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PKR, a *p53* target gene, plays a crucial role in the tumor-suppressor function of *p53*

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Type I IFN-induced expression of dsRNA-activated protein kinase (*PKR*) during viral infection is a well-established antiviral mechanism. However, little is known about the expression of *PKR* in the context of *p53* and about *PKR* involvement in p53-mediated tumor suppression. Here, we report that *PKR* is a *p53* target gene and plays an important role in the tumor-suppressor function of *p53*. Activation of *p53* by genotoxic stress induces a significant level of *PKR* expression by acting on the newly identified *cis*-acting element (ISRE), which is separated from the IFN-stimulated responsive element on the *PKR* promoter, resulting in translational inhibition and cell apoptosis. The genotoxin-mediated inhibition of translation is associated with the *p53/PKR/elF2a* (eukaryotic initiation factor-2α) pathway. To some extent, *p53* activation induced by DNA damage facilitates cell apoptosis by activating *PKR*. *PKR*-knockdown human colon cancer cells grew rapidly in nude mice and proved resistant to anti-cancer drugs. These data indicate that *p53*-mediated tumor suppression can be attributed at least in part to the biological functions of *PKR* induced by *p53* in genotoxic conditions.

p53 tumor suppression | p53RE | translation inhibition and apoptosis

The *p53* tumor suppressor plays a pivotal role in cellular homeostasis via the modulation of cell-cycle arrest, DNA repair, senescence, and apoptosis upon exposure to genotoxic stresses (1–7). Stress-mediated *p53* activation results in the expression of a sizeable number of *p53* target genes, including $p21^{waf1/cip1}$ (8) for G0/G1 arrest, $p21^{waf1/cip1}$ and *14–3-3σ* for G2/M arrest (9), *Bax*, *Noxa*, *Puma*, *Killer/DR5*, *Fas/Apo1*, and others for *p53*-mediated apoptosis (10), a *p53*-inducible gene referred to as "*TIGAR*" for the control of glycolysis and cell survival (2), *DRAM*, a lysosomal protein that induces macroautophagy (11), and *POMC/MSH* keratinocyte-secreting hormone required for UV-induced pigmentation (12).

On the other hand, type I interferons (IFN-α/β), essential cytokines for antiviral activity, sometimes are referred to as "negative growth factors" (13) and manifest anti-oncogenic activities (14). In fact, type I IFNs have been used in the treatment of certain human cancers (15). A recent report demonstrated that the anti-oncogenic activities of IFNs are, in part, accompanied by an increase in the expression of *p53* (16). However, the major proportion of the anti-oncogenic activity of type I IFN is most likely accompanied by the type I IFN-inducible dsRNA-activated protein kinase (*PKR*). IFN-α/β induces the expression of *PKR* through the activation of the highly conserved 13-bp sequence of the IFN-stimulated responsive element (*ISRE*) on the *PKR* promoter (17). *PKR* is one of the best-characterized protein kinases and is involved both in IFN-mediated antiviral activity (18) and in IFN-mediated anti-oncogenic activity (14, 16). Recently, it also was found that *PKR* or viral dsRNA down-regulates *p53* (19, 20). Although the biological functions of *PKR* have been evaluated, it remains unclear whether the induction of *PKR* expression is exclusively dependent on type I IFN. If so, we would be unable to account for *PKR*-associated growth regulation or apoptosis in cells that lack type I IFN.

We found that *PKR* expression was markedly enhanced by genotoxin treatment in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells, regardless of viral infection or IFN treatment. Based on these observations, we investigated whether genotoxin-mediated *PKR* expression is associated with *p53*. Here we report that *p53* induces *PKR* expression by acting on the newly identified *cis*-acting element (*p53RE*) on the *PKR* promoter. *p53* activation caused by DNA-damaging stress results in a significant increase in the expression of *PKR*, followed by the induction of *PKR*-associated biological functions such as inhibition of translation and cell apoptosis, which have been studied in association with the tumor-suppressor functions of *p53*.

