corporation). Vimentin fluorescence images were obtained with an Apotom imaging system (Zeiss, Inc.). AxioVision 4.4 (Zeiss, Inc.) and Photoshop were used to compose Z-stack images. Images were pseudocoloured with Metamorph 7.1.

Invasion assays

Transwell Boyden chambers (8 μ m pore size filter insert) were coated with 2.5 mg/ml Matrigel (BD Biosciences, San Jose, CA). Cells (10^5) were seeded in McCoy's 5A medium containing 0.5% serum in the upper chamber with serum-containing medium in the lower chamber. Tat-C3 (10 μ g/ml) was added to the cells suspension where applicable. After incubation for 24 hours, cells that invaded the Matrigel were stained with 4 μ g/ml of calcein-PBS (Sigma) at 37°C for 1 hour and counted.

Rho activity pull-down assay

RhoA activity assays were performed as described previously (Gadea et al., 2002). Briefly, cells were incubated with recombinant Rhotekin-RBD protein GST beads (Cytoskeleton). GTP-bound RhoA that precipitated was detected by SDS-PAGE and immunoblotted with anti-RhoA antibody.

Statistics

Data are presented as the means \pm standard deviation (s.d.) of the number of experiments (minimum of three performed in duplicate). The statistical significance between experimental values was assessed by Student's *t*-test using SigmaPlot software, and *P*<0.05 was considered to be statistically significant.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/8/1295/DC1>

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