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Dominant effects of Δ40p53 on p53 function and melanoma cell fate

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Abstract

The p53 gene encodes 12 distinct isoforms some of which can alter p53 activity in the absence of genomic alteration. Endogenous p53 isoforms have been identified in cancers; however, the function of these isoforms remains unclear. In melanoma, the frequency of p53 mutations is relatively low compared to other cancers suggesting that these isoforms may play a larger role in regulating p53 activity. We hypothesized that p53 function and therefore cell fate might be altered by the presence of Δ40p53, an embryonic isoform missing the first forty N-terminal amino acids of the full-length protein including the transactivation and Mdm2 binding domains. To test this hypothesis, we transduced tumor and normal cells with a lentivirus encoding Δ40p53. We found that exogenous Δ40p53 caused apoptosis and increased levels of endogenous, activated p53 in both cancerous and non-cancerous cells, which led to significant levels of cell death, particularly in cancer cells. Activated p53 molecules formed nuclear hetero-tetramers with Δ40p53 and altered downstream p53 transcription target levels including p53-induced protein with death domain (PIDD) and cyclin dependent kinase inhibitor, p21. Δ40p53 altered promoter occupancy of these downstream p53 target genes in such a way that shifted cell fate toward apoptosis and away from cell cycle arrest. We show that tumor suppression by p53 can occur via an alternate route that relies on its interaction with Δ40p53.

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

In order to understand the initiation and progression of cancers, numerous tumor suppressors have been screened for the presence of mutations and changes in protein expression (Cheok *et al.*, 2011; Machado-Silva *et al.*, 2010; Robles and Harris, 2010). p53 has been shown to orchestrate an appropriate tumor suppressor function by trans-activating or -suppressing cell cycle and apoptosis genes in response to a particular dose and quality of cellular stress (Beckerman and Prives, 2010; Belyi *et al.*, 2010; Lane and Levine, 2010; Vousden and Prives, 2009). The importance of proper p53 function is emphasized by its high mutation frequency among human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Petitjean *et al.*, 2007) and the overexpression of 'mutant' p53 in certain tumors suggests that some mutations may have a dominant-negative effect on wildtype p53 (Goldstein *et al.*, 2011; Oren and Rotter, 2010). Certain cancers such as melanomas harbor wildtype *TP53*, however, these tumors bypass the regulatory functions of p53 and continue to proliferate and metastasize (Albino *et al.*, 1994; Gwosdz *et al.*, 2006; Li *et al.*, 2006; Montano *et al.*, 1994; Soto *et al.*, 2005; Weiss *et al.*, 1995; Zerp *et al.*, 1999). This poses the question of how melanoma cells continue to proliferate in the presence of wildtype *TP53*.

The *TP53* gene encodes 12 protein isoforms that are missing specific regions of full-length p53 (Marcel *et al.*, 2011) and are capable of altering p53 function (Courtois *et al.*, 2002; Ghosh *et al.*, 2004; Khoury and Bourdon, 2010). Specific p53 isoforms have been identified in both cancer (Anensen *et al.*, 2006; Avery-Kiejda *et al.*, 2008; Boldrup *et al.*, 2007; Bourdon *et al.*, 2005b; Marcel *et al.*, 2010; Takahashi *et al.*, 2012) and non-cancerous tissues (Ungewitter and Scrable, 2010b). One of these isoforms, Δ40p53, is missing the first 40 amino acids encoding the first transactivation domain and can be synthesized primarily by alternative translation initiation in exon 4 (Courtois *et al.*, 2002; Grover *et al.*, 2009; Grover *et al.*, 2008; Grover *et al.*, 2011; Yin *et al.*, 2002) and by alternative splicing of intron 2 (Ghosh *et al.*, 2004). Δ40p53 is uniquely expressed during early embryogenesis (Maier *et al.*, 2004a; Ungewitter and Scrable, 2010b), is associated with less differentiated, proliferative cells, and cannot be found in corresponding adult tissues (Maier *et al.*, 2004a; Medrano *et al.*, 2009b; Takahashi *et al.*, 2012). The overall frequency of Δ40p53 protein expression in cancer, including melanoma, remains unknown.

We previously showed that Δ40p53 is highly and consistently expressed at the protein level in both glioblastoma and gliosis tissues but not in normal brain cortex (Takahashi *et al.*, 2012). Given that Δ40p53 is uniquely found in stem cell populations, the discovery of its endogenous expression in glioblastoma tumors raises the question of whether this isoform profile reflects a subpopulation of less differentiated, proliferative cells within a tumor or an attempt by the host to activate p53 and curb tumor growth. To distinguish between these possibilities, we generated a lentiviral vector that could deliver Δ40p53 to melanoma cells with high efficiency. We hypothesized that, if high expression of Δ40p53 were associated with proliferative, less differentiated cell types, then transduced cells would take on the growth characteristics of rapidly-dividing cells, resulting in increased cell number. Alternatively, if higher than normal expression of Δ40p53 reflected an attempt by the cells to slow down proliferation, then transduced cells would be expected to exhibit characteristics of tumor suppression, such as increased apoptosis or cell cycle arrest,

resulting in reduced numbers of cells. We asked if Δ40p53 could re-activate endogenous p53 function in melanoma cells to favor tumor suppression. Our results are consistent with a model where p53 activation can occur via an alternate route that relies on its interaction with Δ40p53 to modify downstream targets and promote cell death over cell cycle arrest.