Results

p53 Induces a Significant Level of PKR Expression in an IFN-Independent Manner. *PKR*, as well as other *p53* target genes, was up-regulated in HCT116 ($p53^{+/+}$) human colon cancer cells relative to isogenic HCT116 ($p53^{-/-}$) cells (Fig. 1*A*). The transient expression of *p53* induced *PKR* expression at both transcriptional (Fig. 1*B*) and translational levels in a dose-dependent manner (Fig. 1*C*); the quantity of *PKR* induced by *p53* was equivalent to that induced by IFN-α and was equivalent to other *p53* target genes (Fig. 1 *A-C*) such as $p21^{waf1/cip1}$, *Puma*, or *Bax* (4, 7). In the immunocytochemistry and confocal studies, the *p53*-transfected HCT116 $p53^{-/-}$ cells were stained clearly by anti-*PKR* antibody (green), and the level of staining was quite similar to that of cells treated with IFN-α. The cells transfected with empty vector were barely stained (Fig. 1*D*). Even under physiological conditions, *PKR* was significantly induced by genotoxic stresses in $p53^{wt}$ cells but not in $p53^{-/-}$ (i.e., $p53^{null}$) cells or in $p53^{mutant}$ cells (Fig. 1*E*; Fig. S1*A*), suggesting that endogenous *p53* would be sufficient to activate *PKR* expression. Similar results were seen in $p53^{+/+}$ and $p53^{-/-}$ mouse embryo fibroblast (MEF) cells at mRNA (*Left*) and protein (*Right*) levels (Fig. S1*B*). In addition, *PKR* expression induced by doxorubicin treatment was diminished markedly by *PFTα*, a *p53*-specific inhibitor (Fig. 1*F*) (21). These results indicate that *p53* probably is involved in the enhancement of *PKR* expression. On the other hand, Takaoka et al. (16) found that IFN-induced *p53* is crucial for host antiviral defense during vesicular stomatitis virus (VSV) infection. However, the underlying mechanism downstream of *p53* has not been demonstrated. In the present study, as reported previously (16), type 1 IFN induced the expression of *p53* in a dose-dependent manner (Fig. 1*G*). Also, VSV infection induced the expression of type I IFN regardless of *p53* but induced *PKR* expression more

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The authors declare no conflict of interest.

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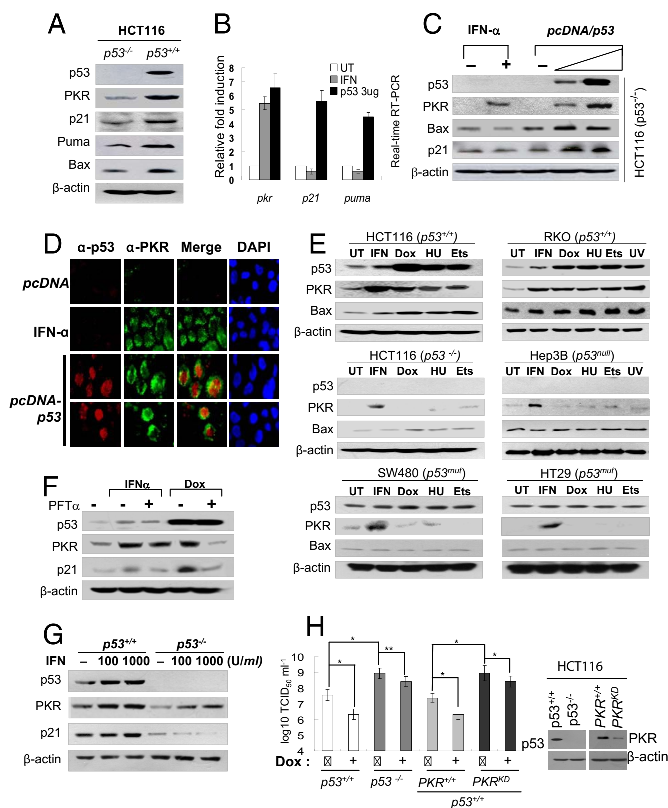

