p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop

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DDR1, discoidin domain receptor 1, belongs to a subfamily of tyrosine kinase receptors with an extracellular domain homologous to *Dictyostellium discoideum* protein discoidin 1. We showed that DDR1 is a direct p53 transcriptional target, and that DNA damage induced a p53-dependent DDR1 response associated with activation of its tyrosine kinase. We further demonstrated that DDR1 activated the MAPK cascade in a Ras-dependent manner. Whereas levels of p53, phosphoserine-15 p53, p21, ARF and Bcl-X_L were increased in response to exogenous overexpression of activated DDR1, dominant-negative DDR1 inhibited irradiation-induced MAPK activation and p53, phosphoserine-15 p53, as well as induced p21 and DDR1 levels, suggesting that DDR1 functions in a feedforward loop to increase p53 levels and at least some of its effectors. Nonetheless, inhibition of DDR1 function resulted in strikingly increased apoptosis of wild-type p53-containing cells in response to genotoxic stress through a caspase-dependent pathway. These results strongly imply that this p53 response gene must predominately act to alleviate the adverse effects of stress induced by p53 on its target cell.

Keywords: apoptosis/cell survival/DDR1/p53/Ras/Raf/MAPK

Introduction

The p53 tumor suppressor gene plays a crucial role in cancer progression, and it is inactivated in the majority of human tumors. Depending on cell context, wild-type (wt) p53 limits cellular proliferation in response to DNA damage and other cellular stresses by inducing cell cycle arrest, apoptosis or senescence (Ko and Prives, 1996; Levine, 1997; Sugrue *et al.*, 1997; Lin *et al.*, 2000; Oda *et al.*, 2000; Vogelstein *et al.*, 2000; Vousden, 2000). It has been shown that inhibition of cell proliferation by p53 is largely due to its ability to transcriptionally activate genes

that directly control cell fate (Ko and Prives, 1996; Levine, 1997; Vogelstein *et al.*, 2000).

We have shown previously that the MAPK cascade can be activated in a sustained manner by p53 (Lee *et al.*, 2000) and that one upstream activator of this pathway is HB-EGF, a growth factor of the EGF family (Fang *et al.*, 2001). These findings, as well as evidence that inhibition of HB-EGF signaling following p53 induction by cellular stresses is pro-apoptotic, support the concept that the p53 program induces pro-survival as well as pro-death cell fate decisions (Fang *et al.*, 2001). In an effort to identify other downstream target genes of p53 and, in particular, those that might be involved in p53-mediated MAPK activation, we performed expression array analysis using tetracycline (tet) regulatable p53-expressing EJ tumor cells that have lost p53 function. We identified the discoidin domain receptor 1 (DDR1) as a p53 response gene, whose expression levels increase markedly in wt-p53-containing cells in response to DNA damage.

The DDR1 was isolated as a novel subfamily of receptor tyrosine kinases (RTK) (Di Marco *et al.*, 1993; Johnson *et al.*, 1993; Alves *et al.*, 1995). DDR1 is characterized by a 160 amino acid segment within the extracellular domain that exhibits strong sequence similarity to the *Dictyostellium discoideum* protein discoidin 1 (Springer *et al.*, 1984), coagulation factors V and VIII, and a *Xenopus laevis* recognition protein, A5 (Takagi *et al.*, 1991), which has an unusually long cytoplasmic juxta-membrane region and a kinase domain that has high homology to that of the NGF receptor, TrkA (Di Marco *et al.*, 1993; Vogel, 1999). DDR1 has been reported to be overexpressed in mammary, ovarian and lung carcinomas, suggesting that it may play a role in the progression of certain carcinomas (Johnson *et al.*, 1993; Zerlin *et al.*, 1993; Laval *et al.*, 1994; Alves *et al.*, 1995; Barker *et al.*, 1995; Perez *et al.*, 1996). Various types of collagen have been identified as ligands capable of activating both DDR receptor kinases (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997). However, the kinetics of DDR receptor activation are very different from those observed in most RTK (Schlessinger, 1997; Shrivastava *et al.*, 1997; Vogel *et al.*, 1997). Whereas activation of DDR1 by collagen is direct, tyrosine phosphorylation of the receptors occurs with delayed but sustained tyrosine kinase activation (Schlessinger, 1997). In addition, DDR activation by collagen can occur in the absence of a functional integrin collagen receptor (Vogel *et al.*, 2000). There is, as yet, relatively little information concerning DDR downstream signaling pathways or functions.

Here, we show that DDR1 is a direct p53 transcriptional target. In response to DNA damage, DDR1 was induced and activated/phosphorylated in a p53-dependent manner. We also present evidence that DDR1 plays a central role in p53 regulation through a positive feedback

Fig. 1. Wt-p53-dependent induction of DDR1. (A) Induction of DDR1 mRNA and protein after tet removal in Saos2-p53 and EJ-p53 cells. Total RNA or protein extracts were prepared from EJ-p53 or Saos2-p53 cells grown in the presence or absence of tet for 0.5, 1, 2 or 3 days. Northern blots were performed sequentially using a 32 P-labeled probe against DDR1, p21, Mdm2 and 36B4 (loading control). Western blotting was performed using antibodies against DDR1, p53, p21 and β -actin. β -actin was used as a loading control. (B) DDR1 induction in response to DNA damage. HCT116 cells with or without p53 were exposed to 1, 2, 2.5 or 5 Gy of γ -irradiation, and total proteins were collected at the indicated times following irradiation. DDR1 protein was significantly increased in HCT116 cells with p53 but not in HCT116-p53 $^{-/-}$ cells. Normal diploid fibroblasts (IMR90) with wt-p53 and two human cancer cell lines (PC3, prostate cancer cell line; Saos2, osteosarcoma cell line) that had lost wt-p53 were exposed to irradiation (5 Gy). Then total RNA and proteins were collected at the indicated times. In p53 $^{+/+}$ or p53 $^{-/-}$ MEF cells, cells were treated with DNA-damaging agents, mitomycin C (MMC, 2.5 μ g/ml) and adriamycin (Adr, 0.25 μ g/ml), at the indicated times.

loop of the DDR1-Ras/MAPK-p53-p21 module. Nonetheless, blocking endogenous DDR1 function in wt-p53-containing cells following genotoxic stimuli resulted in significantly increased apoptosis. These results provide strong evidence that, unlike other known p53 direct target genes, the DDR1 receptor can function as a survival effector directly in the wt-p53-containing cell exposed to genotoxic stress. Since many human cancers maintain wt-p53 status, inhibition of DDR1 function may provide a novel approach to selectively enhancing the targeting of such tumors.

Results

DDR1 is a direct transcriptional target of p53

We showed previously that the Ras/Raf/MAPK signaling pathway is activated in response to p53 and that the ability of p53 to activate this pathway is dependent on its transcriptional activity (Lee *et al.*, 2000). To identify potential p53 transcriptional target genes that might be involved in this signaling response, we used a DNA chip expression array to compare genes expressed in the presence or absence of p53. Affymetrix GeneChips were

used for hybridization. Among upregulated genes detected, the transcript for DDR1 was found to increase in response to p53 induction. A previous study indicated that transient transfection of p53 induced DDR1 mRNA in an osteosarcoma line (Sakuma *et al.*, 1996). However, no evidence was presented as to whether DDR1 was a direct p53 transcriptional target or whether DDR1 could be induced in response to genotoxic stress. To quantitate the level of DDR1 induction, we performed northern and western blot analysis using several different p53 expression systems. As shown in Figure 1A, as early as 12 h after tet removal in Saos2-p53 cells, the transcripts of DDR1 were easily induced and detectable, as well as p53 and p21^{CIP1/WAF1}. In contrast, DDR1 was not induced in Saos2 control cells after tet removal. In addition, western blotting showed that DDR1 was induced in EJ-p53 cells after tet removal with kinetics like those of p53 as well as p21.