Results

Excess Δ40p53 increases the fraction of dead cells in cultures of tumor and normal cells

We used a lentivirus encoding Δ40p53 (Δ40p53V) driven by the spleen focus-forming virus promoter (SFFVp) (Fig. 1A) to overexpress Δ40p53 in four different cell types: A375 melanoma cells, primary glioblastoma xenograft cells, human melanocytes and mouse embryonic fibroblasts (Fig. 1B). A previously described lentivirus encoding GFP (EV, Fig. 1A) or no infection (NI) was used as controls (Demaison *et al.*, 2002). We achieved transduction efficiencies of >95% as determined by expression of a GFP reporter included in both the empty vector and the Δ40p53-encoding lentivirus (Fig. 1A, Supplemental Fig. S1). Using a panel of p53 antibodies that bind specific domains of the p53 protein (Fig. 1C), which we previously developed to detect full-length p53 and its isoforms (Takahashi *et al.*, 2012), we demonstrated that exogenous Δ40p53 was expressed in transduced cells (Fig. 1D). p53 antibodies HR231 and CM1 detected Δ40p53 in A375 melanoma cells transduced with Δ40p53V, but not in uninfected cells (NI) or cells transduced with the empty vector (EV). The N-terminal antibody DO1 detected endogenous p53 expression, but only in Δ40p53V-transduced cells. HR231 also detected endogenous p53 upon higher exposure (data not shown). Approximately five days after infection, there were fewer adherent A375 melanoma and primary glioblastoma cells in wells treated with Δ40p53 lentivirus compared to controls (Fig. 1B, top two panels). To determine if this was due to decreased proliferation or increased cell death, we incubated A375 melanoma cells with ethidium homodimer, a DNA binding molecule that is impermeable to cells with intact cell membranes. The relative number of dead cells was significantly increased in Δ40p53-infected cultures compared to empty vector controls (Fig. 1E). Using trypan blue exclusion, we did not find a significant difference in the number of viable cells between Δ40p53-infected cells and controls (data not shown). We also found decreased numbers of adherent cells in melanocytes and mouse embryonic fibroblasts, but at ten days rather than five days after infection (Fig. 1B, bottom two panels). Thus, Δ40p53 did appear to affect the growth of cultures of both tumor and normal cells by decreasing cell viability.

Δ40p53 causes apoptosis

p53 activates pathways that can result in cell cycle arrest or apoptosis in response to different cellular stressors and damage. To determine if the visible decrease in viable cells in the presence of increased Δ40p53 expression was due to apoptosis or to prolonged cell cycle arrest, we analyzed apoptosis and membrane integrity in Δ40p53-infected cells with PE-conjugated Annexin V and a DNA binding dye, 7AAD, respectively (Fig. 2A, middle row). We found an approximately 3-fold increase in double-positive (late apoptotic) cells, a 2.7-fold increase in Annexin V single-positive (early apoptotic) cells, and a 4.5-fold increase in total Annexin V-positive cells with Δ40p53 infection compared to controls. Consistent with the apoptosis results, we observed a 4.4-fold increase in the proportion of subdiploid cells in

Δ40p53-infected cultures compared to controls, as determined by propidium iodide staining and flow cytometric analysis (Fig. 2A, bottom row). We found a similar increase in apoptosis in primary glioblastoma xenograft cells infected with Δ40p53 (Supplemental Fig. S2). We further confirmed our findings with western blot analysis using antibodies that detect cleaved or full length poly-(ADP-ribose)-polymerase (PARP I), a caspase target that is cleaved during the late phase of apoptosis (Oliver *et al.*, 1998). As shown in Fig. 2B, we found an increase in cleaved PARP I product in Δ40p53-infected lysates, a 2.2-fold increase relative to full-length PARP I, compared to 0.5-fold in both non-infected (NI) and empty vector (EV) controls. We carried out similar experiments in p53-deficient cells and did not observe an increase in the proportion of apoptotic cells in Δ40p53-infected cells compared to EV controls (Supplemental Fig. S3). These results indicate that Δ40p53 reduces cell viability by inducing apoptosis, but only in cells that express full-length p53.

Δ40p53 does not cause cell cycle arrest

To determine if Δ40p53 affected cell cycle progression, we infected A375 melanoma cells with Δ40p53 or empty vector and analyzed the cells by flow cytometry in the presence of propidium iodide. Consistent with our apoptosis results (Fig. 2A and 2B), we observed a 4.5-fold increase in the percentage of subdiploid cells infected with the Δ40p53 lentivirus (Fig. 2C, top row). However, we did not find significant differences in the cell cycle distributions of cycling Δ40p53-infected and empty vector-infected or non-infected A375 cells (Fig. 2D, unshaded area).

Next, we induced cell cycle arrest and determined cell cycle profiles at 0, 3, 6, and 9-hours following 7Gy of γ-irradiation. Representative plots and quantitation at the 3-hour time point are shown in Fig. 2C (bottom row) and Fig. 2D (shaded area), respectively. We did not find differences in cell cycle profiles with Δ40p53 compared to empty vector controls at any timepoints. By 9-hours post-irradiation, we observed a decrease in the percentage of S phase cells across all conditions indicating that both infected and uninfected cells were able to respond to γ-irradiation (data not shown). We obtained similar results in normal melanocytes, where the cell cycle distributions were the same with or without γ-irradiation and in both infected and non-infected cells (Supplemental Fig. S4). We also did not find an increase in the percentage of subdiploid cells in γ-irradiated samples compared to non-irradiated samples (Fig. 2D, % subdiploid). Collectively, these results suggested that Δ40p53 might selectively affect cell death while leaving cell cycle arrest unchanged.