Fig. 1. *p53* induces *PKR* expression. (*A*) Expression of endogenous *PKR* and other *p53* target genes in HCT116 ($p53^{+/+}$ *or* $p53^{-/-}$) cells was assessed by Western blot analysis. (*B*) mRNA levels of *PKR* and other *p53* target genes in the *p53*-transfected (3 μg *pcDNA3-p53*) or IFNα-treated (1,000U/ml for 24 h) HCT116 $p53^{-/-}$ cells and in untreated cells (UT) were examined by real-time quantitative RT-PCR. Results are expressed as means ± SEM ($n = 3$). (*C*) Western blot analysis of *PKR* and other *p53* target genes in *p53*-transfected (1 or 3 μg of *pcDNA3-p53*) or IFNα-treated HCT116 $p53^{-/-}$ cells. (*D*) Immunocytochemistry of *p53*-transfected (1 μg *pcDNA3-p53*) or IFNα-treated (1,000U/ml for 24 h) HCT116 $p53^{-/-}$ cells after staining with anti-*PKR* antibody and anti-rabbit IgG-FITC (green) and anti-*p53* antibody (DO-1) and anti-mouse IgG-Rhodamine (red), together with each isotype control IgG, as described in *Materials and Methods*. (*E*) *PKR* expression in *p53* wild-type, mutant, and null cells under conditions of DNA damage (0.5 μM doxorubicin [Dox], 5 μM etoposide [Ets], and 1 mM hydroxyurea [HU]) for 12 h or 6 h after 20 J/m² UV) and in untreated cells (UT). (*F*) *PKR* expression in RKO cells treated with IFN-α or doxorubicin (Dox) in the presence or absence of 20 μM pifithrin alpha (PFTα), a *p53*-specific inhibitor. (*G*) Western blot analysis of *PKR* and *p53* in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells treated with IFNα for 12 h. (*H*) HCT116 cells ($p53^{+/+}$, $p53^{-/-}$, $p53^{+/+}$/sh-con, or sh-*PKR*) infected for 1 h with VSV (multiplicity of infection = 1) were cultured in presence or absence of doxorubicin (Dox, 1 μM). After 12 h, VSV in the culture supernatant was titrated by the 50% tissue culture infectious dose ($TCID_{50}$) method. *PKR*-knockdown and *p53*-knockout was evaluated (*Right*). *, $P < 0.01$ and **, $P < 0.02$, as compared with indicated control, respectively.