We next analyzed the effects of various genotoxic stresses such as γ -irradiation, actinomycin D, adriamycin or mitomycin C on DDR1 upregulation in wt-p53-containing cells. First, human normal diploid fibroblasts (IMR90) and a human colon cancer cell line (HCT116), both containing wt-p53, were treated with three different doses, 1, 2.5 and 5 Gy, in a γ -irradiator with a mark I¹³⁵ Cs source. The result at all doses was a consistent stepwise increase in DDR1 expression levels (Figure 1B), whereas the expression of β -actin protein remained unchanged. The results from northern and western blot experiments indicate that DDR1 was upregulated as early as 4 h after irradiation in wt-p53-containing cells. The functionality of p53 was monitored by detecting transactivation of the p53 target gene, p21. In contrast, irradiated PC3 and Saos2 cell lines, which contained mutant forms of p53, show no detectable upregulation of DDR1 mRNA and protein (Figure 1B). To further confirm DDR1 increases following DNA damage and to verify that the response was p53 dependent, wt mouse embryonic fibroblasts (MEF) were treated with DNA-damaging agents, actinomycin D, adriamycin and mitomycin C. Exposures to these agents also resulted in a marked induction of DDR1 expression at the mRNA and protein levels (Figure 1B). In contrast, DDR1 expression levels remained unchanged when p53-null MEF was exposed to the agents. These data support the finding that DDR1 is induced by wt-p53 in response to genotoxic stress.

Since the p53 dependence and kinetics of DDR1 expression in response to p53 and DNA damage resemble those of other well-defined p53 target genes, we investigated whether the DDR1 gene contains p53 response elements. The human DDR1 gene is composed of 17 exons (Figure 2A) (Playford *et al.*, 1996; Sakuma *et al.*, 1996). An earlier report indicated that the promoter region of DDR1 contains a consensus sequence of the p53-binding site (Sakuma *et al.*, 1996) but the site lacks a critical nucleotide and does not contain a good consensus half-site. Moreover, a reporter assay revealed that the promoter region containing a potential p53-binding site did not confer p53 responsiveness upon the luciferase reporter (data not shown). These results led us to seek other p53-responsive site(s). We found a potential p53-responsive site of 20 nucleotides in intron 3 of the DDR1 gene, which matched the consensus p53 binding sequence by 90% (Figure 2A). To determine whether this sequence ex-

hibited p53-dependent transcription-enhancing activity, a 0.73 kb DNA fragment (*Mlu*I and *Bgl*II) corresponding to intron 3 was fused to the luciferase reporter construct (subcloned upstream of the SV40 minimal promoter, pGL3prom-DDR1-intron 3) (Figure 2A and C). The same 0.73 kb intron 3 segment containing a mutated p53-BS sequence (G $\rightarrow$ T) (DDR1-intron-mt) was generated and also fused upstream of the SV40 minimal promoter. To examine the p53 responsiveness of the potential p53-responsive site in intron 3, the pGL3-DDR1 intron 3 reporter vector was co-transfected into Saos2 cells (p53-null osteosarcoma cell line) with plasmids expressing wt or mutant p53 (p53V143A). The luciferase activities shown by these constructs were then measured and compared. As a negative control, we tested the pGL3-promoter vector, which served as the backbone for the DDR1-p53RE reporter construct. As a positive control for p53 responsiveness, we used the pGL3-p21^{CIP1/WAF1} promoter construct containing p53-responsive sites.

As shown in Figure 2C, introduction of the pGL3-DDR1-intron 3 construct increased luciferase activity in response to wt-p53 expression but not to mutant p53 or vector alone. Moreover, a point mutation (G to T in p53-BS consensus) in the intron 3 mutant reporter abolished luciferase activity (Figure 2C) in response to wt-p53. Introduction of the pGL3 control vector in Saos2 cells resulted in only a marginal change in activity with/without wt-p53. To determine whether p53 could bind to this putative p53 response element in intron 3, we performed an electrophoretic mobility shift assay (EMSA) and observed the shift in mobility of the 20 bp sequence by analyzing nuclear extracts including wt-p53. Supershift of this band by addition of anti-p53 monoclonal antibody p53 Ab-1 (Oncogene) confirmed that the 20 bp sequence was a p53-binding site (Figure 2B). Moreover, the addition of increasing amounts of the unlabeled wt-DDR1 p53-BS oligo, as well as an oligo containing the p53 binding site from the p21 promoter (El-Deiry *et al.*, 1993), competed with the formation of the electrophoretically shifted complex (Figure 2B), confirming the specificity of p53 binding to the DNA sequence from the DDR1 intron region. These results support the conclusion that DDR1 is a direct p53 transcriptional target.

DDR1 is activated/tyrosine phosphorylated in response to genotoxic stress

We next determined whether DDR1 was activated, as measured by tyrosine phosphorylation, in response to DNA damage such as γ -irradiation via p53. HCT116 cells containing wt-p53 were irradiated (5 Gy) and 12 h later analyzed for levels of tyrosine phosphorylation of endogenous DDR1 receptor by immunoprecipitation with DDR1 antibodies, followed by an immunoblot analysis using PY20 phosphotyrosine antibodies. As shown in Figure 3A, DDR1 receptor expression was dramatically increased after γ -irradiation above an undetectable level in untreated cells. Following irradiation, tyrosine phosphorylation of the induced receptor was readily detectable as well. To further investigate the kinetics of DDR1 activation/phosphorylation by γ -irradiation, we transfected HCT116 cells with DDR1b-myc to exogenously express the myc-tagged receptor. The transfected cells were exposed to γ -irradiation (5 Gy), and cell lysates were

Fig. 2. The DDR1 is directly p53 responsive. (A) Schematic of the genomic structure of the DDR1 gene. Open boxes indicate locations and sizes of the exons. The location of a potential p53-binding site (p53-BS) indicated in intron 3 and the consensus p53-binding sequence are also shown. Required bases for the p53-binding site are in bold (C and G). (B) EMSA of a potential p53-BS in intron 3 of DDR1. An EMSA was performed using oligos corresponding to a potential p53-BS in DDR1 intron 3 (20 bp) and a mutant BS-p53 (GAATCGTCCAGGGCTCGTTC, bold mutated from CAAG) as radiolabeled probes. Saos2-p53 (10 µg) nuclear extract was incubated with 3 ng of probe alone, 30- or 100-fold molar excess of either the unlabeled DDR1 wt-p53-BS (competitor, self) or mutant p53-BS (mutant competitor). Anti-p53 antibody, p53 Ab1 (Pab421), was present in the designated lanes. The arrows indicate the position of the p53-DNA complex (arrow 1) and supershifted complex containing antibody, p53 and DNA (arrow 2). Right panel: competition was also performed using the unlabeled p53 consensus oligo from the human p21 promoter, 5'-AGCTTG-AACATGTCCCAACATGTTGA-3'. (C) Luciferase assay of each construct. The DDR1 intron sequence containing potential p53-BS is activated by wt-p53. pGL3-DDR1-p53-BS-luciferase reporter construct is also shown. The pGL3-promoter-DDR1 p53-BS plasmid (DDR1-intron-wt) or pGL3-DDR1-mutant-BS plasmid (DDR1-intron-mt), and pCMV-β-gal plasmid (for normalization) were co-transfected with either wt-p53 or mutant p53 expression plasmid, as well as the vector (pcDNA3 alone) into Saos2 cells that were grown to 60% confluence in 60 mm diameter dishes 24 h prior to lipofectin transfection. Intron 3 mutant vector (DDR1-intron-mt) contains a point mutation that changes the seventh nucleotide 'G' of p53 BS to 'T'. After transfection, cells were incubated for 48 h. Samples were then collected, and luciferase and β-galactosidase activities were measured.

analyzed at the indicated times (1, 3, 6 and 12 h) by immunoprecipitation with anti-myc followed by immunoblot analysis with anti-myc or anti-pTyr. Of note, activation of the exogenously expressed receptor was not detectable in the absence of irradiation but was readily observed 3–6 h following exposure (Figure 3B). As a control, similar levels of myc-DDR1 were observed in each cell lysate. Taken together, these results demonstrate that the DDR1 receptor is both induced and activated/phosphorylated in response to DNA damage such as γ-irradiation in wt-p53-containing cells.