One of the canonical pathways of tumor suppression by p53 involves ATM/ATR phosphorylation of p53 at serine 15 by ionizing radiation (Banin *et al.*, 1998; Canman *et al.*, 1998; Khanna *et al.*, 1998; Siliciano *et al.*, 1997). The choice between cell cycle arrest and apoptosis is affected by the level of p53 and post-translational modifications at residues such as serine 15 (Hollstein and Hainaut, 2010; Vousden and Prives, 2009). We compared the level of serine 15 phosphorylation in non-infected cells to cells infected with Δ40p53V or EV by western blot analysis at 0, 3, 6, and 9-hours after irradiation (Fig. 2E, ser15). Exogenous Δ40p53 expression was confirmed using antibodies HR231, CM1, and pAb1801 (Fig. 2E, bottom panels). We found that serine 15 phosphorylation was increased in cells infected with Δ40p53V even in the absence of ionizing radiation (Fig. 2E, 0h). Serine 15

phosphorylation was increased as early as 3-hours and sustained to 9-hours post irradiation in both uninfected and infected cells, but to significantly higher levels in Δ40p53 expressing cells (Fig. 2E, ser15). Total full-length p53 as detected by DO1 was similarly affected (Fig. 2E, DO1). Although there were increased levels of phospho-p53 in Δ40p53 expressing cells compared to EV control, there was no significant difference within each group over time (Supplemental Fig. S5). We observed similar effects of Δ40p53 and γ-irradiation in a second melanoma cell line (WM266) and in human melanocytes (Supplemental Fig. S6). These results suggested that Δ40p53 enhanced the activation of p53 in response to genotoxic stress through ATM/ATR-dependent phosphorylation of serine 15.

Given that p53 can be activated by γ-irradiation through the canonical ATM/ATR-serine15 phosphorylation pathway, we hypothesized that, Δ40p53, similar to γ-irradiation, might act through ATM/ATR to increase serine-15 phosphorylated p53 levels. To test the dependence of serine 15 phosphorylation on the activity of the ATM/ATR pathway, we again infected cells with Δ40p53V, but this time in the presence of CGK733, an ATM/ATR inhibitor (Bhattacharya *et al.*, 2009; Crescenzi *et al.*, 2008). Inhibition of ATM/ATR kinase activity prevented serine 15 phosphorylation in EV and NI controls, but had no effect on the level of serine 15 phosphorylation in Δ40p53-transduced cells (Fig. 3A). This suggests that Δ40p53V acts through a mechanism that is different from that of ATM/ATR activation seen with γ-irradiation. We also compared the levels of other kinases and phosphatases known to affect serine 15 phosphorylation, but did not find any significant changes that could account for the increased phosphorylation seen in Δ40p53-infected cells (Fig. 3B). What we did find, however, was a strong, Δ40p53-dependent increase in serine 15 phosphorylation under conditions that induce ER stress and the unfolded protein response (UPR) (Ito *et al.*, 2006; Shenkman *et al.*, 2007). As shown in Fig. 3C, blocking protein translation with cycloheximide led to a gradual increase in the level of serine 15 phosphorylated p53 concurrent with the decline of control proteins, such as cyclin B1, and virally-encoded Δ40p53 (Fig. 3C, CM5). Together with previous results, these data suggest that activation of p53 by Δ40p53, which is reflected in the phosphorylation of serine 15, is primarily in response to proteotoxic rather than genotoxic stress brought on by high levels of Δ40p53 relative to full-length p53.

Δ40p53 increases the expression of apoptotic gene, PIDD, and suppresses expression of cell cycle arrest gene, p21

p53 exerts its tumor suppressor activity and cellular response to damage primarily as a transcription factor. We therefore asked if the presence of excess Δ40p53 might alter the levels of p53 transcription target gene expression. To discover candidate p53 targets altered by the introduction of Δ40p53, we used a PCR array containing 84 known p53 targets to compare fold changes in Δ40p53-transduced cells relative to EV infected cells (Supplemental Figure S7A, upper panel 0Gy). When comparing Δ40p53V samples to EV, we found that the majority of transcript levels relative to EV were within a 3-fold dynamic range based on a $\log_{10}$-scale (Fig. 4A, black circles). However, there were eight target genes outside this dynamic range (Fig. 4A, red circles), and 5 of these are involved in apoptosis (Supplemental Figure S7B). Based on these data, we focused on the expression of individual genes involved in apoptosis (PIDD, PCBP4, APAF1, and Bax) and on two cell cycle arrest

genes (p21 and GADD45), which exhibited reduced expression in the array. Single gene transcript levels determined by qPCR revealed an increase in the apoptotic gene, PIDD, in Δ40p53 infected cells compared to EV controls (Fig. 4B). These data were consistent with the increased apoptosis observed in the presence of Δ40p53V (Fig. 2A and 2B).

Next, we screened 84 known p53 targets by PCR array 3-hours after exposure to 7Gy γ-irradiation in A375 melanoma cells infected with Δ40p53V or EV (Supplemental Figure S7A, bottom panel 7Gy). Changes in p21 and PIDD were verified by single-gene qPCR (Fig. 4C).

Δ40p53 oligomerizes with endogenous activated p53 in the nucleus

To determine the mechanism by which Δ40p53 could alter p53 target gene expression and increase apoptosis, we first asked if both activated p53 and Δ40p53 localized to the nucleus. We fractionated Δ40p53V or EV-infected A375 melanoma cells and determined the subcellular localization of Δ40p53 and full-length p53 by western blot analysis (Fig. 5A). Δ40p53, serine 15 phosphorylated p53, and total full-length p53 were exclusively found in the nucleus. The small amount of endogenous p53 detected in the cytoplasmic fraction is most likely due to carry-over from the nuclear fraction, as indicated by the presence of NP62, a nuclear protein. We could not detect any Δ40p53 in the cytoplasmic fraction. To determine if Δ40p53 formed hetero-tetramers with endogenous activated p53, we treated nuclear fractions of Δ40p53-infected cells with glutaraldehyde to cross link proteins and looked for higher molecular weight oligomers by western blot analysis as previously described (Powell *et al.*, 2008; Ungewitter and Scrable, 2010a). Using antibodies that detect activated p53 (serine 15 phosphorylated p53), full length p53 (DO1 and CM1), and Δ40p53 (CM1), we found bands at molecular weights corresponding to p53 tetramers, p53/Δ40p53 tetramers, p53 dimers, and p53/Δ40p53 dimers in glutaraldehyde-treated, but not untreated, nuclear extracts (Fig. 5B; compare upper and lower panels). p53-specific antibodies that recognize epitopes in the N-terminus of the protein (ser15 and DO1) detected two bands, one migrating above the 150kD marker in glutaraldehyde treated samples and the other at 50kD corresponding to p53 monomers.

