Cell

Mutant p53 Drives Invasion by Promoting Integrin Recycling

Patricia A.J. Muller,[1] Patrick T. Caswell,[1] Brendan Doyle,[1] Marcin P. Iwanicki,[2] Ee H. Tan,[1] Saadia Karim,[1] Natalia Lukashchuk,[1,3] David A. Gillespie,[1] Robert L. Ludwig,[1] Pauline Gosselin,[2,4] Anne Cromer,[2] Joan S. Brugge,[2] Owen J. Sansom,[1] Jim C. Norman,[1,*] and Karen H. Vousden[1,*]
[1]The Beatson Institute for Cancer Research, Switchback Road, Bearsden, Glasgow G61 1BD, UK
[2]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
[3]Present address: The Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK
[4]Present address: UPMC Univ Paris 06, UMR 7150, Equipe Traduction Cycle Cellulaire et Développement, Station Biologique de Roscoff, Roscoff 29682 France
*Correspondence: j.norman@beatson.gla.ac.uk (J.C.N.), k.vousden@beatson.gla.ac.uk (K.H.V.)
DOI 10.1016/j.cell.2009.11.026

SUMMARY

p53 is a tumor suppressor protein whose function is frequently lost in cancers through missense mutations within the *Tp53* gene. This results in the expression of point-mutated p53 proteins that have both lost wild-type tumor suppressor activity and show gain of functions that contribute to transformation and metastasis. Here, we show that mutant p53 expression can promote invasion, loss of directionality of migration, and metastatic behavior. These activities of p53 reflect enhanced integrin and epidermal growth factor receptor (EGFR) trafficking, which depends on Rab-coupling protein (RCP) and results in constitutive activation of EGFR/integrin signaling. We provide evidence that mutant p53 promotes cell invasion via the inhibition of TAp63, and simultaneous loss of p53 and TAp63 recapitulates the phenotype of mutant p53 in cells. These findings open the possibility that blocking alpha5/beta1-integrin and/or the EGF receptor will have therapeutic benefit in mutant p53-expressing cancers.

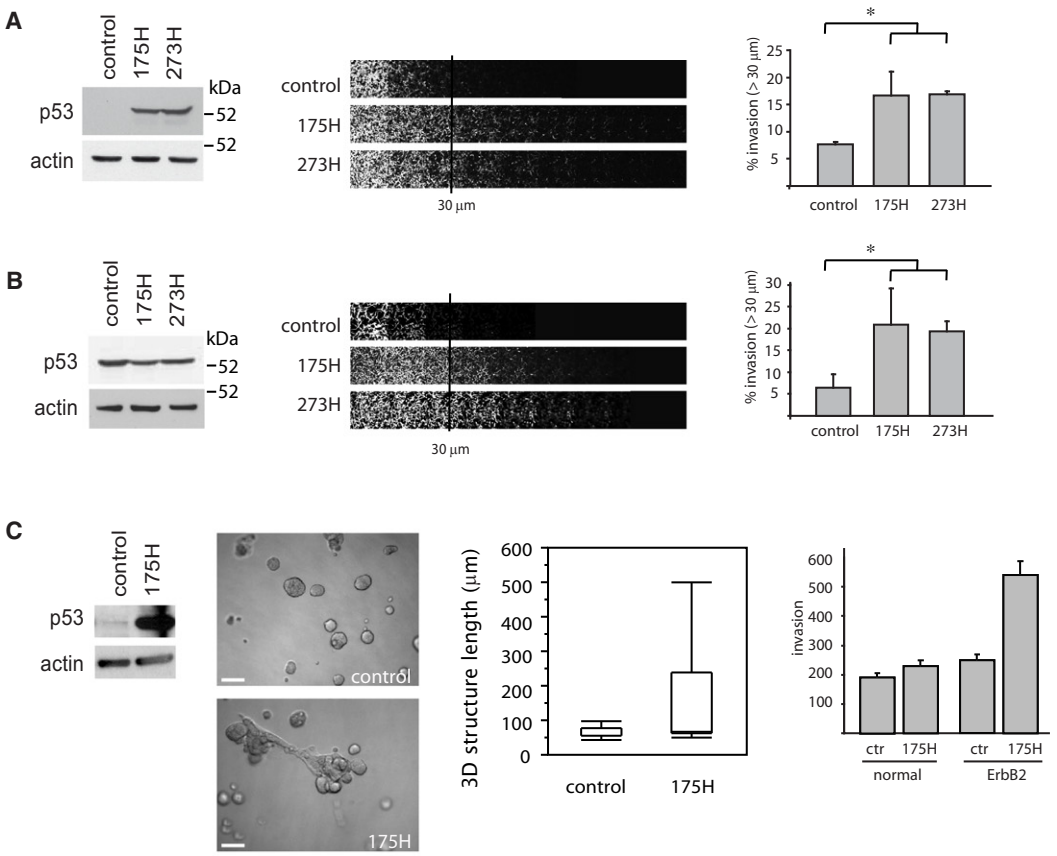
INTRODUCTION

Wild-type p53 is activated in response to oncogenic stress signals and functions primarily to prevent cancer development (Vogelstein et al., 2000). To a large part, this activity of p53 is mediated by its ability to function as a transcription factor and induce a permanent inhibition of cell growth, through the activation of either senescence or apoptotic cell death (Vousden and Prives, 2009). The p53 gene is mutated in around 50% of all human cancers and even those cancers that retain wild-type p53 frequently show defects in the pathways leading to its activation (Vogelstein et al., 2000). Thus almost all cancers have defects in the p53 response, underscoring the importance of loss of wild-type p53 activity for successful tumor development. Consistent with this, mice deleted of p53 are highly cancer prone (Attardi and Jacks, 1999).

It is clear that a key consequence of p53 mutation is the loss of wild-type p53 activity. However, in contrast to the loss-of-function mutations that occur in most other tumor suppressors, p53 commonly suffers point mutations resulting in the expression of a full-length protein with a single amino acid substitution (Petitjean et al., 2007). These mutations tend to be clustered at 6 hot spots within the central sequence-specific DNA-binding region of the protein and result in loss of DNA binding, either by mutation of a DNA-contacting residue (such as amino acid 273) or by perturbing the structure of the DNA-binding domain (such as mutations at residue 175) (Cho et al., 1994). Although expressed at low levels in normal tissue, these mutant p53 proteins are frequently stabilized and expressed at high levels in tumors—indeed high expression of p53 in cancer tissue is predictive of the presence of mutant p53 (Lane, 1994).

Inactivating point mutations of p53 may be selected in cancers because they act as trans-dominant inhibitors of wild-type p53 coexpressed in the same cell (Blagosklonny, 2000). However, there is also clear evidence that mutant p53 proteins acquire a transforming activity that is independent of wild-type p53 and so constitutes a gain of function (Sigal and Rotter, 2000). Studies in mice confirmed that mutation of p53 is not equivalent to p53 loss and showed that mutant p53 can drive the development of different types of tumors with a more invasive phenotype than those that arise following loss of p53 (Lang et al., 2004; Olive et al., 2004). Similarly, in human cancers mutant p53 expression has been linked with a poorer prognosis (Petitjean et al., 2007). This new appreciation of mutant p53 function raises the possibility that the mutant protein may be a good target for the design of novel therapies aimed at inhibiting cancer dissemination, rather than the appearance of the primary tumor. Critical to such approaches will be an understanding of how mutant p53 drives this invasive behavior.

In this study we have identified a key mechanism by which mutant p53 can promote invasive behavior of cells, through a gain of function that is independent of the loss of wild-type p53 function. We show that mutant p53 can drive both random cell motility and increased invasiveness through the enhancement of integrin recycling pathways that have been shown previously to promote invasive behavior (Caswell et al., 2007, 2008).



D

Genotype	Mice with invasive tumors
$APC^{fl/+}$ $p53^{fl/+}$	25% (5/20)
$APC^{fl/+}$ $p53^{R172H/+}$	100% (10/10)

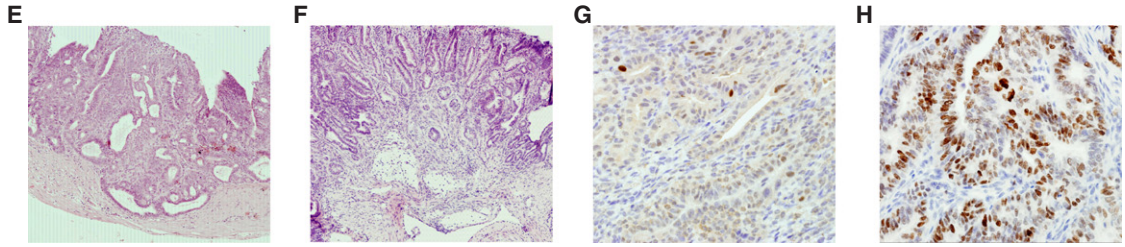


Figure 1. Mutant p53 Expression Drives Invasion In Vitro and In Vivo

(A and B) Expression of mutant p53 in polyclonal vector control and mutant p53 (175H and 273H) H1299 (A) and RPE (B) cells assessed by western blot (left panels). Actin expression was used as loading control in a parallel blot. The invasion of mutant p53 H1299 cells into fibronectin-supplemented Matrigel was quantified (middle panel and right panel) as described in the Experimental Procedures. Values are means ± standard error of the mean (SEM) of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(C) Expression of p53 in MCF10A cell lines containing a vector control or mutant p53 175H (left panel). These cells were seeded into collagen 1-supplemented Matrigel (second panel, scale bars represent 100 μm) and the number of invasive structures was quantified (third panel, measurements of at least 15 individual acini in three independent experiments). Invasion of MCF10A or ErbB2-expressing MCF10A cells after infection with control (ctr) or mutant p53 175H vectors was examined using Matrigel-coated chambers (right panel). These data represent 3–5 independent experiments.