Effects of p53-induced and activated DDR1 on downstream signaling pathways

To investigate whether the DDR1 receptor might be involved in the p53-mediated MAPK signaling, human 293T cells were transfected with DDR1 (pcDNA3-DDR1b-myc) or vector (pcDNA3). As shown in Figure 4A, exogenously expressed DDR1 was tyrosine

phosphorylated with slow but sustained kinetics in response to collagen IV stimulation, and MAPK (p-MAPK) was activated with similar kinetics. As reported previously (Vogel *et al.*, 2000), MAPK was not detectably activated upon DDR1 stimulation by collagen I (data not shown). It is well established that truncated forms of RTK that lack intracellular catalytic domains can function as dominant-negative (DN) mutant proteins by oligomerizing with endogenous wt RTKs (Hunter, 2000; Schlessinger, 2000; Simon, 2000). A truncated DDR1 mutant that lacks the catalytic domain has been shown to act as a DN, inhibiting DDR1 tyrosine phosphorylation in response to collagen in a dose-dependent manner (Bhatt *et al.*, 2000; Vogel *et al.*, 2000). Thus, we generated such a DN-DDR1 and transfected it into 293T cells together with an expression construct containing myc-tagged full-length wt-DDR1. Transfected cells were then stimulated with collagen IV for up to 4 h. As shown in Figure 4A, tyrosine phosphorylation of DDR1 was significantly inhibited by the

Fig. 3. DDR1 is activated/phosphorylated in response to genotoxic stress. **(A)** Tyrosine phosphorylation of endogenous DDR1 proteins following γ -irradiation. p53^{+/+} HCT116 cells were exposed to γ -irradiation (5 Gy) and incubated for 12 h. Cells were then lysed and DDR1 proteins were immunoprecipitated. The amount of endogenous DDR1 proteins in the immunoprecipitate was determined by western blot analysis using DDR1 antibodies (lower blot). The levels of tyrosine phosphorylation of endogenous DDR1 receptor in the immunoprecipitate were measured by western blotting using PY20 phosphotyrosine antibodies. **(B)** Tyrosine phosphorylation of exogenous DDR1 proteins by γ -irradiation treatment. HCT116 cells were transiently transfected with 2 μ g of pcDNA3-DDR1-myc. Sixteen hours after transfection, cells were left untreated (control; no irradiation, -IR) or treated with γ -irradiation (5 Gy). At the indicated times after treatment, cells were lysed and myc-tagged proteins were immunoprecipitated, and the levels of tyrosine phosphorylation were determined by western blot analysis using PY20 phosphotyrosine antibodies. The quantity of DDR1-myc proteins in the myc immunoprecipitate was determined by western blot analysis using myc-tagged antibodies.

DN-DDR1 construct, as were the levels of phosphorylated MAPK compared with levels induced by DDR1 alone. These results confirm that MAPK was a downstream effector in the DDR1 pathway.

Through the use of DN mutants of Ras and Raf, whose products block the functions of the MAPKs within this cascade, we next sought to identify upstream effectors involved in activating the MAPK signaling by DDR1 activation. When DDR1 and either a DN mutant form of Ras (N17Ras) or Raf1 (DN-Raf-Flag), a direct downstream effector of Ras, were transiently transfected into 293T cells stimulated with collagen IV, activation levels of ERK were substantially decreased (Figure 4B). These findings demonstrated that DDR1 activates MAPK through Ras and Raf, as has been demonstrated for p53-induced MAPK activation (Lee *et al.*, 2000).

It is well established that Ras activation of the MAPK cascade can induce p53 through the p19/ARF pathway (Serrano *et al.*, 1997; Lin *et al.*, 1998; Michael and Oren,

Fig. 4. Sustained activation of the Ras/raf/MAPK cascade by DDR1 receptor. **(A)** 293T cells were transiently transfected with vector (pcDNA3), DDR1 (pcDNA3-DDR1-myc) or DDR1 + DN-DDR1 (pcDNA3-DDR1-myc + pcDNA3-DN-DDR1-myc). Twenty-four hours after transfection, cells were serum starved for an additional 24 h. Collagen IV (final concentration 10 μ g/ml) was added and total lysates were prepared at the times (0, 0.5, 1, 2, 3 or 4 h) indicated at the top of each lane. Immunoblots were performed using antibodies against myc tag, phospho-MAPK/ERK and β -actin, respectively. The levels of tyrosine phosphorylation of DDR1 receptor protein were analyzed by immunoprecipitation with DDR1 antibodies, followed by western blot analysis using PY20 phosphotyrosine antibodies. Expression of DDR1-myc was detected by using myc-tagged antibodies. Lysates from transfected cells stimulated with collagen IV were immunoprecipitated by DDR1 antibodies. **(B)** Inhibition of DDR1-induced MAPK activation by DN-Ras and DN-Raf. 293T cells were transfected with vector, DDR1-myc, DDR1b plus DN-Raf, or DDR1b plus DN-Ras as indicated at the top of each lane. Lysates were prepared at 48 h post-transfection. Immunoblots were performed using antibodies against phospho-MAPK, total MAPK, myc tag (DDR1b-myc) or flag tag (DN-Raf).

2002). Therefore, it was possible that exogenous DDR1 expression and activation might cause accumulation of p53, p21 and ARF through activation of the Ras/MAPK cascade. Infection of adenovirus-expressing DDR1 (Ad-DDR1) into wt-p53-containing cells, IMR90, MCF7 and HCT116, in the presence of collagen IV led to high levels of exogenous DDR1 expression and tyrosine phosphorylation along with increased levels of both phosphorylated MAPK and AKT. Under these conditions, Mdm2 and ARF expression levels also increased associated with increased levels of p53, phosphoserine-15 p53 (pSer15-p53) and p21 (Figure 5A). Moreover, in all three lines tested, the expression and activation of DDR1 increased expression levels of Bcl-X_L, an anti-apoptotic protein, while pro-apoptotic proteins including Noxa (Figure 5A) and Puma

(data not shown) were expressed at similar levels with or without DDR1.