Using CM1, which recognizes epitopes present in both p53 and Δ40p53, we found that all detectable Δ40p53 (migrating above the 37kD marker in 0% glutaraldehyde samples) was bound in tetramers with p53. These tetramers migrated above the 150kD marker in glutaraldehyde-treated samples and were detected by all 3 antibodies. We could detect p53 monomers but no Δ40p53 monomers in glutaraldehyde treated samples. A band migrating at 200kD above the p53/Δ40p53 tetramer band (best visualized with CM1 in NI and EV controls) indicated the apparent migration of endogenous p53 tetramers and was used as a reference point. Likewise in the CM1 lanes, a band at approximately 100kD in control lanes was used as a reference point for endogenous p53 dimers. The band migrating below 100kD in the Δ40p53V lane, therefore, must correspond to p53/Δ40p53 dimers. From these data, it is clear that Δ40p53 oligomerizes with full-length p53 and, more importantly, forms tetramers with activated p53 in the nucleus. This suggested a molecular model by which the presence of Δ40p53 might affect the expression of p53 target genes (identified by PCR array and qPCR analysis) by modifying the ability of activated tetramers to bind DNA.

Δ40p53 increases promoter occupancy of PIDD and p21

p53 isoforms, including Δ40p53, have been shown to modulate promoter occupancy of p53 gene targets by full-length p53, a known transcription factor, and subsequently alter the expression of downstream targets (Bourdon *et al.*, 2005a; Mills, 2005; Ungewitter and Scrable, 2010a). We hypothesized that the increase in PIDD and decrease in p21 transcript levels could be due to altered promoter occupancy by Δ40p53/p53 complexes. We tested this hypothesis by infecting A375 melanoma cells with either EV or Δ40p53V and used chromatin immunoprecipitation (ChIP) assays to determine promoter occupancy at the *LRDD* and *CDKN1A* genes encoding PIDD and p21, respectively. p53 was immunoprecipitated from chromatin complexes using serine 15 phosphorylated p53, pAb421, and 9282 antibodies. Rabbit IgG was used as a control. At the PIDD promoter (Fig. 5C), immunoprecipitating polyclonal p53 antibody 9282 revealed significantly increased occupancy in Δ40p53-infected cells compared to cells infected with the empty virus. Similarly, analysis of p21 promoter occupancy demonstrated a significant increase in p53 molecules bound in the presence of Δ40p53V compared to EV with p53 antibodies pAb421 (Fig. 5D) and 9282 (Supplemental Fig. S8). We did not find a significant difference in promoter occupancy using the serine 15 phosphorylated p53 antibody (Supplemental Fig. S8).

In summary, we found that exogenous Δ40p53 increases p53-dependent cell death by apoptosis in both cancer and normal cells without altering cell cycle arrest. Consistent with previous studies, γ-irradiation did not induce cell cycle arrest (Kaufmann *et al.*, 2008), nor did it change the fraction of dead cells. These data suggest that the decision to undergo apoptosis is favored in the presence of Δ40p53 and cannot be amended by exogenous stimuli such as γ-irradiation. Δ40p53 increased levels of endogenous, activated (serine 15 phosphorylated) p53 in A375 melanoma cells, which could be enhanced by proteotoxic agents, such as cycloheximide. Increased levels of endogenous p53 were not due to phosphorylation by ATM/ATR as seen with γ-irradiation. Δ40p53 increased transcript levels of apoptotic targets, such as PIDD, while suppressing the expression of cell cycle arrest genes, such as p21. We found that Δ40p53 formed nuclear hetero-tetramers with activated p53 and increased promoter occupancy at both PIDD and p21 genes.

Discussion

Δ40p53 is an isoform of p53 that is normally expressed only during embryogenesis and in the stem cell compartment of adult tissues (Medrano *et al.*, 2009a; Ungewitter and Scrable, 2010b). Recently, we have also identified Δ40p53 as the only consistently expressed p53 isoform in glioblastoma multiforme (Takahashi *et al.*, 2012), the most common brain tumor in adults. To gain insight into the role of Δ40p53 in cancer, we utilized a lentiviral system to overexpress Δ40p53 in tumors, such as melanoma, and normal counterparts (melanocytes) with or without functional p53. We chose an overexpression model to parallel the p53 expression profiles previously described in glioblastoma multiforme (Takahashi *et al.*, 2012). Our results are summarized in the model presented in Fig. 5E. We found that Δ40p53 shifted melanoma cell fate in a p53-dependent manner to favor apoptosis over cell cycle arrest, even in the presence of γ-irradiation, a known inducer of DNA damage and cell cycle

arrest. Δ40p53 increased endogenous, activated p53 levels in melanoma cells and increased expression of downstream p53 targets such as PIDD and suppressed others such as p21. Δ40p53 formed nuclear tetramers with endogenous, serine 15 phosphorylated p53 and directly altered promoter occupancy of PIDD and p21. Endogenous p53 phosphorylation by Δ40p53 was dependent on proteotoxic and not genotoxic damage, which led to significant levels of cell death. Our results are consistent with a role for Δ40p53 in the reactivation of p53-dependent tumor suppression.