RESULTS

Expression of Tumor-Derived Mutant p53 Proteins Promotes Invasive Behavior in Cancer and Normal Cells

In light of in vivo studies suggesting that mutant p53 can drive enhanced metastasis, we examined the consequences of mutant p53 expression in a number of tissue culture systems that model such behavior. Initially, H1299 lung cancer cells were employed because they are null for p53 expression and thus preclude the possibility that any effects of mutant p53 are due to the dominant-negative inhibition of wild-type p53. Polyclonal cell lines were established that stably express p53 mutants frequently found in human cancers—either p53 175H or p53 273H—and these were compared to polyclonal H1299 cells transfected with an empty vector construct (Figure 1A, left panel). Wild-type p53 was not included in these assays because its expression resulted in cell-cycle arrest and apoptosis, with only rare outgrowth of p53-expressing cells that are likely to have undergone alterations to allow them to tolerate any wild-type p53 expression. Analysis of the ability of these cells to invade into fibronectin-supplemented reconstituted basement membrane (Matrigel) showed that expression of either p53 mutant significantly enhanced their migration (Figure 1A). Increased migration was also evident following transient expression of mutant p53, indicating that invasiveness was not acquired indirectly during the establishment of the stably expressing cell populations (Figure S1A available online). To establish whether mutant p53 expression could drive invasion in other cell types, we examined untransformed human epithelial cell systems. A series of polyclonal TERT-immortalized human retinal pigment epithelial (RPE) cells expressing either p53 175H or p53 273H were established and compared to cells transfected with an empty vector. Despite ectopic expression from a cytomegalovirus (CMV) promoter, the levels of p53 protein in the stable mutant p53 transfected cells were not higher than endogenous wild-type p53 expression (Figure 1B). As seen with the H1299 cells, expression of mutant p53 promoted the invasion of RPE cells into Matrigel (Figure 1B). We also examined the effects of stable expression of mutant p53 in MCF10A cells, a primary mammary epithelial cell line (Figure 1C). These cells form growth-arrested structures—also termed acini—when cultured in Matrigel supplemented with collagen (Figure 1C, second panel). After oncogenic stimulation such as expression of V12Ras or ErbB2 (NeuT) or coexpression of ErbB1 and TGF-β, these cells can be induced to form invasive structures (Muthuswamy et al., 2001; Seton-Rogers et al., 2004). Similar invasive structures were formed in cells expressing the 175H mutant of p53 (Figure 1C, third panel), and there was a slight enhancement in their invasion into Matrigel (in Boyden chamber assays), which was strongly augmented by coexpression of ErbB2 (Figure 1C, right panel). Taken together, these studies showed that mutant p53 proteins drive invasion and migration in a number of cell culture models.

The oncogenic and metastatic potential of H1299 cells as xenografts in vivo was assessed following injection into the mammary fat pad of nude mice (Figure S1B). As described previously (Cantor et al., 2007), H1299 cells are tumorigenic in this model, with primary tumors arising after 30 days. Tumors originating from mutant p53-expressing cells were more invasive than those formed from control cells (Figure S1B), and micrometastases in the lung were only detected in mice that had been injected with cells expressing mutant p53 (Figure S1C). These results are consistent with studies showing a similar role for mutant p53 in xenograft studies (Adorno et al., 2009) and in mice expressing mutant p53 in all tissues (Lang et al., 2004; Olive et al., 2004). To investigate the effect of targeted expression of mutant p53 in the adult mouse more closely, we generated mice with intestine-specific alterations in *APC* and *Tp53* (Sansom et al., 2004). Here *AhCre* transgenic mice carrying a single conditional inactivatable $Apc^{fl/+}$ allele were intercrossed to mice carrying either an inducible knockout *Tp53* allele (*AhCre* $APC^{fl/+}$, $Tp53^{fl/+}$) or an inducible knockin allele of mutant p53 (*AhCre* $APC^{fl/+}$, $Tp53172^{H/+}$). In this model, intestinal tumor formation is associated with the loss of the remaining wild-type Apc allele (Sansom et al., 2006). Both cohorts of mice developed intestinal tumors with similar incidence and latency (Log Rank p = 0.815), but there was a clear difference in incidence of invasive adenocarcinomas. Coordinate deletion of one allele of *APC* and *Tp53* gave rise to primarily intestinal adenomas, with evidence of invasive adenocarcinomas in only 25% of mice (Figure 1D), similar to that seen previously in $APC^{fl/+}$ $Tp53^{+/+}$ mice (Marsh et al., 2008; Sansom et al., 2006). However, all mice where mutant p53 was introduced (*AhCre* $APC^{fl/+}$, $Tp53172^{H/+}$) developed invasive tumors (Figures 1D–1F) with pronounced nuclear staining for mutant p53 (Figures 1G and 1H). These results support the ability of mutant p53 (as opposed to loss of p53) to enhance invasion and metastasis.

Mutant p53 Drives Random Migration and Disordered Lamellipodia

To investigate further the behavior of mutant p53-expressing H1299 cells, we examined their migration in scratch-wound assays. Control H1299 cells migrated persistently following wounding and followed paths that were largely perpendicular to the wound edge (Figure 2A). By contrast, mutant p53-expressing cells moved erratically into the wound and displayed a marked loss of persistence, although their migration speed was unaltered (Figure 2A, Figure S2, and Movies S1–S3).

(D) Invasion of tumors arising in *AhCre* $APC^{fl/+}$ $p53^{fl/+}$ mice and *AhCre* $APC^{R172H/+}$ $p53^{fl/+}$ mice. No difference in median survival was seen between the two groups (Log-Rank p = 0.815).

(E and F) Representative photomicrographs of invasive tumors seen in the *AhCre* $APC^{R172H/+}$ $p53^{fl/+}$ mice. (E) shows a superficially invasive adenocarcinoma with extension of the tumor to the junction between the longitudinal and circumferential muscle layers. (F) shows a more advanced tumor, with mucin lakes infiltrating throughout the full thickness of the muscle wall (both H&E, 100×).

(G and H) p53 staining demonstrating accumulation of nuclear mutant p53 in one of the invasive tumors from *AhCre* $APC^{fl/+}$ $p53^{fl/+}$ and *AhCre* $APC^{fl/+}$ $p53^{R172H/+}$ mice (p53 immunohistochemistry, 400×).

See also Figure S1.

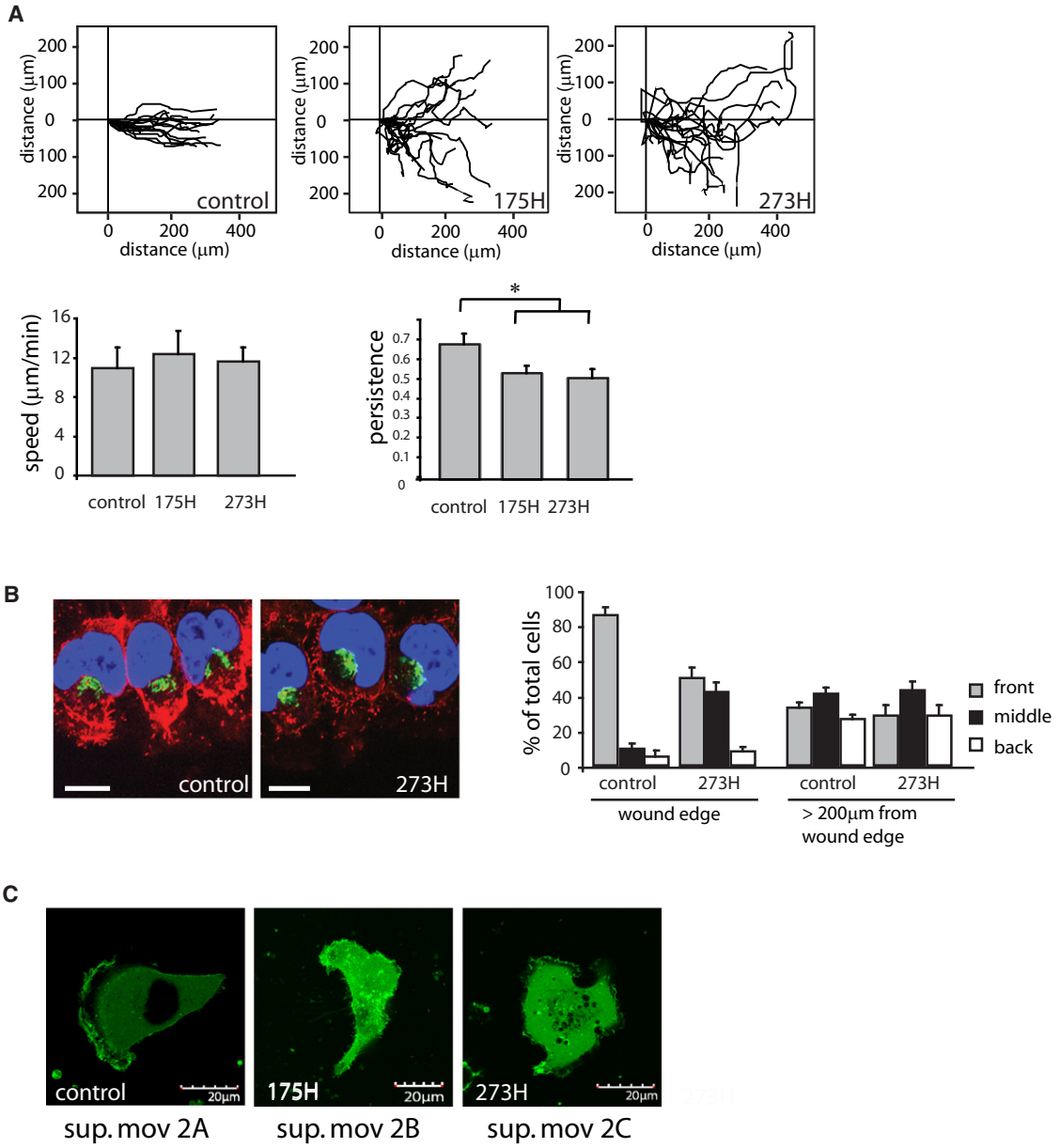


Figure 2. Mutant p53 Expression Leads to Loss of Persistent Migration and Polarity