To assess whether DDR1 induction by genotoxic stress had similar effects on induced p53 levels, DDR1 function was inhibited in wt-p53 cells including IMR90, HCT116 and MEF by expressing DN-DDR1. Normal diploid fibroblasts (IMR90) and HCT116 cells were infected with Ad-GFP or Ad-DN-DDR1, followed by exposure to γ -radiation (5 Gy). MEF cells were exposed to adriamycin (0.3 μ g/ml) after infection with either Ad-GFP or Ad-DN-

DDR1. Twelve hours after irradiation or adriamycin treatment, cells were collected for immunoblotting analysis using DDR1, phospho-MAPK, p53, pSer15-p53, p21 and Mdm2 antibodies. As shown in Figure 5B, these genotoxic treatments led to increased p53, pSer15-p53, p21 and Mdm2 levels as well as increased DDR1 expression associated with MAPK activation. Inhibition of DDR1 function by DN-DDR1 inhibited irradiation-induced MAPK phosphorylation associated with a detectable decrease in DDR1, p53, pSer15-p53, p21 and Mdm2

Fig. 5. Effects of p53-induced and -activated DDR1 on downstream signaling pathways. (A) Activated DDR1 induces accumulation of p53, p21 and ARF in wt-p53-containing cells. IMR90, MCF7 and HCT116 cells were used for this study. Immunoblot analyses of cellular lysates corresponding to cells infected with control GFP-expressing (C) or DDR1-expressing (D) adenoviruses were performed using antibodies against p53, pSer15-p53, DDR1, phospho-MAPK, p21, p19/ARF, Mdm2, Bcl-X_L, Noxa and β -actin. (B) The effects of DDR1 on p53-downstream signaling after genotoxic stress. IMR90 and HCT116 cells were infected with Ad-GFP (GFP) or Ad-DN-DDR1 (DN), followed by exposure to γ -irradiation (5 Gy). Wt-MEFs were also infected with Ad-GFP (GFP) or Ad-DN-DDR1 (DN), followed by exposure to adriamycin (0.3 μ g/ml). Twelve hours after irradiation or adriamycin treatment, cell lysates from treated or untreated cells were collected for western blot analysis using DDR1, p53, pSer15-p53, phospho-MAPK, p21, Mdm2 and β -actin (loading control) antibodies.

Fig. 6. Effects of DDR1 on p53-mediated apoptosis. (A) Effect of DN-DDR1 expression on p53-induced apoptosis and MAPK activation. Saos2 cells lacking functional p53 were co-infected with Ad-LacZ, Ad-DDR1b or Ad-DN-DDR1b for 24 h prior to co-infection with Ad-p53 or Ad-LacZ for an additional 24 h. At the time of co-infection with Ad-p53, cells were treated for 24 h with a MEK inhibitor, U0126, at a concentration of 20 μ M or DMSO as a control solvent. The left panel shows a western blot analysis using lysates prepared from cells co-infected with adenoviruses. Western blot analysis was performed using antibodies against p53, myc tag, DDR1, phospho-MAPK, p21, Mdm2, Bcl-X_L and β -actin. The middle panels show the patterns of apoptosis analysis by FACSscan. The M1 cell population represents apoptotic cells from each sample. The percentages of these cells were calculated and are represented in the right panel. Error bars indicate $\pm$ SD of three independent experiments with duplicate plates. (B) Antisense inhibition of DDR1 in p53-induced apoptosis. Saos2 cells were co-infected with Ad-p53 and Ad-AS-DDR1. The left panel shows a western blot analysis using lysates prepared from cells co-infected with adenoviruses. Western blot analysis was performed using antibodies against p53, DDR1, phospho-MAPK, p21 and β -actin. An AS-DDR1 cDNA construct (AS-DDR1) containing the 0.9 kb *XhoI* fragment of DDR1 was made in the same adenovirus expression system and amplified. Co-expression of p53 and AS-DDR1 cDNA suppressed p53-induced DDR1 expression by ~60–70%, as compared with the level of DDR1 induced by p53 alone. The right panels show the patterns of apoptosis analysis by FACSscan. p21 was used as a control target gene of p53. (C) Effects of inhibitors on cell survival after co-expression of p53 and DDR1 or DN-DDR1. Saos2 cells were co-infected with Ad-p53 or Ad-LacZ with Ad-DDR1 or Ad-DN-DDR1, then treated for 24 h with the indicated inhibitors, U0126 (20 μ M) and/or LY294002 (10 μ M), followed by Trypan Blue staining. The right panel represents the effect of a constitutively active Raf (Raf22W) on DN-DDR1-mediated cell death. Saos2 cells were transfected with a plasmid expressing Raf22W and, 24 h later, cells were co-infected with Ad-p53 or Ad-LacZ with Ad-DN-DDR1 or Ad-GFP for an additional 24 h, followed by Trypan Blue staining. The percentage of dead cells was calculated and compared. Error bars are means $\pm$ SD of two independent experiments with duplicate plates.

levels. Taken together, these findings indicate that DDR1 is an upstream regulator of the Ras/Raf/MEK/MAPK cascade as well as of AKT activation, and that DDR1 induction/activation by genotoxic insult results in a positive feedback loop to increase p53.

Induction of DDR1 increases cell survival in response to p53-mediated apoptosis and genotoxic stress

To investigate the biological functions of p53-mediated DDR1 induction, we determined the effects of DDR1

inhibition on p53-induced cellular responses such as apoptosis by expression of the DN form of DDR1 (DN-DDR1) or antisense (AS)-DDR1. An AS-DDR1 cDNA construct containing the 0.9 kb *XhoI* fragment of DDR1 was made in the same adenovirus expression system and amplified. Co-expression of p53 and AS-DDR1 repressed p53-induced DDR1 expression by ~60–70%, as compared with the level of DDR1 expression in response to p53 alone. Expression levels from each adenovirus co-infection were examined by western blot analysis and are shown in Figure 6A and B (left panels). Following

infection of Saos2 cells that lack functional p53 with Ad-DDR1-myc (Ad-DDR1-myc) or p53 (Ad-p53), high levels of DDR1 expression and an increased level of phospho-MAPK were observed (Figure 6A). Co-infection of Ad-p53 and Ad-DDR1-myc further enhanced the level of an active form of MAPK, while co-expression of p53 and DN-DDR1 abrogated p53-induced MAPK activation (Figure 6A). Of note, DN-DDR1 expression in p53-expressing cells reduced p21 and Mdm2 expression, as was observed when DN-DDR1 was expressed in irradiated IMR90 or HCT116 cells (Figure 5B). DN-DDR1 expression in p53-expressing cells also reduced Bcl-X_L expression. Co-expression of p53 and DN-DDR1 or AS-DDR1 resulted in a dramatic increase in p53-induced apoptosis (DN-DDR1, ~13.4–32%; AS-DDR1, ~15–33%) in p53-null Saos2 cells compared with that induced by co-expression of p53 with *LacZ* or wt-DDR1 (Figure 6A and B, right panels). In contrast, co-expression of p53 and wt-DDR1 lowered the level of p53-induced apoptosis from 13.4 to 6.6% (Figure 6A, right panel).

In order to determine whether these DDR1-mediated protective effects correlated with the upregulation of MAPK and/or AKT signaling, Saos2 cells co-infected with adenoviruses expressing p53 and DDR1 or p53 and DN-DDR1 were treated with the MEK inhibitor U0126 and/or the PI3 kinase inhibitor LY294002, which was shown to block PI3 kinase-dependent AKT phosphorylation and kinase activity. First, exposure of Saos2 cells expressing p53 and DDR1 to this MAPK inhibitor increased the level of apoptosis significantly (Figure 6A, right panel), indicating that the inhibition of MAPK activation decreased the protective effects of DDR1. Next, Saos2 cells co-infected with adenoviruses expressing p53 and DDR1 or p53 and DN-DDR1 were exposed to DMSO, U0126, LY294002 or both U0126 and LY294002. Control cells were co-infected with adenoviruses expressing *LacZ* and DDR1 or *LacZ* and DN-DDR1, and treated with the same inhibitors including the control solvent, DMSO. Then, cells were collected for Trypan Blue exclusion to measure the population undergoing apoptosis. Figure 6C (left panel) shows that the exposure of cells expressing p53 or p53 and DDR1 to the MEK inhibitor U0126 and/or PI3 kinase inhibitor LY294002 increased the percentage of dead cells. Of note, the treatment of cells expressing p53 or DDR1 with the MEK inhibitor resulted in higher cell death rates as compared with the treatment of the same cells with the PI3 kinase inhibitor LY294002. To further reinforce the DDR1-mediated protective effect through the activation of the Ras/Raf/MEK/MAPK cascade, a constitutive active Raf mutant, Raf-22W (Shields *et al.*, 2002), was transiently transfected into Saos2 cells expressing p53 and DN-DDR1. As shown in Figure 6C (right panel), expression of Raf-22W significantly reduced DN-DDR1-mediated cell death from ~45 to 27%. These results demonstrate that DDR1 protects cells from p53-mediated apoptosis through activation of MAPK signaling.