Although p53 is the most frequently inactivated tumor suppressor in human tumors, certain types of cancer, such as melanoma, have relatively few *TP53* mutations (Albino *et al.*, 1994; Gwosdz *et al.*, 2006; Li *et al.*, 2006; Montano *et al.*, 1994; Soto *et al.*, 2005; Weiss *et al.*, 1995; Zerp *et al.*, 1999). Despite high levels of wildtype protein expression, studies have showed that p53 fails to execute downstream functions such as cell cycle arrest and apoptosis in melanoma primarily due to a failure in transactivating target genes (Avery-Kiejda *et al.*, 2011; Houben *et al.*, 2011; Knopf *et al.*, 2011). Consequently, there have been a number of studies that have focused on the idea of re-activating or 'rescuing' p53 function in cells with wildtype *TP53* alleles by introducing exogenous p53 (Kichina *et al.*, 2003; Lane *et al.*, 2010) or by inhibiting Mdm, which has been found to be overexpressed in melanomas (Danovi *et al.*, 2004; Gembarska *et al.*, 2012; Ji *et al.*, 2012; Muthusamy *et al.*, 2006; Terzian *et al.*, 2010). Our results reveal a way in which endogenous p53 can be activated and directed to increase apoptosis in tumor cells. Validation of these results using in vivo tumor models would be necessary to determine any therapeutic utility of these findings.

Materials and Methods

Lentiviral vectors and transduction

A375 melanoma cells (ATCC, Manassas) and melanocytes (Life Technologies, Grand Island) were cultured according to manufacturer's protocols. Primary glioblastoma cells and mouse embryonic fibroblasts (MEFs) were cultured as previously described (Carlson *et al.*, 2011; Maier *et al.*, 2004b). The Δ40p53 fragment was PCR amplified and cloned into the pSIN construct (gift of Dr. Yasuhiro Ikeda, Mayo Clinic) and lentivirus produced as previously described (Demaison *et al.*, 2002). Transduction efficiency >95% (based on GFP fluorescence) was achieved in all infected cell types prior to carrying out downstream assays (approximately five days post infection for cancer cells and ten days for non-transformed cells).

Western blot analysis

Western blot analyses as previously described (Ungewitter and Scrable, 2010b). p53 antibody include: DO1 and HR231 (Santa Cruz Technologies, Inc., Santa Cruz), pAb421 and pAb1801 (Calbiochem/EMD Chemicals, Gibbstown), phospho-p53 (ser15) (Cell Signaling, Danvers), CM1 (Vector Labs, Burlingame), and GAPDH (Ambion, Foster City). PARP I antibodies (Promega, Madison) (Budihardjo *et al.*, 1998). Serine 15 phosphorylation kinase and phosphatase antibodies: p-AMPKα, p-mTOR, p-RSK2, p70 S6K, CDK5,

CDC25A (Cell Signaling, Danvers). Cyclin B1 (Cell Signaling, Danvers); Np62 (BD Transduction Laboratories, San Jose).

ATM/ATR inhibition and cycloheximide treatment

Infected A375 cells were treated with ATM/ATR inhibitor, CGK733 (10μM), or vehicle for approximately 60 hours and harvested for western blot analysis. Infected cells were treated with cycloheximide (100μg/mL) or vehicle and harvested at 1, 2, 3, 6, and 12 hours.

Subcellular fractionation

Infected A375 cells were harvested for nuclear and cytoplasmic fractionation using the Qproteome Cell Compartment Kit (Qiagen, Germantown). For tetramer detection, nuclear fractions were treated with 0.1% glutaraldehyde and analyzed as previously described (Ungewitter and Scrable, 2010b).

Cell cycle and apoptosis assays

The fraction of dead cells was determined by ethidium homodimer binding (Life Technologies, Grand Island), percentage of apoptotic cells by Annexin V: PE Apoptosis Detection Kit I (BD Pharmingen, San Jose), and cell cycle profiles by propidium iodide staining. PARP I products were detected by western blot analysis. Cells were γ-irradiated with 7Gy and harvested at 3, 6, or 9-hours post irradiation. Flow data were analyzed with ModFit and CellQuest.

Quantitative PCR and Chromatin Immunoprecipitation Assays

RNA harvested using a miRNeasy Mini Kit (Qiagen, Valencia) was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island). iQ SYBR Green Supermix was used for single gene PCR (Biorad, Hercules). The RT^2 Profiler PCR array was used (SABiosciences, Valencia) for candidate p53 target discovery. Chromatin immunoprecipitation analysis was carried out using SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling, Danvers). Immunoprecipitating antibodies: serine 15 phosphorylated p53, 9282, and rabbit IgG (Cell Signaling, Danvers); pAb421 (Calbiochem/EMD Chemicals, Gibbstown).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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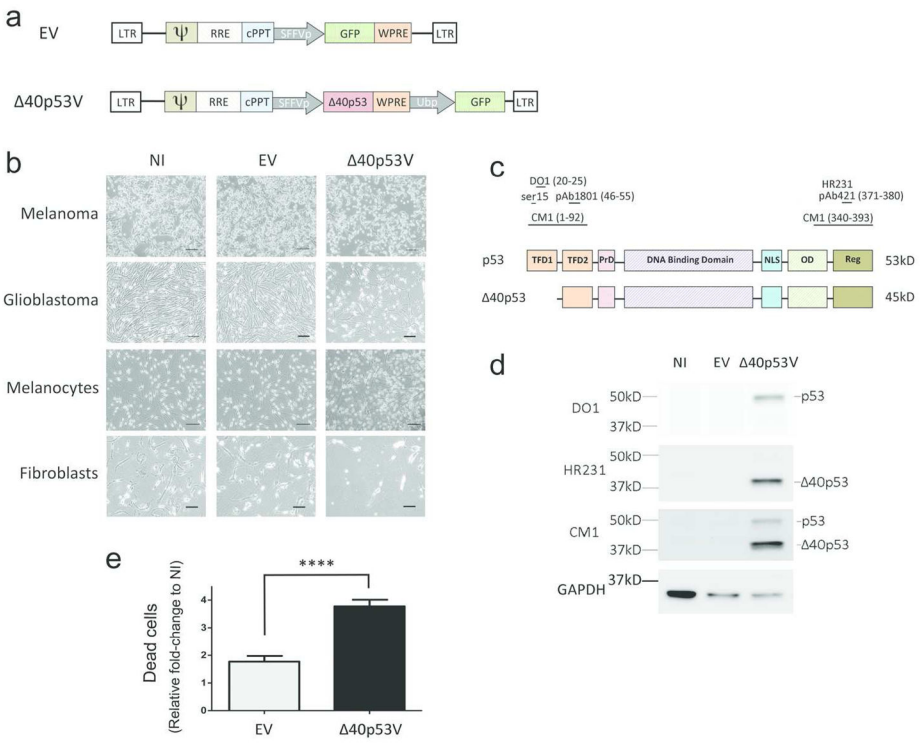