(A) Vector control and mutant p53-expressing H1299 cells were observed by time-lapse video microscopy, the movement of individual cells was followed using cell-tracking software, and this is presented as overlays of representative trajectories described by cells during their migration into the wound. The persistence (lower right panel) and speed (lower left panel) of migration were extracted from the track-plots. Persistence is defined as the ratio of the vectorial distance traveled to the total path length described by the cell. Values are means ± SEM of >100 track-plots from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(B) Vector control and mutant p53 H1299 cells were grown in monolayers and wounded. The localization of the Golgi (green) was determined using immune fluorescence and cells were counterstained with phalloidin (red) and DAPI (blue). Scale bars indicate 10 μm. The localization of the Golgi complex in >100 cells at the edge of the wound and in cells further away from the wound is depicted in the right panel.

(C) Stills taken from movies (Figures S2A, S2B, and S2C) showing the motility of vector control and mutant p53 (175H and 273H) H1299 cells following transfection with GFP-actin. The data are representatives of three independent experiments of a total of 18 movies. Scale bars represent 20 μm.

See also Figure S2 and Movies S1–S6.

Cells orient the Golgi complex to face the direction of migration when they move into scratch wounds (Kupfer et al., 1982), and in around 80% of control H1299 cells the Golgi complex faced the wound edge (Figure 2B). Conversely, mutant p53 cells did not consistently orientate their Golgi toward the wounded area (Figure 2B), indicating the possibility that their random

migration may be associated with an inability to properly establish an axis of polarity with respect to the wound's edge.

Fluorescence time-lapse imaging of GFP-actin revealed that migrating H1299 cells adopted a fan-like morphology in which an actin-rich lammellipodium advanced in an organized, processive fashion, with retrograde movement of filaments back from the leading edge being visible (Figure 2C and Movies S4–S6). However, the morphology of mutant p53-expressing cells was markedly altered, with an apparent collapse of the lamellipodium and instead the presence of disorganized actin-rich ruffles that displayed alternately protruding and retracting behavior and that were not concertedly oriented toward the wound edge (Figure 2C and Movies S4–S6). Taken together these data indicate that expression of mutant p53 proteins in these cells alters their morphology to favor disorganized random movement on two-dimensional (2D) surfaces, and this is accompanied by increased invasiveness in 3D.

Mutant p53 Promotes β1-Integrin-Dependent Cell Motility and Invasion

The invasive capacity of H1299 and RPE cells was dependent on the presence of EGF as a chemoattractant (Figure 3A) and fibronectin (Figure 3B) in the Matrigel, suggesting a role for epidermal growth factor receptor (EGFR) and a fibronectin-binding integrin (such as α5β1) in mutant p53-dependent invasion. We therefore examined the effect of a specific α5-integrin-blocking antibody (mAb16) and an EGFR inhibitor (PD153035) on the invasion of mutant p53-expressing H1299 cells into fibronectin-containing Matrigel. Whereas these inhibitors had no effect on the invasiveness of control cells, the ability of mutant p53 to drive invasion was ablated by inhibition of either α5β1 or EGFR (Figure 3C). Consistently, blockade of β1-integrin (with the AIIB2 antibody that inhibits all β1-integrin heterodimers, including α5β1) also abolished the formation of invasive structures by MCF10A cells (Figure 3D). The ability of mutant p53 to drive random migration of H1299 cells was also opposed by inhibition of EGFR or α5β1, which reverted the migratory behavior of mutant p53-expressing cells to that seen in control cells (Figures 3E and S3). Therefore the altered migration and enhanced invasion driven by mutant p53 depends on EGFR signaling and the ability of β1-integrins to engage their ligands.

There is considerable crosstalk between integrins and the signaling of receptor tyrosine kinases (RTKs) (Schwartz and Ginsberg, 2002), so we examined the effect of mutant p53 expression on integrin and EGFR signaling in trypsinized cells that were either allowed to remain in suspension or were replated onto fibronectin-coated dishes. Western blotting indicated that EGFR phosphorylation and activation of AKT (both basally and following plating onto fibronectin) were markedly higher in mutant p53-expressing cells, but activity of the MAPK pathway, as evidenced by phosphorylation of ERK, was less strongly affected (Figure 3F). These results predict that cells with mutant p53 would show more active signaling downstream of EGFR, particularly to AKT. To investigate this directly, we stained tissue microarrays of human colorectal cancer samples for p53 and compared this with the levels of active phospho-AKT. We used high p53 staining as an indication of the presence of potential gain-of-function p53 mutations—such as the ones under study here. In contrast to loss-of-function mutations, these p53 mutants are stable in cancer cells and accumulate in the nucleus (Soussi, 2000). Consistent with our model, we detected a clear correlation between elevated p53 staining and high levels of phospho-AKT (Figure 3G).

Enhanced Recycling and Invasion Is Dependent on Rab-Coupling Protein

Integrin function and signaling are dictated by the way in which the heterodimers are trafficked through the endosomal pathway (Caswell and Norman, 2008; White et al., 2007). We have found that α5β1 and EGFR recycle to the plasma membrane in complex with one another, and a consequence of enhanced cotrafficking of α5β1 and EGFR is random migration on 2D surfaces and increased invasion into fibronectin-containing matrices. This is associated with enhanced signaling from EGFR to the proinvasive kinase, AKT (Caswell et al., 2008). We therefore compared the internalization and recycling of a number of receptors in control H1299 cells with those that express mutant p53. Whereas the rate of receptor internalization was unaltered (Figure S4A), mutant p53 increased the rate at which internalized α5β1 and EGFR (but not the transferrin receptor) returned to the plasma membrane (Figure 4A and Figure S4B). Furthermore, the ability of mutant p53 to enhance α5β1 recycling was not a consequence of altered EGFR signaling, as knockdown of this RTK did not alter α5β1 trafficking (Figure S4C).

We have previously reported that enhancement and coordination of α5β1 and EGFR trafficking depend on their association with the Rab11 effector Rab-coupling protein (RCP, Rab11-FIP1) (Caswell et al., 2008). Consistent with this, we found that p53-driven recycling of α5β1 and EGFR was completely dependent on RCP, whereas receptor trafficking in control cells was unaffected by knockdown of RCP (Figure 4B). Furthermore, knockdown of RCP also opposed the ability of p53 to drive invasion into Matrigel (Figure 4C) and restored the 2D migration pattern (Figure 4D) and actin dynamics (Figure 4E and Movies S7–S14) of mutant p53-expressing cells to that of control, p53 null H1299 cells.

The incorporation of RCP into a complex with α5β1 and EGFR is associated with enhanced recycling of the receptors (Caswell et al., 2008). Interestingly, we found that although the interaction of EGFR with RCP was not affected, the presence of mutant p53 strongly promoted interaction between α5β1 and RCP (Figure 4F). Importantly, no interaction was observed between mutant p53 and RCP, suggesting that mutant p53 indirectly facilitates the recruitment of RCP to α5β1 (Figure 4F) to drive invasion.

Endogenous Mutant p53 Promotes RCP-Dependent Invasion

The results presented above depended on ectopic expression of mutant p53. The levels of mutant p53 expressed in our engineered H1299 cells were not higher than those seen in a series of tumor cell lines expressing endogenous mutant p53 (Figure 5A), suggesting that the phenotypes observed were not due to gross overexpression of the mutant p53. However, we wished to assess the contribution of endogenous mutant p53 to invasive behavior and so we used A431 cells, which were