We also tested the effects of DDR1 overexpression in p53-null cells in response to DNA damage-induced apoptosis. Using the DDR1 adenovirus, DDR1 was overexpressed in p53-null cells, including MEF p53^{-/-} and Saos2, then treated with DNA-damaging agents, adriamycin, γ -irradiation or staurosporin. Cells were then collected for FACS analysis and Trypan Blue exclusion;

sub-G₁ DNA content was analyzed by FACScan. As shown in Figure 7A, exposure to γ -radiation (IR; 5 Gy) after exogenous DDR1 overexpression caused the sub-G₁ population to decrease from ~15 to 8.2% in p53^{-/-} MEF cells, while no significant difference in cell cycle profiles was observed in Ad-GFP-infected control cells. In addition, the percentage of dead cells observed by Trypan Blue staining decreased from ~14 to 4.5% with 0.3 μ g/ml adriamycin (Figure 7A, right panel). As shown in Figure 7B, exposure to staurosporin caused the apoptotic population to decrease from ~29% in Ad-GFP-infected Saos2 cells to ~8.8% in the presence of DDR1. As a control, no significant difference in cell cycle profiles was observed in control cells (no drug treatment).

Since DDR1 can be induced under physiological stress conditions and such induction requires wt-p53 (Figure 1), we next sought to determine the functional consequence of DDR1 induction in wt-p53 cells by inhibiting DDR1 function using DN-DDR1 as well as AS-DDR1 (AS-DDR1). p53^{+/+} MEF and HCT116 cells containing wt-p53 were infected with Ad-DN-DDR1 or Ad-*LacZ* and exposed to γ -irradiation (5 Gy) or adriamycin (0.3 μ g/ml). Cells were then collected for FACS analysis and Trypan Blue exclusion to measure the population undergoing apoptosis. p53^{+/+} MEF cells were also infected with Ad-DN-DDR1, followed by treatment with γ -irradiation. As shown in Figure 8A, DDR1 inactivation resulted in a marked increase in cell death. The percentage of dead cells analyzed by FACS increased from ~23% of Ad-GFP infected wt-MEFs to ~44% of Ad-DN-DDR1 infected wt-MEFs with γ -irradiation. Similarly, adriamycin-induced apoptosis was significantly enhanced in cells expressing DN-DDR1 (Figure 8A, right panel), while exogenously expressed wt-DDR1 resulted in a decrease of cell death from ~23% of GFP-infected cells to ~4.4% with γ -irradiation. When HCT116 cells expressing DN-DDR1 or AS-DDR1 were exposed to γ -irradiation or adriamycin, the sub-G₁ population increased from ~11.9 to 25.9% (DN-DDR1) and ~22.6% (AS-DDR1) (Figure 8B). These data indicate that DDR1 induction protects cells from apoptosis in a p53-dependent manner and that impairment of DDR1 expression or function leads to a pronounced increase of DNA damage-induced apoptosis. To determine whether the death-enhancing effects of DDR1 inhibition were through a caspase-dependent pathway, similar experiments were carried out in the presence of the general caspase inhibitor Z-VAD-fmk. As shown in Figure 8C, addition of Z-VAD-fmk blocked enhanced cell death induced by DDR1 inhibition. Moreover, exogenous DN-DDR1 expression in HCT116 followed by irradiation resulted in a further increase in cleavage of procaspase 3 to the active caspase 3 as compared with control irradiated HCT116 cells, whereas activation of caspase 3 was effectively blocked in DDR1-overexpressing HCT116 cells (Figure 8C).

Discussion

We report here that DDR1 expression levels increase dramatically and rapidly in response to the tumor suppressor p53 or to DNA damage stimuli, which induce p53. We identified a p53 response element within intron 3 of the gene. This element was shown to form a complex

Fig. 7. Protective effect of DDR1 overexpression on genotoxic stress-induced apoptosis in p53^{-/-} cells. (A) p53^{-/-} MEF cells were infected with Ad-GFP or Ad-DDR1, treated with γ -irradiation (5 Gy) and incubated for 24 h. The apoptotic population (indicated by M1) was then measured by FACS analysis. p53^{-/-} MEF cells were also infected with Ad-LacZ, DDR1 or DN-DDR1 for 24 h, then treated with adriamycin for 24 h followed by Trypan Blue staining. The percentage of dead cells was calculated and compared. Error bars indicate means $\pm$ SD of three independent experiments with duplicate plates. (B) Saos2 (p53-null) cells were infected with either Ad-GFP (control) or Ad-DDR1 for 24 h, then treated with staurosporin (0.2 μ M) for 9 h, followed by FACS analysis.

with p53, and a luciferase reporter with this element responded in a p53-specific manner. All of these findings imply that DDR1 is a direct p53 target gene. We show further that DDR1 is functionally activated, as determined by its tyrosine phosphorylation, which was also stimulated in a p53-dependent manner. DDR1 activation contributed to p53 activation of downstream mediators including the Ras/MEK/MAPK cascade and AKT, as demonstrated by the inhibition of these responses in the presence of DN-DDR1. HB-EGF has been shown previously to contribute to MAPK and AKT activation in response to p53 (Fang *et al.*, 2001). Whereas a growth factor might act in a paracrine as well as autocrine manner, DDR1 must function in the target cell exposed to cellular stresses, which upregulate p53.

The Ras/MEK/MAPK pathway is known to exert a role in the regulation of the p53-Mdm2 autoregulatory module

(Serrano *et al.*, 1997; Lin *et al.*, 1998; McMahon and Woods, 2001; Michael and Oren, 2002). In normal cells, expression of oncogenic Ras induces p53, p16 and p19/ARF, leading to features of senescence (Serrano *et al.*, 1997; Lin *et al.*, 1998; Lin and Lowe, 2001; McMahon and Woods, 2001; Ferbeyre *et al.*, 2002). However, the effect of ARF on the ability of Mdm2 to degrade p53 is believed to be cell type dependent and not strictly linear (Ries *et al.*, 2000; Sherr and Weber, 2000; McMahon and Woods, 2001; Ferbeyre *et al.*, 2002; Michael and Oren, 2002). Our previous demonstration that p53 causes sustained MAPK activation, combined with our present findings that DDR1 activates MAPK, predict a positive feedback loop in which p53 induction of DDR1 activates the Ras/raf/MAPK cascade, causing accumulation of p19^{ARF} followed by increased p53 and p53 targets such as p21. In fact, exogenous DDR1 overexpression was found to increase

p19^{ARF}, p53, p21 and Mdm2 levels. Moreover, in wt-p53 cells exposed to DNA damage, inhibition of DDR1 function by DN-DDR1 was associated with less robust increases in DDR1, p53 and p21 protein levels, suggesting that this feedforward loop plays a functional role in the p53 response. A recent study established the role of p19^{ARF} in the p53-mediated arrest responses induced by

specific genotoxic stresses including ionizing radiation (Khan *et al.*, 2000). This study provided strong evidence that p19^{ARF} contributes to the ability of MEFs to undergo p53-dependent cell cycle arrest in response to γ -irradiation, and the lack of damage-induced arrest in p19^{ARF}−/− cells correlated with decreased basal p53 levels (Khan *et al.*, 2000). We propose that DDR1 may be

an important regulator that keeps p53 in an active state through the p53→DDR1→Ras/Raf/MAPK→ARF→p53 autoregulatory module when cells are under stressed conditions (Figure 9).