Figure 1. Δ40p53 increases the relative number of dead cells

(A) Lentiviral constructs encoding Δ40p53 and green fluorescence protein (GFP) (Δ40p53V) or GFP alone (EV). Spleen Focus-Forming Virus promoter (SFFVp); Ubiquitin promoter (Ubp); Long terminal repeat (LTR); packaging signal (ψ); Rev-responsive element (RRE); HIV central polypurine tract (cPPT); Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). **(B)** Representative images of cells infected with EV, Δ40p53V, or no lentivirus (NI). Scale bar represents 1μM. **(C)** p53 and Δ40p53 domain structures and antibody epitopes. Δ40p53 is missing transcription factor domain 1 (TFD1), but retains transcription factor domain 2 (TFD2), proline-rich domain (PrD), DNA binding domain, nuclear localization signal (NLS), oligomerization domain (OD), and regulatory domain (Reg). **(D)** Western blot analysis of A375 melanoma cells infected with EV, Δ40p53V, or NI. Δ40p53V was detected by HR231 and CM1 but not DO1. **(E)** Relative fold-change of dead cells in EV- and Δ40p53V-infected cells normalized to NI control. ****p<0.0001.

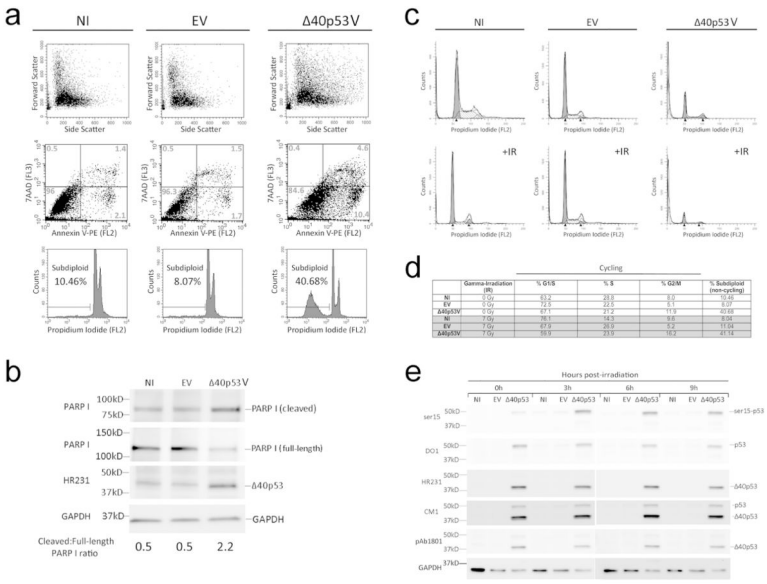

Figure 2. Δ40p53 causes apoptosis, not cell cycle arrest

(A) Flow cytometric analysis of apoptotic and subdiploid populations in A375 cells infected with EV, Δ40p53V, or NI. Percentages within each quadrant represent fraction of total cells. Bottom panel percentages indicate subdiploid fraction of cells. At least 20,000 events were collected per experimental sample. Representative plots shown. **(B)** Western blot analysis of poly-(ADP-ribose) polymerase (PARP I) cleavage. **(C)** Cell cycle profiles of infected A375 cells in the presence of propidium iodide following 0 or 7Gy of γ-irradiation (+IR). **(D)** Cell cycle distribution of infected vs. uninfected cells with (shaded area) or without γ-irradiation (non-shaded area). Subdiploid (non-cycling) percentages are shown separately. **(E)** Western blot analysis of p53 expression in infected A375 cells with γ-irradiation (7Gy). Cells were harvested at 0, 3, 6, and 9-hours post-irradiation.