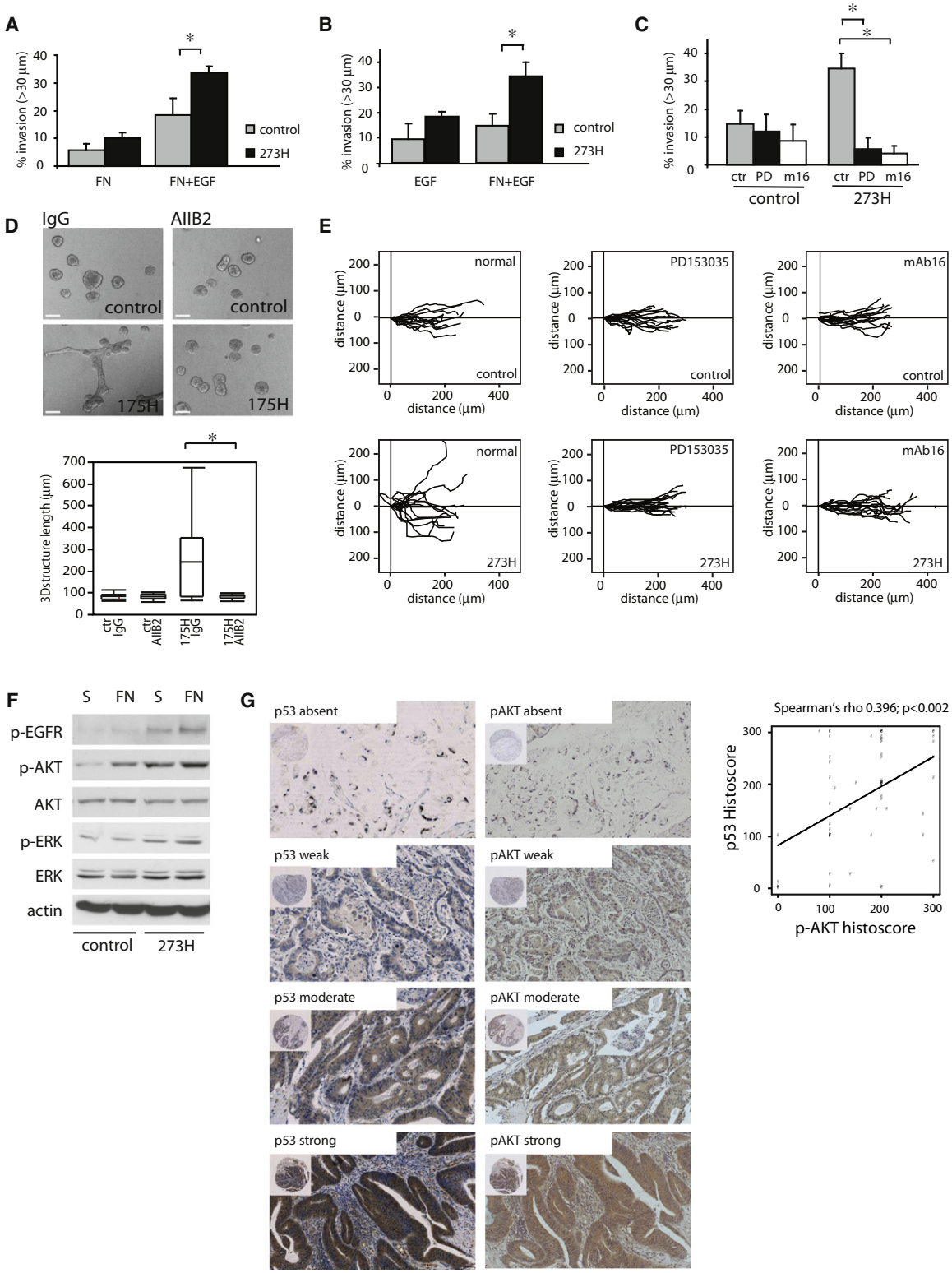


Figure 3. Invasive Behavior of Mutant p53-Expressing Cells Is Dependent on Integrin/EGFR Signaling

(A and B) The dependence of mutant p53-driven invasion on fibronectin or EGF was determined by plating H1299 vector control or mutant p53 (273H) cells in the absence of EGF as chemo-attractant (A) or in the absence of fibronectin (B). Values are means ± SEM of 9 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

able to invade efficiently into Matrigel supplemented with fibronectin (data not shown and Figure 5B). Previous studies have shown that the invasion of A431 cells injected into nude mice was greatly impaired after β1-integrin knockdown (Brockbank et al., 2005). Accordingly, inhibiting EGFR or α5-integrin significantly impeded the invasion of A431 cells (Figure 5B). Importantly, siRNA-mediated reduction of endogenous mutant p53 or RCP levels efficiently blocked the invasion of these cells (Figure 5C). Moreover, knockdown of mutant p53 expression resulted in a reduced recycling of α5β1 and EGFR (Figure 5D), and an impaired interaction of RCP with α5β1 (Figure 5E), consistent with a role for endogenous mutant p53 in recruitment of RCP, α5β1, and EGFR1 into a physical complex that facilitates the return of these receptors to the cell surface.

To confirm that endogenous mutant p53 promotes invasive behavior, we turned to MDA MB231 cells, which express high levels of a DNA-binding domain p53 mutant (R280K) that has lost wild-type activity (Epstein et al., 1998) (Figure 5F). MDA MB231 cells invade efficiently into both Matrigel and collagen supplemented with fibronectin, and siRNA depletion of either endogenous mutant p53 or RCP significantly reduced this invasion (Figure 5G). Taken together, the results in A431 and MDA MB231 cells support the conclusion that endogenous levels of mutant p53 drive tumor cell invasion through an RCP-dependent mechanism.

Invasive Activity of Mutant p53 Correlates with Inhibition of p63 Function

Previous studies have identified two mechanisms that contribute to the gain of function of mutant p53, one that requires the N-terminal trans-activation domain of p53 (Lin et al., 1995; Matas et al., 2001), and the other reflecting its interaction with family members p63 or p73 (Li and Prives, 2007). As reported previously, we detected an interaction between p63 or p73 and mutant p53 (Figure 6A) (Gaiddon et al., 2001; Strano et al., 2002), with a stronger interaction of the 175H conformational mutant compared to the 273H DNA-contacting mutant (Figure 6A). The interaction between mutant p53 and p63 can be enhanced in response to TGF-β (Adorno et al., 2009). However, we did not detect such an increased interaction (Figure S5A) and we did not observe a significant effect of TGF-β treatment or inhibition on migration (data not shown), suggesting that our cells are not sensitive to these effects of TGF-β.

A series of N-terminal and C-terminal truncations within the context of p53 175H (Figure 6B) were constructed and examined. Several previous studies have shown that the interaction between mutant p53 and p63 is mediated through the central DNA-binding domain (Gaiddon et al., 2001; Strano et al., 2002), and we confirmed that both N- and C-terminal deletions of p53 175H retained p63-binding activity (Figure S5B). To test the effect of these p53 proteins on p63 activity, we assessed their ability to inhibit the ability of the TA isoform of p63 (containing the full-length trans-activation domain within the N terminus) to drive expression from a p63-responsive promoter (Figure 6C). As shown previously, mutant p53 expression inhibited TAp63 activity, through a mechanism that did not require the N terminus of mutant p53. Interestingly, however, the strength of interaction between the proteins as assessed by coprecipitation was not a strong indicator of the effectiveness of p63 inhibition by mutant p53. For example, p53 273H reproducibly decreased TAp63-dependent transcriptional activity more effectively than p53 175H, despite apparently binding more weakly. Conversely, the C-terminal truncations of p53 175H and p53 273H (Δ347 and Δ370) were less effective in inhibiting TAp63 without a clear defect in binding (Figures 6B and 6C).

Next, we tested the ability of the p53 truncation mutants to promote invasion. Deletion (Δ43) of the N terminus of p53 in the context of either 175H or 273H did not disrupt the activity of mutant p53 in this assay (Figure 6D), showing that this trans-activation domain of p53 is not required. Similarly, a double point mutant within this domain (22/23) also retained invasive activity (data not shown). In contrast, C-terminal deletions (Δ347 and Δ370) impaired the ability of the 175H and 273H p53 mutants to drive invasion (Figure 6D). Thus, the activity of each of the p53 mutants in the invasion assay correlated with their ability to inhibit the transcriptional function of TAp63 (Figure 6C).

Loss of p63 Promotes Invasion and Random Migration

Our results suggested that the activity of mutant p53 may reflect an inhibition of p63 or p73. Loss of p63 or p73 can enhance invasion and transformation in the absence of p53 (Flores et al., 2005; Lang et al., 2004), and so we tested whether loss of p63 or p73 could substitute for the expression of mutant p53 in our assays. p63 is expressed as three alternatively spliced isoforms (α, β, and γ) from two promoters that generate full-length or truncated N termini (TA and ΔN) (Bourdon, 2007). Using a series of reverse transcriptase reactions, we determined that H1299 cells express

(C) The invasion of mutant p53 (273H) H1299 cells into Matrigel in the presence of a monoclonal antibody directed against α5-integrin (Mab16) or an EGFR inhibitor (PD 153035), using EGF as chemo-attractant. Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