Paradoxically, we observed that exogenous expression of DN-DDR1 or AS-DDR1, which inhibited activation of downstream effectors, resulted in strikingly increased apoptosis of wt-p53-containing cells in response to DNA-damaging agents through a caspase-dependent pathway. These results imply that, on balance, the induction and activation of DDR1 in response to p53 in the cells analyzed favor a cellular outcome of cellular arrest rather than apoptosis in response to p53 or genotoxic stress. Based on these findings as well as the protective effects of DDR1 exogenous expression in both wt-p53 and null cells exposed to DNA-damaging agents, we propose a multi-step model for the functional interaction between DDR1 and p53 or p21. According to this model (Figure 9), DDR1 induction and activation increase p53 expression levels, which could directly enhance p53 pro-apoptotic or p53-mediated survival in cells subjected to genotoxic stresses. Moreover, the results indicate that the reduction in p21^{WAF1} expression appears to correlate with the apoptosis-enhancing effects of DDR1 inhibition. A recent report provides strong evidence that p21 expression can protect from apoptosis without altering p53 activity (Seoane *et al.*, 2002).

Targeted deletions of *DDR1* in the mouse result in a severe post-natal growth reduction, resulting in smaller size than in control littermates, but with no apparent embryonic phenotype (Vogel *et al.*, 2001). However, the mice are unable to bear offspring because of a lack of proper blastocyst implantation in the uterine wall. It would be of interest to determine the effect of this knockout on cellular responses to DNA stresses in different tissues. Moreover, the generation of DDR1 conditional knockout mice and double knockout mice for DDR1 and p53 could provide further understanding of mechanisms underlying DDR1-associated functions in various p53 responses.

Collagen has been shown to activate DDR receptors in a fundamentally different manner than that of typical high-affinity growth factors, which bind and activate their receptors. The slow kinetics of tyrosine phosphorylation of the DDR1 receptor in response to collagen stimulation has suggested a low-affinity interaction between collagen and DDR receptor (Schlessinger, 1997). We observed that the induction of DDR1 by γ -irradiation was accompanied by its sustained tyrosine phosphorylation. Moreover, DDR1

activation in wt-p53-containing cells by exposure to γ -irradiation was achieved independently of increased collagen levels, since expression levels of collagen (I and IV) were not increased by p53 or DNA damage (data not shown). These findings raise the possibility that some other ligand(s) for DDR1 may be induced in response to p53 or DNA damage, but the mechanism of activation remains to be resolved.

Our evidence demonstrating p53 pro-survival functions mediated by the induction and activation of DDR1, as well as by growth factors such as HB-EGF, counteracts the notion that p53 acts only to promote growth arrest/cell death (Fang *et al.*, 2001). A recent paper has reported that inducible expression of oncogenic Raf in normal epithelial cells strongly induced autocrine expression of the EGF-like growth factors HB-EGF, TGF α and amphiregulin, which were directly implicated in the ability of sustained Raf/MAP kinase pathway stimulation to protect cells from apoptosis (Schulze *et al.*, 2001). Whereas HB-EGF might be postulated to play only a paracrine role in tissue repair (Raab and Klagsbrun, 1997), our present findings concerning p53 activation of DDR1 imply a p53 survival function directly in the cell subjected to genotoxic stress. Such pro-survival functions probably depend not only on the intensity and duration of the p53 response, but on cell

Fig. 9. Model for the role of DDR1 in p53-mediated tumor suppression. p53 induction and activation of DDR1 activates the Ras/raf/MAPK cascade and AKT pathway. DDR1 activation of the Ras/raf/MAPK cascade predicts a positive feedback loop in which p53 induction of DDR1 activates the Ras/raf/MAPK cascade, causing further accumulation of p53, p21, p19^{ARF} and Mdm2. These signaling effects and other unknown downstream effectors promote cell survival in stressed environments.

Fig. 8. Inhibition of DDR1 function enhances genotoxic stress-mediated apoptosis in p53^{+/+} cells. Cells were infected with Ad-GFP, Ad-DN-DDR1 or Ad-AS-DDR1 at a m.o.i. of 100. After 24 h of infection, cells were exposed to DNA-damaging agents, γ -irradiation (5 Gy) and adriamycin (0.3 μ g/ml), and incubated for 24 h, and then analyzed by FACS analysis as well as Trypan Blue staining. (A) Effects of DDR1 inhibition on DNA damage-induced apoptosis in p53^{+/+} MEF cells. p53^{+/+} MEF cells (passage 4–5) were infected with adenovirus expressing *LacZ*, DDR1 or DN-DDR1 for 24 h, followed by treatment with γ -irradiation (IR; 5 Gy). Twenty hours after γ -irradiation, cells were collected for FACS analysis. The experiments were repeated at least three times; representative data are shown. For Trypan Blue exclusion studies, p53^{+/+} MEF cells were infected with Ad-*LacZ*, DDR1 or DN-DDR1 for 24 h, then treated with adriamycin (0.3 μ g/ml) for an additional 48 h, followed by Trypan Blue staining. The percentage of dead cells was compared. (B) Effects of DDR1 inhibition on apoptosis after γ -irradiation or adriamycin in HCT116 cells containing p53^{+/+}. Cells were infected with Ad-*LacZ*, Ad-DN-DDR1 or Ad-AS-DDR1 at a m.o.i. of 100. After 24 h of infection, cells were exposed to γ -irradiation at 5 Gy or adriamycin (0.3 μ g/ml) and incubated for an additional 24 h, and then analyzed by FACS analysis as well as Trypan Blue staining (lower panels). Cells were collected for FACS analysis and sub-G₁ DNA content was analyzed by FACSscan. DNA content, as measured by propidium iodide fluorescence, is depicted on the x-axis. (C) Cell death-enhancing effect of DDR1 inhibition is caspase dependent. HCT116 cells containing wt-p53 were infected with Ad-GFP or Ad-DN-DDR1. After 24 h of infection, cells were pretreated with DMSO or Z-VAD-fmk (50 μ M), a general caspase inhibitor, for 1 h prior to DNA damage (γ -radiation, 5 Gy), and incubated for an additional 24 h, and then analyzed by FACS analysis. The lower panel shows the activity of caspase 3 in respective cell extracts determined by western analysis.

context as well. For example, the balance between pro- and anti-apoptotic effects in the case of DDR1 might conceivably be influenced by the availability of its ligand(s). Our demonstration of the ability to impair such p53 pro-survival responses with inhibitors of MAPK or AKT signaling offers novel therapeutic possibilities. For example, such inhibitors might find clinical application in sensitizing tumors with functional p53 to targeted therapy such as localized ionizing radiation or in selective killing of normal endothelial cells involved in tumor neo-angiogenesis.

Materials and methods

Cell lines and culture conditions

EJ-p53, EJ-CAT, Saos2-p53 and Saos2 control cells were cultured in the presence or absence of tet (1–2 µg/ml) in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C as described previously (Lee *et al.*, 2000). Human MCF7, EJ, 293T, Saos2, HCT116, HCT116-p53^{-/-}, HCT116-p21^{-/-}, PC3 and HeLa cells were maintained in DMEM plus 10% FBS. IMR90 human normal diploid fibroblasts were cultured in DMEM plus 15% FBS. Receptor activation was achieved by the addition of soluble collagen (I or IV; Sigma) at 10 µg/ml. For drug treatment, cells were grown to ~50% confluency prior to exposure to a DNA-damaging agent, mitomycin C at concentrations of 2.5 µg/ml or adriamycin at concentrations of 0.25 µg/ml, for 24 h. HCT116 or Saos2 cells were treated with 20 µM PD98059. After 24 or 48 h, cells were collected and analyzed with FACSscan (Becton-Dickinson) using Cellquest software.