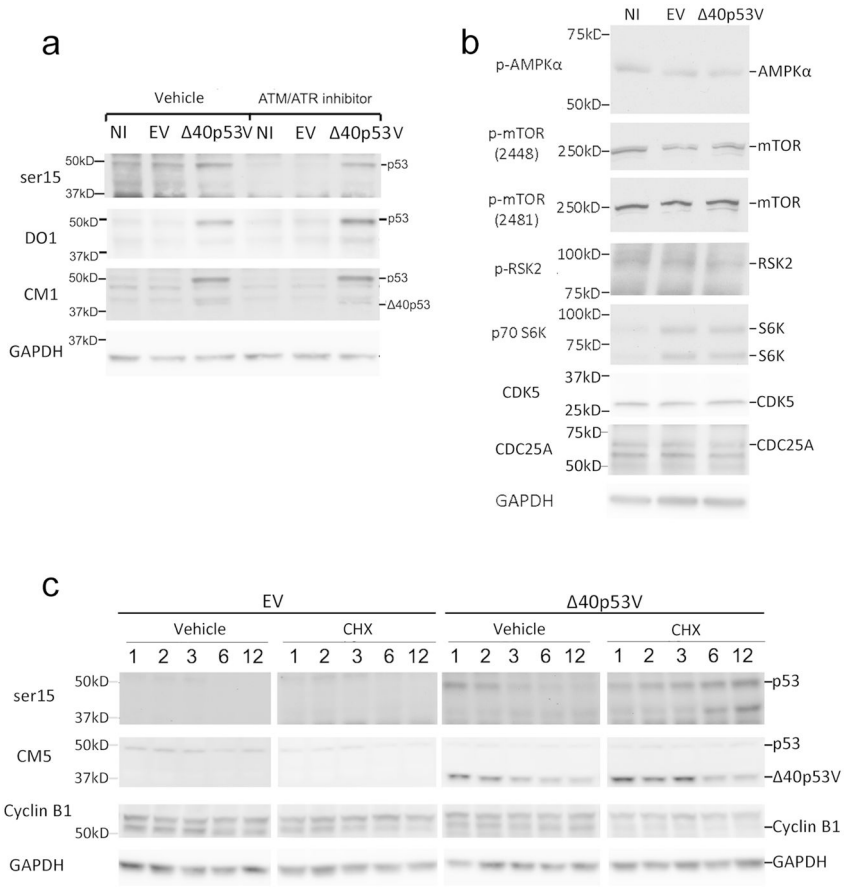

Figure 3. Δ40p53 activates p53 under proteotoxic, but not genotoxic, conditions

(A) Serine 15 phosphorylated p53 in A375 cells infected with EV, Δ40p53V, or NI treated with ATM/ATR inhibitor CGK733 (10μM). **(B)** Kinases and phosphatases affecting p53 serine 15 phosphorylation in Δ40p53V, EV, and NI in A375 cells. Phosphorylated AMP-activated protein kinase alpha (p-AMPKα); phosphorylated mammalian target of rapamycin (p-mTOR); phosphorylated ribosomal protein S6 kinase 2 (p-RSK2); p70 ribosomal S6 kinase (p70 RSK2); cyclin dependent kinase 5 (CDK5) and cell division cycle 25 homolog A (CDC25A). **(C)** Serine 15 phosphorylated p53 in Δ40p53-infected A375 cells treated with cycloheximide (CHX). Cells were infected with EV or Δ40p53V and treated with 0 or 100μg/mL cycloheximide for 1, 2, 3, 6, or 12-hours. p53 and Δ40p53V detected by ser15 and CM5. Cyclin B1 levels shown to determine effectiveness of cycloheximide treatment.

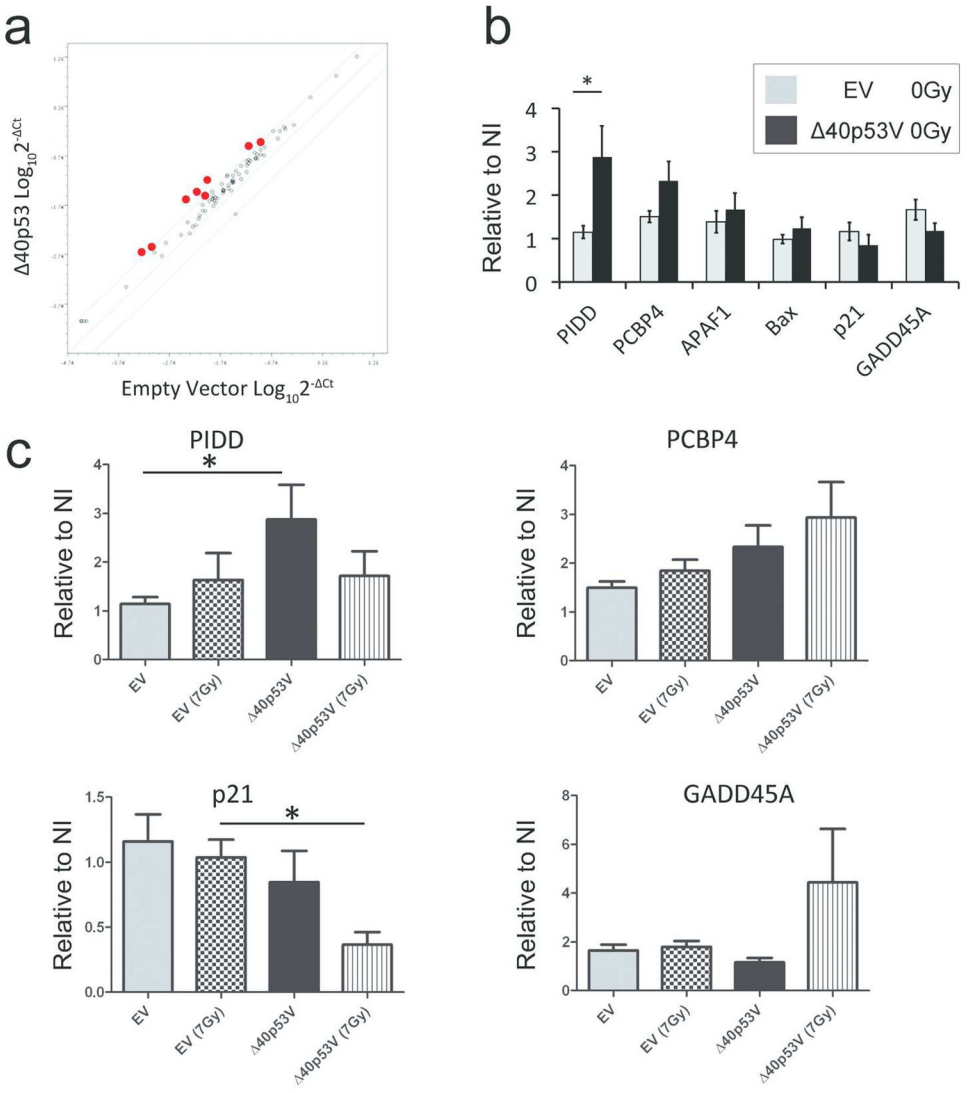

Figure 4. Δ40p53 increases expression of apoptotic gene, PIDD, and suppresses expression of cell cycle arrest gene, p21