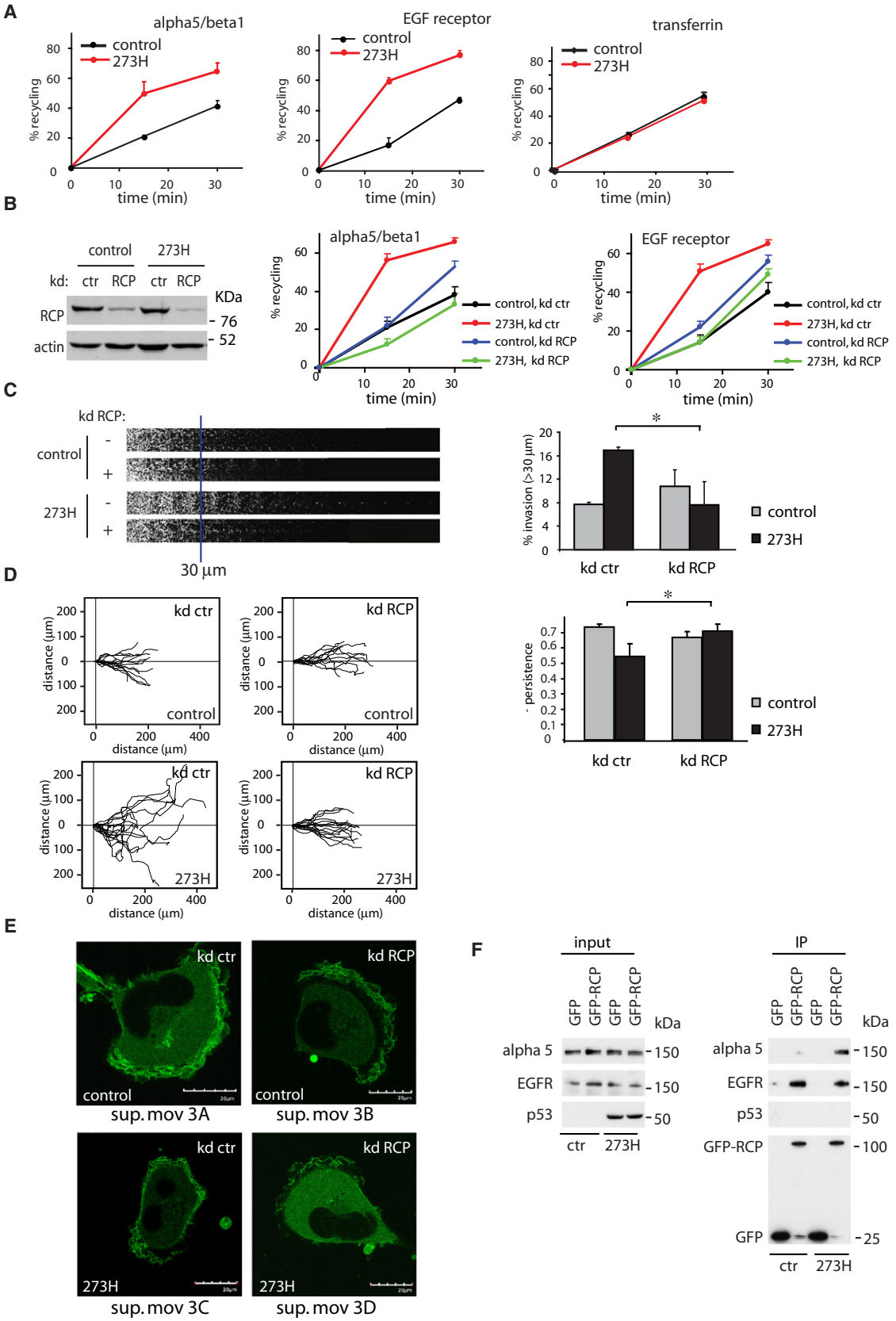
(D) Vector control or mutant p53 (175H) MCF10A cells were seeded into collagen 1-supplemented Matrigel in the presence of a β1-integrin blocking antibody (AIIB2) or control immunoglobulin and invasive structures were quantified as for Figure 1. Scale bars represent 100 μm.

(E) Representative track-plots were extracted from movies of vector control and mutant p53 (175H and 273H)-expressing H1299 cells migrating into scratch wounds under normal conditions or in the presence of Mab16 or PD 153035.

(F) The activity of the EGFR was determined in attachment assays in which mutant p53 (273H) and vector control H1299 cells were trypsinized, serum-starved, and then plated onto fibronectin (FN) coated surfaces or kept in suspension (S) for 30 min. Cells were lysed and phosphorylation of EGFR (residue 845), AKT (residue 437), and ERK 1/2 was determined by immunoblot analysis using phospho-specific antibodies. Actin expression was used as loading control in a parallel blot.

(G) Immunostaining of 60 primary human colon cancer sections with antibodies against p53 and p-AKT. Staining was scored blind as absent, weak, moderate, or strong and the weighted histoscore used to determine the Spearman correlation between p53 levels and p-AKT staining (right panel).

See also Figure S3.


A
alpha5/beta1
EGF receptor
transferrin
control
273H
% recycling
time (min)
B
control
273H
kd: ctr RCP ctr RCP
KDa
RCP
actin
- 76
- 52
control, kd ctr
273H, kd ctr
control, kd RCP
273H, kd RCP
C
kd RCP:
control
273H
30 µm
% invasion (>30 µm)
kd ctr
kd RCP
D
kd ctr
kd RCP
control
273H
distance (µm)
- persistence
E
sup. mov 3A
sup. mov 3B
sup. mov 3C
sup. mov 3D
F
input
IP
GFP
GFP-RCP
kDa
alpha 5
EGFR
p53
GFP-RCP
GFP
ctr
273H

mainly TAp63 α and β (data not shown), so we used siRNAs targeted to these isoforms to reduce p63 expression in these cells (Figure 7A). Inhibition of p63 expression in H1299 cells resulted in enhanced invasion into Matrigel to levels similar to those seen following mutant p53 expression (Figure 7A), although reduction of p63 expression did not further affect invasion by mutant p53-expressing cells. Also, an siRNA resulting in a partial knockdown of p63 caused only a partial activation of invasion (Figure 7A). Furthermore, knockdown of p63 expression led to random migration in p53 null H1299 cells, as seen in cells expressing mutant p53 (Figure 7B). By contrast, modulation of p73 expression by siRNA knockdown in these cells did not obviously affect invasion (Figure 7A) or random migration (Figure 7B). As seen following mutant p53 expression, knockdown of p63 resulted in increased recycling of α5β1-integrin and EGFR (Figure 7C). Finally, we also found that overexpression of TAp63α reversed the effects of mutant p53 expression on the ability of cells to invade into Matrigel (Figure 7D). These results indicate that inactivation of p63 cooperates with loss of wild-type p53 to drive integrin recycling, invasion, and random migration and suggest that these activities of mutant p53 may reflect its ability to inactivate TAp63. It is important to note, however, that the effects of modulating p63 are highly context dependent, with previous studies showing that inhibition of p63 expression in MCF10A cells (that express only ΔNp63) results in cell detachment and death (Carroll et al., 2006).

DISCUSSION

This study describes a gain of function of mutant p53 in promoting both random cell migration and invasion. Underlying these activities is the ability of mutant p53 to promote recycling of integrins and EGFR in a way that is dependent on RCP—and importantly knock down of RCP prevented p53-driven recycling but had no effect on the basal receptor trafficking in these cells. Mutant p53 promoted enhanced binding of integrin to RCP, and since the effect of mutant p53 on integrin recycling did not depend on EGFR, it seems likely that—as seen in cells treated with osteopontin (Caswell et al., 2008)—the RTK is recycled to the plasma membrane as a consequence of the integrin/RCP interaction, and not vice versa.

Interestingly, RCP has recently been identified as an oncogene that can contribute to the aggressiveness of breast cancers by regulating invasion and metastatic potential (Zhang et al., 2009). Previously we found that RCP-dependent α5β1/EGFR trafficking augments EGFR phosphorylation, leading to increased AKT signaling (Caswell et al., 2008). Consistent with this, mutant p53 drives constitutive activation of EGFR and its communication with AKT (but not ERK). As predicted by this model, IHC staining of primary human colons showed a correlation between high expression of p53 (an indicator of the presence of mutant p53) and strong phospho-AKT staining. These results suggest that mutant p53 specifically drives RCP-dependent recycling of integrin and EGFR (and possibly other receptors), resulting in alteration of AKT signaling, which contributes to increased invasion and metastasis.

Our results support a role for the mutant p53/p63 interaction in controlling invasion, consistent with previous studies showing that loss of p63 and/or p73 in the context of p53 deletion might be functionally equivalent to the expression of mutant p53 (Flores et al., 2005; Lang et al., 2004). p63 plays an important role in cell adhesion and survival (Carroll et al., 2006), and the ability of TGF-β to enhance the interaction of p63 with mutant p53 has been shown to correlate with invasion (Adorno et al., 2009). Although the activities of mutant p53 described here do not depend on TGF-β, it will be of interest to determine whether enhanced integrin recycling driven by mutant p53 might also affect TGF-β signaling.

Despite compelling evidence that p63 is an important target for mutant p53, it is clear that expression of mutant p53 is not equivalent to loss of p63 under all circumstances—as illustrated by MCF10A cells that show enhanced invasion following expression of mutant p53, but loss of attachment, detachment, and death in response to p63 depletion (Carroll et al., 2006). This may be due to which isoforms of p63 are expressed, since the function of TAp63 that is expressed in H1299 cells is likely to be different from that of ΔNp63 expressed in MCF10A cells. Intriguingly, mutant p53 expression also prevents the loss of integrin expression that normally occurs following matrix detachment of MCF10A cells, and it is tempting to evoke a role for additional activities of mutant p53 that may be independent of p63 (Brosh and Rotter, 2009).

Our results show that the ability of mutant p53 proteins to contribute to the development of invasive and metastatic cancers in vivo is paralleled by their ability to enhance RCP-dependent recycling of integrin, thereby promoting trafficking and

Figure 4. RCP Is Required for Mutant p53-Mediated Integrin and EGFR Recycling

(A) The recycling of α5β1, EGFR, or the transferrin receptor in vector control or mutant p53 (273H) H1299 cells was determined as described in the Experimental Procedures. Values are means ± SEM of 9 replicates from three independent experiments.

(B) Vector control or mutant (273H) H1299 cells were transfected with siRNAs targeting RCP or a nontargeting control (ctr). RCP levels were determined by western blotting (left panel), and actin expression was used as loading control. The recycling of α5β1 or EGFR was determined as for (A) (right panels).