Cell transfections and adenovirus infection

Full-length cDNAs for DDR1b-myc as well as DN mutants of Ras (H-RasN17) (Lange-Carter and Johnson, 1994) and Raf (K375M; Dent *et al.*, 1995) were subcloned into pcDNA3 and used for transient transfection studies. HEK 293T cells were transfected with the indicated plasmids using Lipofectamine plus or 2000 (Gibco). In transient co-transfection experiments, the total number of plasmids was kept constant with empty vector. The expression construct for the full-length wt mouse or human DDR1 is as described previously (pcDNA3-DDR1b-myc; Shrivastava *et al.*, 1997). A DN form of DDR1 was prepared as described previously (Bhatt *et al.*, 2000; Vogel *et al.*, 2000) but a myc tag was added to the C-terminus by PCR. The DN form of DDR1b is a truncated form of DDR1 that lacked the catalytic domain (K529). DN-DDR1 mutant was generated by amplifying the C-terminal portion of the cDNA (pcDNA3-DDR1b-myc) by PCR using a pair of oligo primers, 5'-TACTCGAGG-TCACCGTCTCTCAGAA-3' (sense containing *XhoI* site for cloning) and 5'-CATTCTAGTTGTGGTGTGCC-3', and digested with *XhoI*. The PCR fragment was ligated into pcDNA3-DDR1b-myc to replace the wt catalytic C-terminus with the truncated fragment. Ad-wt-DDR1b or DN-DDR1b was generated as reported previously (He *et al.*, 1998). Cells were infected with DDR1b, DN-DDR1, p53 or *LacZ* viruses at a multiplicity of infection (m.o.i.) of 100 for Saos2 and 10 for HCT116. Plasmids for recombinant adenovirus expression were generously provided by B.Vogelstein. A number of different cell types, including Saos2, HCT116 p53^{+/+} or p53^{-/-}, MEF p53^{+/+} or p53^{-/-}, and IMR90, were infected with recombinant adenoviruses. As a similar approach to a DN-DDR1 mutant, Ad-AS-DDR1 cDNA was generated. Prior to adenovirus generation of AS-DDR1, AS-DDR1 cDNA was expressed in Saos2-p53 or EJ-p53 to test potential inhibition of DDR1 induction following p53 expression. Among several different AS-DDR1 constructs, one containing a 0.9 kb *XhoI* restriction fragment significantly suppressed p53-mediated DDR1 induction. This construct was used to generate an adenovirus-mediated transfer of AS-DDR1 expression. Specificity was tested by co-transient transfection with p53 as well as wt-DDR1 using western blot analysis.

Expression array screening

Affymetrix GeneChips were used for hybridization. Two sets of human expression array (human genome U95A; Affymetrix Inc.) were hybridized with fluorescently labeled rRNA probes derived from total RNAs extracted from EJ-p53 cells grown in the presence or absence of tet for 2 days.

Northern blot analysis

Total RNA was extracted, denatured and subjected to electrophoresis through a 1% agarose-formaldehyde gel (20 µg of total RNA per lane) and transferred to a nylon membrane (Bio-Rad). Hybridization was performed with ³²P-labeled probes prepared by the randomly primed DNA-labeling method for the indicated genes.

Immunoprecipitation and western blot analysis

IMR90, 293T or HCT116 cells were transiently transfected with wt-DDR1, DN-DDR1 or control plasmid. Cells were washed twice with ice-cold PBS with 2 mM sodium vanadate and lysed in EBC lysis buffer as described previously (Fang *et al.*, 2001). Lysates were cleared by centrifugation at 14 000 r.p.m. for 20 min at 4°C. Protein concentrations were then determined using a BCA protein assay kit (Pierce). Immunoprecipitation was performed using 500 µg of cell extracts using DDR1 (Santa Cruz) or myc-tagged (Santa Cruz) antibodies, mixed at 4°C overnight. Protein A beads (Sigma; 20 µl) were added for 1 h; the beads were washed three times with NET-N buffer and subjected to SDS-PAGE followed by immunoblotting with the reciprocal antibodies. Approximately 40 µg of total cellular protein per sample were subjected to SDS-PAGE and transferred to an Immobilon (Millipore) polyvinylidene difluoride filter. Antibodies included 421 monoclonal for p53, the Ab-1 and 3 monoclonal for Mdm2, the Ab-1 monoclonal for p21 (Oncogene Research Products), and phosphoserine-15 p53, monoclonal antibodies for MAPK and phospho-specific MAPK (Cell Signaling).

FACS analysis

Cells were pelleted at 1000 r.p.m. and washed once with 10 ml of ice-cold PBS. The resultant pellets were resuspended in 1 ml of cold PBS. Ethanol (80%) pre-chilled at -20°C was added dropwise with periodic vortexing to mix the cells. The resultant mixture was kept on ice for 60 min. Cells were permeabilized in reagent consisting of 0.5% Triton X-100, 230 µg/ml RNase A and propidium iodide to 50 µg/ml in PBS. Samples were kept at 37°C for 30 min, followed by flow cytometry analysis (Becton Dickinson FACSscan). Data were processed with VERITY ModFit v5.2-MS-Windows software for DNA distribution analysis.

Luciferase assays

Reporter plasmid DNA (pGL3-promoter; Promega) containing the DDR1 intron 3 region with p53-responsive site was transiently transfected into Saos2 cells. Approximately 2 × 10⁶ cells were co-transfected as indicated. Cells were harvested 48 h after transfection, and luciferase activity was measured using a luciferase assay kit (Promega). The assay was normalized by co-transfection of a pCMV-β-gal plasmid and measurement of β-galactosidase activities.

EMSAs

All procedures for nuclear extract isolation were conducted at 4°C. For each 100 mm dish, cells were washed three times with 10 ml of PBS. Cells were then scraped into 500 µl of lysis buffer (20 mM HEPES pH 7.5, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSO, 50 µM leupeptin, 50 µg/ml aprotinin) and centrifuged at 500 g for 5 min. Pellets were resuspended in 200 µl of nuclear extraction buffer (lysis buffer containing 500 mM NaCl) and incubated for 1 h. Samples were centrifuged at 18 000 g for 10 min. For EMSA experiments, Saos2 cells were infected with adeno-p53 virus expressing wt-p53 at a m.o.i. of 100. Nuclear extracts from these cells were incubated for 30 min at room temperature with the appropriate ³²P-labeled double-stranded oligo or, in some samples, with monoclonal anti-p53 antibodies (Pab421; Oncogene Science). After incubation, each reaction mix was subjected to electrophoresis in a native 4% polyacrylamide gel using 0.5% TBE. The gels were exposed for autoradiography at -80°C from several hours to overnight.

