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 Dictyostelium 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-X₁, 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; Vouwen, 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 Dictyostelium 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 juxtamembrane 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 [35S]-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 μg/ml) and adriamycin (Adr, 0.25 μg/ml), at the indicated times.

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 p21CIP1/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-}

**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
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
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., 2002).

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, 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, phosphosine-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-XL, 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 μg/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, phospho-AKT, p21, p19ARF, Mdm2, Bcl-XL, 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 μg/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.](image)

![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 μM 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-XL 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 ± 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 Xhol 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 μM) and/or LY294002 (10 μM), followed by Trypan Blue staining. The right panel represents the effect of a constitutively active Raf (RaR22W) 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 ± SD of two independent experiments with duplicate plates.](image)
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-XL 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 constitutively 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 FACSscan. As shown in Figure 7A, exposure to γ-irradiation (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 µg/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 µg/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
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 p19ARF followed by increased p53 and p53 targets such as p21. In fact, exogenous DDR1 overexpression was found to increase

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 ± 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 μM) for 9 h, followed by FACS analysis.
p19ARF, 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 p19ARF in the p53-mediated arrest responses induced by specific genotoxic stresses including ionizing radiation (Khan et al., 2000). This study provided strong evidence that p19ARF 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 p19ARF−/− 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 p21WAF1 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 (Seoune 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 DDR1 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, TGFα and amphiregulin, which were directly implicated in the ability of sustained Raf/MAPK 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/crf/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, p19ARF 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 μg/ml), and incubated for 24 h, and then analyzed by FACScan 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 LusZ, 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 FACScan 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-LusZ, DDR1 or DN-DDR1 for 24 h, then treated with adriamycin (0.3 μg/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-LusZ, 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 μg/ml) and incubated for an additional 24 h, and then analyzed by FACScan analysis as well as Trypan Blue staining (lower panels). Cells were collected for FACScan analysis and sub-G1 DNA content was analyzed by FACScan. 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 μM), a general caspase inhibitor, for 1 h prior to DNA damage (γ-irradiation, 5 Gy), and incubated for an additional 24 h, and then analyzed by FACScan 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 neoangiogenesis.

Materials and methods

**Cell lines and culture conditions**

EJ-p53, EJ-CAT, Saso2-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% confluence 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. For 24 or 48 h, cells were collected and analyzed with FACScan (Becton-Dickinson) using CellQuest software.

**Cell transfections and adenovirus infection**

Full-length cDNAs for DDR1-β-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-DDR1-β-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 DDR1β 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-DDR1-β-myC) by PCR using a pair of oligo primers, 5’-TACTCGAGG-TCACGCGTCCCTCGAGA-3’ (sense containing XhoI site for cloning) and 5’-CATTCTAGTGTTGTTGGTCCT-3’, and digested with XhoI. The PCR fragment was ligated into pcDNA3-DDR1-β-myC to replace the wt catalytic C-terminus with the truncated fragment. Ad-wt-DDR1β or DN-DDR1β was generated as reported previously (He et al., 1998). Cells were infected with DDR1β, 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-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 RNA 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 plasmids. 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 RDD1 (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 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 phosphoerin-15 p53, monoclonal antibodies for MAPK and phospho-specific MAPK (Cell Signaling).

**FACS analysis**

Cells were pelleted at 1000 rpm and washed once with 10 ml of ice-cold PBS. The resultant pelleted cells were resuspended in 1 ml of cold PBS. Ethanol (90%) 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 FACScan). 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 × 105 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 mM 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 adenovirus-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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