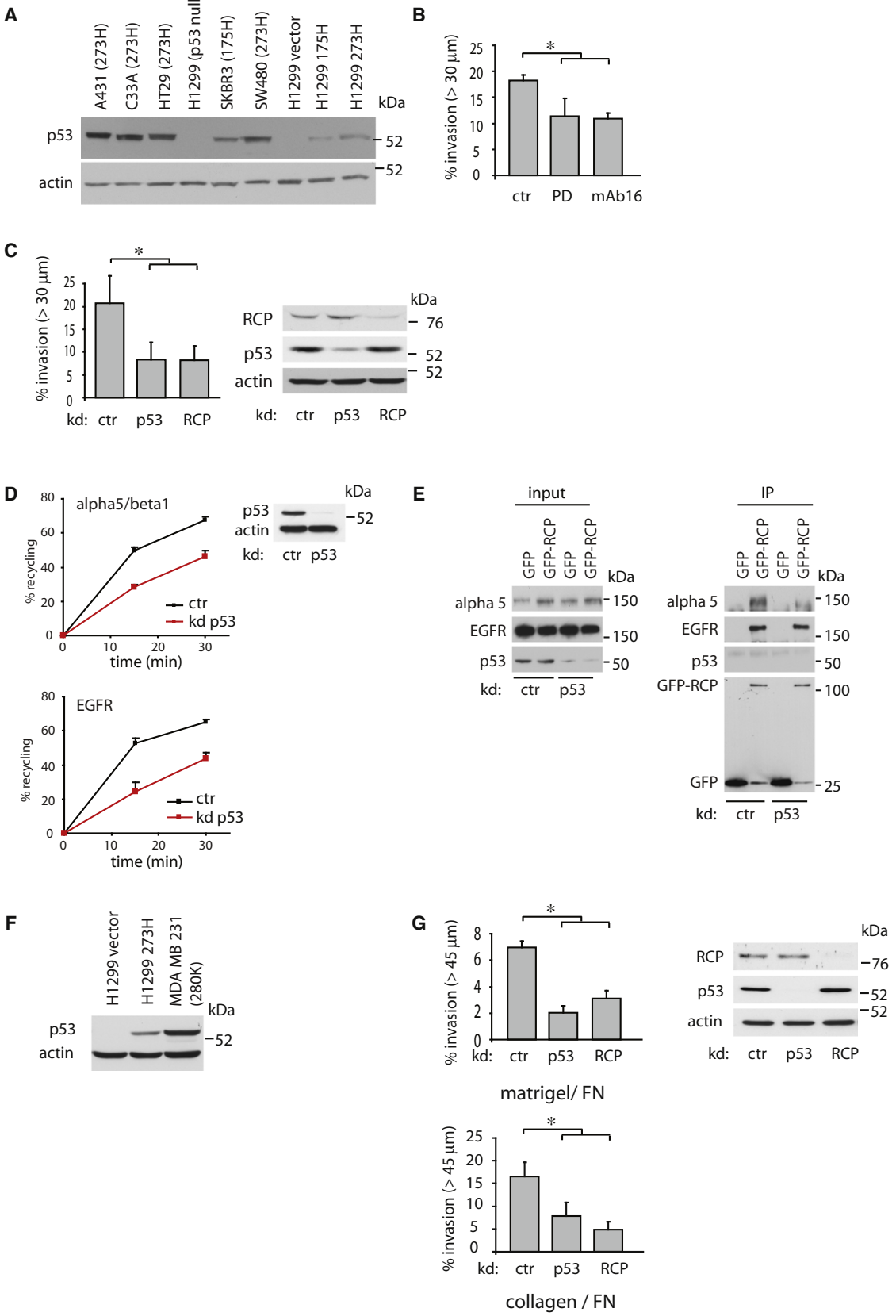
(C) The invasion into Matrigel of vector control or mutant p53 (273H) H1299 cells that were transfected with siRNAs targeting RCP or a nontargeting control (right panel). Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(D) Representative track-plots showing the effect of RCP knockdown on the migration of vector control and mutant p53 (175H and 273H)-expressing H1299 cells migrating into scratch wounds (left panels). Values of migrational persistence were extracted from the track-plots as for Figure 2A (lower right panel). Values are means ± SEM from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(E) Stills from Movies S7–S10, showing the motility of mutant p53 (273H) cells that were transfected with siRNAs targeting RCP in combination with GFP-actin and monitored by a 5 min time-lapse microscopy 1.5 hr after scratching the wound. Scale bars represent 20 μm.

(F) Immunoprecipitation of GFP from vector or mutant p53-expressing H1299 cells transfected with GFP or GFP-RCP. The input protein levels (left panel) and those present in immunoprecipitates (right panel) were determined by western blotting.

See also Figure S4 and Movies S7–S14.


A
A431 (273H)
C33A (273H)
HT29 (273H)
H1299 (p53 null)
SKBR3 (175H)
SW480 (273H)
H1299 vector
H1299 175H
H1299 273H
kDa
p53
actin
52
52
B
% invasion (> 30 µm)
ctr
PD
mAb16
C
% invasion (> 30 µm)
kd: ctr p53 RCP
RCP
p53
actin
76
52
52
D
alpha5/beta1
% recycling
time (min)
ctr
kd p53
EGFR
E
input
IP
GFP
GFP-RCP
alpha 5
EGFR
p53
GFP-RCP
GFP
150
150
50
100
25
F
H1299 vector
H1299 273H
MDA MB 231 (280K)
G
% invasion (> 45 µm)
matrigel/ FN
collagen / FN

signaling of growth factor receptors. This can drive both random migration and invasion, and the precise response to mutant p53 expression will be influenced by the cellular context. While mutant p53 has other activities that can impinge on cell behavior and survival (Brosh and Rotter, 2009), our study provides an understanding of the molecular details of at least one aspect of the invasive behavior of mutant p53, an important first step to the design of therapies that might interfere with this activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Constructs

H1299, A431, SK-BR3, SW480, MDA MB231, RPE, and MCF10A cells were cultured as described in the Supplemental Experimental Procedures. For the generation of stable cell lines, H1299, RPE, and MCF10A cells were transfected with an empty vector (PCB6+) or constructs expressing p53175H or 273H (in PCB6+; 72R polymorphism) with effectene reagent (QIAGEN). Cells were selected by medium containing 600 μg/ml G418. p53 mutants have been previously described or created using site-directed mutagenesis, as detailed in the Supplemental Data.

Inverted Invasion Assays

Matrigel or collagen assays were performed as described previously (Caswell et al., 2008) and in the Supplemental Experimental Procedures. Briefly, cells were seeded on the base of matrigel or collagen-filled transwell chambers and invasion toward a gradient of 10% FCS and 25 ng/ml EGF was measured by confocal microscopy in serial sections.

Immunoprecipitation and Immunblot Analyses

p53 and GFP-RCP were immunoprecipitated from lysates of transfected cells using the 1801 and anti-GFP (Abcam) antibodies, respectively. Details of the protocol and source of antibodies used for immunoblotting are provided in the Supplemental Experimental Procedures.

Scratch-Wound Assays, Fluorescence Time-Lapse, and Immunofluorescence

Movement of cells into a wound scratched into a monolayer and grown in 1% FCS was monitored with a phase contrast 20× objective using an inverted microscope (Nikon TE2000). Images were obtained every 10 min for the duration of 16 hr.

Cells transfected with GFP-actin and siRNAs were scratched and actin behavior assessed using an inverted confocal fluorescence microscope (Fluoview FV1000, Olympus), taking pictures every 10 s for 5 min. Immunofluorescent imaging of the Golgi was carried out using a GM-130 antibody.

Tumor Formation In Vivo

Female CD1 nude mice were obtained from Charles River (Margate Kent) and all experiments were carried out according to Home Office regulations. To study igenesis, in total 20 mice were divided in 2 groups of 10 mice that were injected with stably transfected vector control H1299 or mutant p53 273H H1299. 1×10^6 cells were washed in HBS and diluted in Matrigel (1:1) and 100 μl of this mixture was injected into the fat pad. Every 2 to 3 days diameters were measured and when they reached a 1.7 cm diameter, primary tumors, mammary lymph nodes, mammary tissue, and lungs were harvested for immune histochemistry. Tissues were fixed in paraformaldehyde and stained with hematoxylin and eosin (H&E).

For intestinal tumorigenesis experiments, AhCre, $Apc^{580\ s}$ (referred to as Apc^{fl}) (Sansom et al., 2004), $p53^{fl}$ (Jonkers et al., 2001), and Lox stop lox $p53^{R172H}$ (Olive et al., 2004) alleles were used. Mice had been backcrossed to C57BlJ for at least five generations. Mice were given three injections of β-napthoflavone in a single day at 6–8 weeks of age to induce near 100% recombination within the intestinal epithelium (Sansom et al., 2006). Intestinal tumors develop in this model through loss of the remaining wild-type Apc allele. Mice were aged until they developed signs of intestinal disease, namely paling of feet due to anemia, weight loss, and hunching. At this stage, intestines were removed, flushed with PBS, and opened *en face*. Tumor number and size were then scored macroscopically and intestines fixed as previously described (Sansom et al., 2004).

Tissue Microarrays

Zymed's MaxArray Human Colon Carcinoma Tissue Microarray slides (Invitrogen, 75-4033) containing 60 tissue samples were deparaffinized and rehydrated according to manufacturer's instructions. Staining intensities were analyzed using the Spearman's rank correlation coefficient.

Receptor Internalization and Recycling Assays

Internalization and recycling of α5β1, EGFR, and transferrin were determined in cells transfected with the appropriate siRNAs and serum starved for 30–45 min before the start of the experiment, as previously described (Caswell et al., 2008; Roberts et al., 2001).