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References

- Alves,F., Vogel,W., Mossie,K., Millauer,B., Hofler,H. and Ullrich,A. (1995) Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. *Oncogene*, **10**, 609–618.
- Barker,K.T., Martindale,J.E., Mitchell,P.J., Kamalati,T., Page,M.J., Phippard,D.J., Dale,T.C., Gusterson,B.A. and Crompton,M.R. (1995) Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours. *Oncogene*, **10**, 569–575.
- Bhatt,R.S., Tomoda,T., Fang,Y. and Hatten,M.E. (2000) Discoidin domain receptor 1 functions in axon extension of cerebellar granule neurons. *Genes Dev.*, **14**, 2216–2228.
- Dent,P., Reardon,D.B., Morrison,D.K. and Sturgill,T.W. (1995) Regulation of Raf-1 and Raf-1 mutants by Ras-dependent and Ras-independent mechanisms *in vitro*. *Mol. Cell. Biol.*, **15**, 4125–4135.
- Di Marco,E., Cutuli,N., Guerra,L., Cancedda,R. and De Luca,M. (1993) Molecular cloning of trkE, a novel trk-related putative tyrosine kinase receptor isolated from normal human keratinocytes and widely expressed by normal human tissues. *J. Biol. Chem.*, **268**, 24290–24295.
- El-Deiry,W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
- Fang,L., Li,G., Liu,G., Lee,S.W. and Aaronson,S.A. (2001) p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. *EMBO J.*, **20**, 1931–1939.
- Ferbeyre,G., de Stanchina,E., Lin,A.W., Querido,E., McCurrach,M.E., Hannon,G.J. and Lowe,S.W. (2002) Oncogenic Ras and p53 cooperate to induce cellular senescence. *Mol. Cell. Biol.*, **22**, 3497–3508.
- He,T.C., Zhou,S., da Costa,L.T., Yu,J., Kinzler,K.W. and Vogelstein,B. (1998) A simplified system for generating recombinant adenoviruses. *Proc. Natl Acad. Sci. USA*, **95**, 2509–2514.
- Hunter,T. (2000) Signaling—2000 and beyond. *Cell*, **100**, 113–127.
- Johnson,J.D., Edman,J.C. and Rutter,W.J. (1993) A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain. *Proc. Natl Acad. Sci. USA*, **90**, 5677–5681.
- Khan,S.H., Moritsugu,J. and Wahl,G.M. (2000) Differential requirement for p19^{ARF} in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. *Proc. Natl Acad. Sci. USA*, **97**, 3266–3271.
- Ko,L. and Prives,C. (1996) p53: puzzle and paradigm. *Genes Dev.*, **10**, 1054–1072.
- Lange-Carter,C.A. and Johnson,G.L. (1994) Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science*, **265**, 1458–1461.
- Laval,S., Butler,R., Shelling,A.N., Hanby,A.M., Poulson,R. and Ganesan,T.S. (1994) Isolation and characterization of an epithelial-specific receptor tyrosine kinase from an ovarian cancer cell line. *Cell Growth Differ.*, **5**, 1173–1183.
- Lee,S.W., Fang,L., Igarashi,M., Ouchi,T., Lu,K.P. and Aaronson,S.A. (2000) Sustained activation of Ras/Raf/mitogen-activated protein kinase cascade by the tumor suppressor p53. *Proc. Natl Acad. Sci. USA*, **97**, 8302–8305.
- Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.
- Lin,A.W. and Lowe,S.W. (2001) Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. *Proc. Natl Acad. Sci. USA*, **98**, 5025–5030.
- Lin,A.W., Barradas,M., Stone,J.C., van Aelst,L., Serrano,M. and Lowe,S.W. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev.*, **12**, 3008–3019.
- Lin,Y., Ma,W. and Benchimol,S. (2000) Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. *Nat. Genet.*, **26**, 122–127.
- McMahon,M. and Woods,D. (2001) Regulation of the p53 pathway by Ras, the plot thickens. *Biochim. Biophys. Acta*, **1471**, M63–M71.
- Michael,D. and Oren,M. (2002) The p53 and Mdm2 families in cancer. *Curr. Opin. Genet. Dev.*, **12**, 53–59.
- Oda,E., Ohkim,R., Murasawa,H., Nemoto,J., Shibue,T., Yamashita,T., Tokino,T., Taniguchi,T. and Tanaka,N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, **288**, 1053–1058.
- Perez,J.L., Jing,S.Q. and Wong,T.W. (1996) Identification of two isoforms of the Cak receptor kinase that are coexpressed in breast tumor cell lines. *Oncogene*, **12**, 1469–1477.
- Playford,M.P., Butler,R.J., Wang,X.C., Katso,R.M., Cooke,I.E. and Ganesan,T.S. (1996) The genomic structure of discoidin receptor tyrosine kinase. *Genome Res.*, **6**, 620–627.
- Raab,G. and Klagsbrun,M. (1997) Heparin-binding EGF-like growth factor. *Biochim. Biophys. Acta*, **1333**, F179–F199.
- Ries,S., Biederer,C., Woods,D., Shifman,O., Shirasawa,S., Sasazuki,T., McMahon,M., Oren,M. and McCormick,F. (2000) Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19^{ARF}. *Cell*, **103**, 321–330.
- Sakuma,S., Saya,H., Tada,M., Nakao,M., Fujiwara,T., Roth,J.A., Sawamura,Y., Shinohe,Y. and Abe,H. (1996) Receptor protein tyrosine kinase DDR is up-regulated by p53 protein. *FEBS Lett.*, **398**, 165–169.
- Schlessinger,J. (1997) Direct binding and activation of receptor tyrosine kinases by collagen. *Cell*, **91**, 869–872.
- Schlessinger,J. (2000) Cell signaling by receptor tyrosine kinases. *Cell*, **103**, 211–225.
- Schulze,A., Lehmann,K., Jefferies,H.B., McMahon,M. and Downward,J. (2001) Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev.*, **15**, 981–994.
- Seoane,J., Le,H.V. and Massague,J. (2002) Myc suppression of the p21^{Cip1} Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature*, **419**, 729–734.
- Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D. and Lowe,S.W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16^{INK4a}. *Cell*, **88**, 593–602.
- Sherr,C.J. and Weber,J.D. (2000) The ARF/p53 pathway. *Curr. Opin. Genet. Dev.*, **10**, 94–99.
- Shields,J.M., Rogers-Graham,K. and Der,C.J. (2002) Loss of transgelin in breast and colon tumors and in RIE-1 cells by Ras deregulation of gene expression through Raf-independent pathways. *J. Biol. Chem.*, **277**, 9790–9799.
- Shrivastava,A. *et al.* (1997) An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol. Cell*, **1**, 25–34.
- Simon,M.A. (2000) Receptor tyrosine kinases: specific outcomes from general signals. *Cell*, **103**, 13–15.
- Springer,W.R., Cooper,D.N. and Barondes,S.H. (1984) Discoidin I is implicated in cell–substratum attachment and ordered cell migration of *Dictyostelium discoideum* and resembles fibronectin. *Cell*, **39**, 557–564.
- Sugrue,M.M., Shin,D.Y., Lee,S.W. and Aaronson,S.A. (1997) Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proc. Natl Acad. Sci. USA*, **94**, 9648–9653.
- Takagi,S., Hirata,T., Agata,K., Mochii,M., Eguchi,G. and Fujisawa,H. (1991) The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. *Neuron*, **7**, 295–307.
- Vogel,W. (1999) Discoidin domain receptors: structural relations and functional implications. *FASEB J.*, **13**, S77–S82.
- Vogel,W., Gish,G.D., Alves,F. and Pawson,T. (1997) The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol. Cell*, **1**, 13–23.
- Vogel,W., Brakebusch,C., Fassler,R., Alves,F., Ruggiero,F. and Pawson,T. (2000) Discoidin domain receptor 1 is activated independently of β_1 integrin. *J. Biol. Chem.*, **275**, 5779–5784.
- Vogel,W.F., Aszodi,A., Alves,F. and Pawson,T. (2001) Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. *Mol. Cell. Biol.*, **21**, 2906–2917.
- Vogelstein,B., Lane,D. and Levine,A.J. (2000) Surfing the p53 network. *Nature*, **408**, 307–310.
- Vousden,K.H. (2000) p53: death star. *Cell*, **103**, 691–694.
- Zerlin,M., Julius,M.A. and Goldfarb,M. (1993) NEP: a novel receptor-like tyrosine kinase expressed in proliferating neuroepithelia. *Oncogene*, **8**, 2731–2739.

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