(A) Potential p53 gene targets altered by Δ40p53V. Log-scale dot plot of gene target levels in Δ40p53V versus EV. Eight genes (red circles) fell outside of the 3-fold dynamic range (delineated by parallel lines); see Supplemental Fig. S7. **(B)** Relative expression of p53 gene targets. Quantitative PCR analysis of selected p53 targets in A375 cells infected with EV (grey bars) and Δ40p53V (black bars). p53-induced protein with a death domain (PIDD); Poly(rC)-binding protein 4 (PCBP4); Apoptotic protease activating factor 1 (APAF1); Bcl-2– associated X protein (Bax); Cyclin dependent kinase 1 (p21); Growth arrest and DNA-damage-inducible protein alpha (GADD45A). *p<0.05. **(C)** Relative expression of p53 targets (PIDD, PCBP4, p21, and GADD45A) in Δ40p53-infected, γ-irradiated A375 cells. *p<0.05.

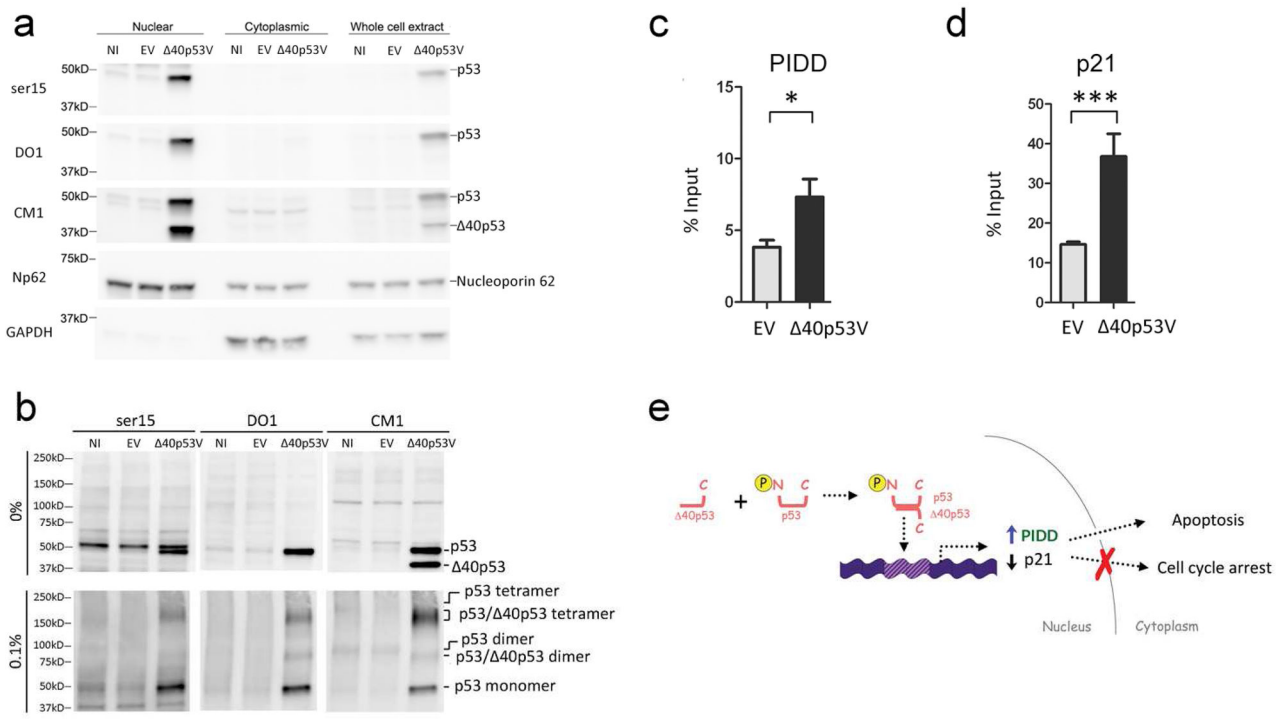

Figure 5. Δ40p53 oligomerizes with endogenous activated p53 in the nucleus and increases promoter occupancy of PIDD and p21

(A) Nuclear and cytoplasmic fractions of A375 cells infected with Δ40p53V. Nucleoporin 62 (Np62) and GAPDH were used as nuclear and cytoplasmic markers, respectively. **(B)** Higher order oligomers in A375 melanoma cells infected with Δ40p53V. Nuclear fractions of cells infected were treated with glutaraldehyde (0.1%). p53 promoter occupancy at PIDD **(C)** and p21 **(D)** promoters. p53 antibodies were used to immunoprecipitate p53, Δ40p53, and activated p53 from Δ40p53V-infected A375 cells for ChIP analysis (see Supplemental Fig. S8). *p<0.05; ***p<0.001. **(E)** A model for how Δ40p53 alters cell fate. Endogenous p53 can be activated and directed by Δ40p53 to favor apoptosis over cell cycle arrest (even with γ-irradiation) in tumor cells. Our results are consistent with a role for Δ40p53 in the reactivation of p53-dependent tumor suppression.