Quantitative RT-PCR

To assess mRNA levels, RNA was isolated from cells using a QIAGEN kit or Trizoll reagent, and cDNA synthesized using the Invitrogen kit. For the RT-PCR reaction, 5 μl of the 40 times diluted cDNA was used in combination with 10 μl Sybr green Mastermix, 1 μl of each oligo (10 μM) as described in the Supplemental Experimental Procedures.

p63 Luciferase Reporter Assays

Cells were transfected with 300 ng K14 Firefly luciferase and 30 ng Renilla luciferase constructs, together with p63 and p53 expression constructs as described in the figure legends and Supplemental Experimental Procedures. Firefly luciferase measurements were corrected for Renilla luciferase values and expressed as relative light units (RLU).

Figure 5. Downregulation of Endogenous Mutant p53 Expression Reduces RCP-Dependent Invasion

(A) The exogenous expression of mutant p53 in H1299 cells compared with the expression of endogenous mutant p53 in various cell lines. Actin expression was used as loading control in a parallel blot.

(B) The invasion of A431 cells in the presence of PD153035 or Mab16 into Matrigel. Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(C) A431 cells were transfected with siRNAs targeting p53 or RCP and their invasiveness into Matrigel was determined. The expression of p53 and RCP is depicted in the right panel (on parallel blots), with actin used as loading control, on the RCP blot. Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(D) A431 cells were transfected with siRNAs targeting endogenous mutant p53 and the recycling of α5β1 or EGFR, and p53 expression levels were determined.

(E) Immunoprecipitation of GFP from control or mutant p53 knockdown A431 cells transfected with GFP-RCP or GFP. The input protein levels (left panel) and those present in immunoprecipitates (right panel) were determined by western blotting.

(F) The endogenous expression of mutant p53 in MDA MB231 cells compared to the expression of mutant p53 in transfected H1299 cells. Actin was used as loading control.

(G) The invasion of MDA MB231 cells transfected with siRNA directed against p53 or RCP into Matrigel (top) or collagen (bottom). Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test. The expression of p53 and RCP is depicted in the right panel (on parallel blots); actin was used as loading control on the RCP blot.

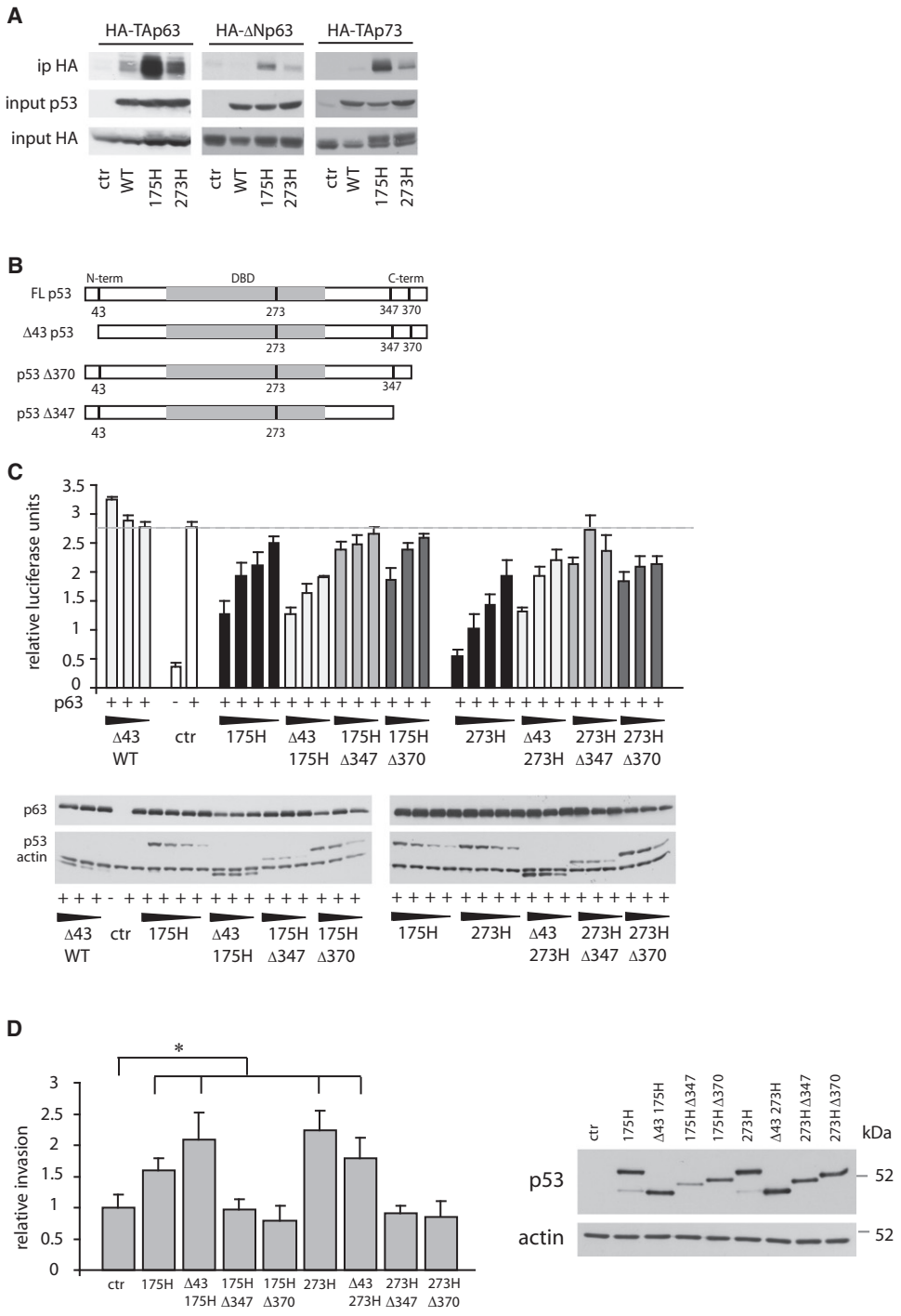


Figure 6. Mutant p53 Proteins that Drive Invasion Inhibit p63 Activity

(A) Immunoprecipitation of p53 in H1299 cells transfected with HA-TAp63 alpha, HA-ΔNp63 alpha, or HA-p73 in combination with a vector control, wild-type (WT) p53, p53 175H, or p53 273H.

(B) Cartoon depicting the position of the targeted deletions in mutant p53.

(C) H1299 cells were transfected with K14 Firefly luciferase, TK Renilla luciferase, and p63 in combination with decreasing concentrations of the indicated p53 constructs. Firefly luciferase activity was corrected for Renilla luciferase expression and the values are means ± SEM from three independent experiments. The expression of HA-p63 and the expression of (mutant) p53 were determined in immunoblot analysis and actin was used as loading control, on a parallel blot.

(D) The invasion into Matrigel of H1299 cells (left panel) transfected with the indicated p53 proteins (right panel). Invasion is expressed relative to p53-negative control cells. Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

See also Figure S5.

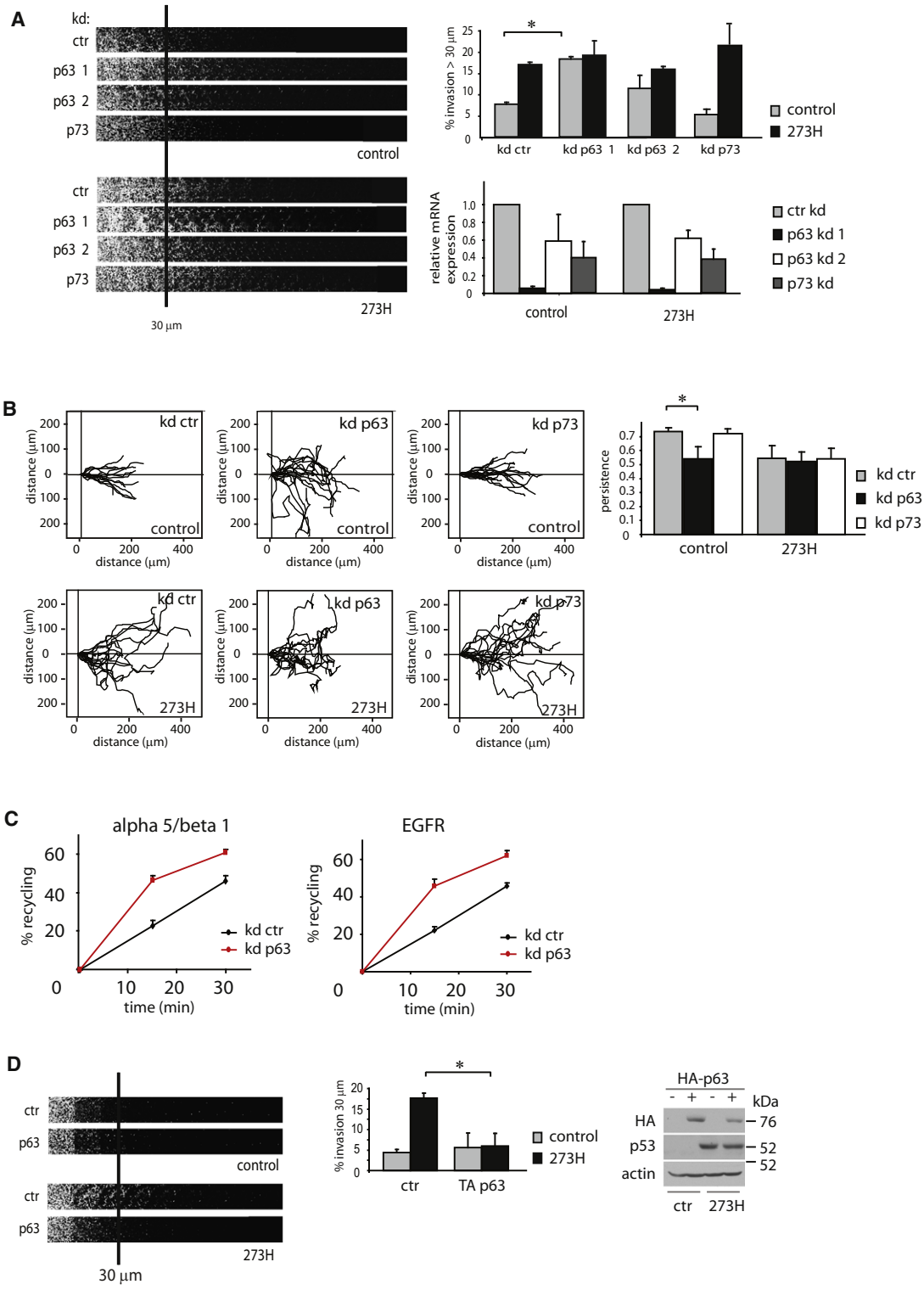


Figure 7. p63, but Not p73, Controls Invasion and Persistence of Migration

(A) The invasion of control or mutant p53 (273H) H1299 cells that were transfected with two different siRNAs targeted against p63 or an siRNA targeted against p73 was measured (left panel) and quantified (upper right panel). Values are means ± SEM of 18 replicates from three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test. The mRNA expression of p63 was determined by RT-PCR analysis (lower right panel). The relative expression in control cells was designated as 1 and values were corrected for GAPDH expression.

SUPPLEMENTAL DATA

Supplemental Data include five figures, Supplemental Experimental Procedures, and 14 movies and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01438-X.

ACKNOWLEDGMENTS

This work in K.H.V.'s, J.C.N.'s, and O.J.S.'s laboratories is supported by Cancer Research UK, and the work in J.S.B.'s laboratory by BCRF and NIGMS (GM064346). P.A.J.M. is a recipient of a Rubicon Fellowship from the Netherlands Organisation for Scientific Research. The p53R172H mice were obtained from the MMHRC and the p53 flox mice kindly provided by Anton Berns. We would like to thank Derek Miller for assistance and advice with the in vivo studies and Sam Lawn and Lyne McGarry for help with the Matrigel invasion assay. We are also grateful to Gerry Melino for the p63-responsive reporter construct.

Received: April 17, 2009
Revised: September 11, 2009
Accepted: November 10, 2009
Published: December 24, 2009

REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., et al. (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87–98.

Attardi, L.D., and Jacks, T. (1999). The role of p53 in tumour suppression: lessons from mouse models. Cell. Mol. Life Sci. *55*, 48–63.

Blagosklonny, M.V. (2000). p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. FASEB J. *14*, 1901–1907.

Bourdon, J.C. (2007). p53 and its isoforms in cancer. Br. J. Cancer *97*, 277–282.

Brockbank, E.C., Bridges, J., Marshall, C.J., and Sahai, E. (2005). Integrin beta1 is required for the invasive behaviour but not proliferation of squamous cell carcinoma cells in vivo. Br. J. Cancer *92*, 102–112.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat. Rev. Cancer *9*, 701–713.

Cantor, J.P., Iliopoulos, D., Rao, A.S., Druck, T., Semba, S., Han, S.Y., McCorkell, K.A., Lakshman, T.V., Collins, J.E., Wachsberger, P., et al. (2007). Epigenetic modulation of endogenous suppressor expression in lung cancer xenografts suppresses igenicity. Int. J. Cancer *120*, 24–31.

Carroll, D.K., Carroll, J.S., Leong, C.O., Cheng, F., Brown, M., Mills, A.A., Brugge, J.S., and Ellisen, L.W. (2006). p63 regulates an adhesion programme and cell survival in epithelial cells. Nat. Cell Biol. *8*, 551–561.

Caswell, P., and Norman, J. (2008). Endocytic transport of integrins during cell migration and invasion. Trends Cell Biol. *18*, 257–263.

Caswell, P.T., Spence, H.J., Parsons, M., White, D.P., Clark, K., Cheng, K.W., Mills, G.B., Humphries, M.J., Messent, A.J., Anderson, K.I., et al. (2007). Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. Dev. Cell *13*, 496–510.

Caswell, P.T., Chan, M., Lindsay, A.J., McCaffrey, M.W., Boettiger, D., and Norman, J.C. (2008). Rab-coupling protein coordinates recycling of alpha5-beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. J. Cell Biol. *183*, 143–155.

Cho, Y., Gorina, S., Jeffery, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 suppressor-DNA complex: understanding igenic mutations. Science *265*, 346–355.

Epstein, C.B., Attiyeh, E.F., Hobson, D.A., Silver, A.L., Broach, J.R., and Levine, A.J. (1998). p53 mutations isolated in yeast based on loss of transcription factor activity: similarities and differences from p53 mutations detected in humans. Oncogene *16*, 2115–2122.

Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Predisposition in mice mutant for p63 and p73: evidence for broader suppressor functions for the p53 family. Cancer Cell *7*, 363–373.

Gaiddon, C., Lokshn, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-reulate p63 and p73 through a direct interaction with the p53 core domain. Mol. Cell. Biol. *21*, 1874–1887.

Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. *29*, 418–425.

Kupfer, A., Louvard, D., and Singer, S.J. (1982). Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. Proc. Natl. Acad. Sci. USA *79*, 2603–2607.

Lane, D.P. (1994). p53 and human cancers. Br. Med. Bull. *50*, 582–599.

Lang, G.A., Iwakuma, T., Suh, Y.-A., Liu, G., Rao, A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., et al. (2004). Gain-of-function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861–872.

Li, Y., and Prives, C. (2007). Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene *26*, 2220–2225.

Lin, J., Teresky, A.K., and Levine, A.J. (1995). Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene *10*, 2387–2390.

Marsh, V., Winton, D.J., Williams, G.T., Dubois, N., Trumpp, A., Sansom, O.J., and Clarke, A.R. (2008). Epithelial Pten is dispensable for intestinal homeostasis but suppresses adenoma development and progression after Apc mutation. Nat. Genet. *40*, 1436–1444.

Matas, D., Sigal, A., Stambolsky, P., Milyavsky, M., Weisz, L., Schwartz, D., Goldfinger, N., and Rotter, V. (2001). Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J. *20*, 4163–4172.

Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J., and Brugge, J.S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat. Cell Biol. *3*, 785–792.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain-of-function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847–860.

Petitjean, A., Achatz, M.I., Borresen-Dale, A.L., Hainaut, P., and Olivier, M. (2007). TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene *26*, 2157–2165.

Roberts, M., Barry, S., Woods, A., van der Sluijs, P., and Norman, J. (2001). PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early

(B) Representative track-plots showing the effect of p63 and p73 knockdown on the migration of vector control and mutant p53 (175H and 273H)-expressing H1299 cells migrating into scratch wounds (left panels). Values are means ± SEM of three independent experiments. * indicates $p < 0.05$ as determined by a Student's t test.

(C) Vector control H1299 cells were transfected with an siRNA targeting p63 and the recycling of $\alpha5\beta1$ or EGFR was determined. Values are means ± SEM from three independent experiments.

(D) The invasion into Matrigel of vector control or mutant p53 (273H) H1299 cells that were transfected with HA TA-p63 (left and middle panel). Values are means ± SEM of 18 replicates from four independent experiments. * indicates $p < 0.05$ as determined by a Student's t test. Expression of p63 and p53 was determined by western blotting (right panel); actin was used as a loading control on a parallel blot.

endosomes is necessary for cell adhesion and spreading. Curr. Biol. *11*, 1392–1402.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., et al. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. *18*, 1385–1390.

Sansom, O.J., Meniel, V., Wilkins, J.A., Cole, A.M., Oien, K.A., Marsh, V., Jamieson, T.J., Guerra, C., Ashton, G.H., Barbacid, M., et al. (2006). Loss of Apc allows phenotypic manifestation of the transforming properties of an endogenous K-ras oncogene in vivo. Proc. Natl. Acad. Sci. USA *103*, 14122–14127.

Schwartz, M.A., and Ginsberg, M.H. (2002). Networks and crosstalk: integrin signalling spreads. Nat. Cell Biol. *4*, E65–E68.

Seton-Rogers, S.E., Lu, Y., Hines, L.M., Koundinya, M., LaBaer, J., Muthuswamy, S.K., and Brugge, J.S. (2004). Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. Proc. Natl. Acad. Sci. USA *101*, 1257–1262.

Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 suppressor: the demons of the guardian of the genome. Cancer Res. *60*, 6788–6793.

Soussi, T. (2000). The p53 suppressor gene: from molecular biology to clinical investigation. Ann. N Y Acad. Sci. *910*, 121–137.

Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M.G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M., et al. (2002). Physical interaction with human derived p53 mutants inhibits p63 activities. J. Biol. Chem. *277*, 18817–18826.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H., and Prives, C. (2009). Blinded by the light: the growing complexity of p53. Cell *137*, 413–431.

White, D.P., Caswell, P.T., and Norman, J.C. (2007). alpha v beta3 and alpha5-beta1 integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration. J. Cell Biol. *177*, 515–525.

Zhang, J., Liu, X., Datta, A., Govindarajan, K., Tam, W.L., Han, J., George, J., Wong, C., Ramnarayanan, K., Phua, T.Y., et al. (2009). RCP is a human breast cancer-promoting gene with Ras-activating function. J. Clin. Invest. *119*, 2171–2183.