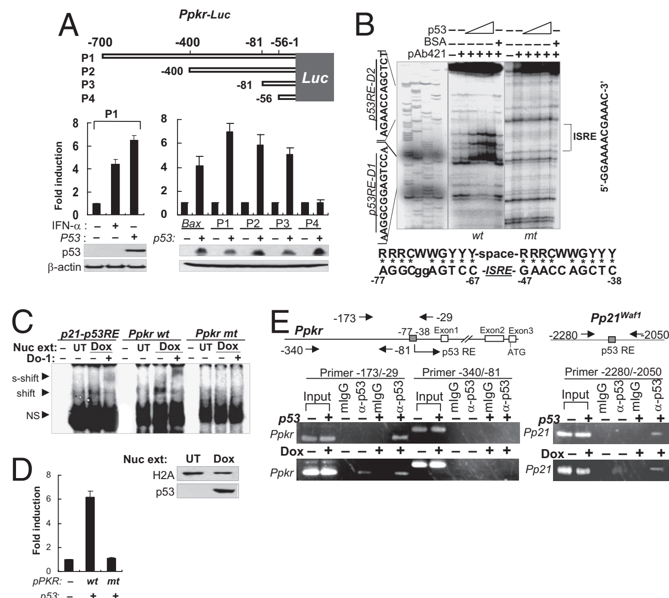

Fig. 2. *p53* acts directly on the *PKR* promoter. (*A*) Promoter deletion assay using luciferase reporters (*Ppkr-Luc*). Luciferase activities were assessed in *p53*-transfected HCT116 ($p53^{-/-}$) cells. Results are reported as mean ± SEM ($n = 3$). (*B*) *DNase I* footprinting with ^{32}P-labeled wild-type (*wt*) and mutant (*mt*, sequence shown in Fig. S4) *PKR* promoter fragments (-160/-29) and p53 protein (BaculoV-p53). Consensus *p53RE* sequence is aligned with the identified *Ppkr-p53RE* sequence (*Lower*). (*C*) EMSA using nuclear extracts (Nuc ext) obtained from doxorubicin-treated HCT116 $p53^{+/+}$ cells (Dox), ^{32}P-labeled $PKR_{81/-29}$/*p21* promoter fragments, and α-*p53* antibody (DO-1). Nuclear extracts were assessed with anti-histone 2A (H2A) antibody and DO-1 (*Lower*). UT, untreated; NS, nonspecific. (*D*) Luciferase reporter assay with wild-type (*wt*) and mutant (*mt*) *PKR* promoters (*Ppkr*) used in EMSA in panel *C* and in Fig. S4*B*. Luciferase assay was performed 48 h after transfection of HCT116 $p53^{+/+}$ cells with wild-type or mutant *PKR* promoter (*Ppkr*-81/-29)-attached luciferase reporters together with *p53*-expressing plasmid (1 μg *pCDNA*-p53). Data shown are from 3 independent experiments and are expressed as means ± SEM. (*E*) ChIP assay with chromatins obtained from *p53*-transfected HCT116 ($p53^{-/-}$) cells or from doxorubicin-treated (0.5 μM for 12 h) $p53^{+/+}$ cells (Dox), and DO-1 (α-*p53*) or control mouse IgG (mIgG).

efficiently in $p53^{+/+}$ cells than in $p53^{-/-}$ cells (Fig. S2). The antiviral activity of *p53* was abrogated by *PKR* knockdown (Fig. 1*H*). These results indicate that the antiviral function of IFN-induced *p53* is associated with the expression of *PKR* downstream of *p53*.

***p53* Acts Directly on the *PKR* Promoter.** We then attempted to determine whether *p53* acts directly on the *PKR* promoter. In the luciferase reporter assay, using *PKR* promoter (*Ppkr*)-conjugated luciferase (*Ppkr-luc*), transient expression of *p53* induced strong luciferase activity in the $Ppkr_{full}$-*luc*–transfected cells (Fig. 2*A*, *Lower left*), suggesting that *p53* may act on the *PKR* promoter. A series of promoter deletion assays showed that the putative *p53*-responsive region is located around -81/-1 of the 5′-franking region of the *PKR* promoter (Fig. 2*A*, *Lower right*). Further promoter deletion assays narrowed this region to -81/-38 (Fig. S3).

To verify the location of *p53RE* on the *PKR* promoter, we conducted a *DNase* I footprinting assay. Two protected regions and their sequences were identified on positions -77/-67(*p53RE-D1*) and -47/-38 (*p53RE-D2*) of the *PKR* promoter, whereas these footprints were not detected by mutation in the region (Fig. 2*B* and Fig. S4*A*). As shown at the bottom of Fig. 2*B*, the putative *pkr-p53RE* sequence located on both sides of *ISRE* has some mutations at each site and an unusually long spacer (19 bp instead of less than 13 bp) between the 2 half-sites, as compared with the well-established *p53RE* consensus sequence (22).

In an EMSA using baculovirus-expressed p53 protein (BaculoV-p53) and *Ppkr* oligonucleotide ($Ppkr_{-81/-29}$) encompassing the putative *p53*-responsve region (*pkr-p53RE*), the *Ppkr* oligonucleotide was shifted by *p53* binding and also was supershifted by anti-*p53* Ab (DO-1), whereas the mutant oligonucleotide containing *pkr-p53RE* was not shifted or supershifted (Fig. S4 *A* and *B*). In the cross-competitive EMSA, *p53* binding to ^{32}P-labeled $Ppkr_{-81/-29}$ was not blocked completely by the unlabeled *p21-p53RE* competitor until a 500-fold molar excess was used, whereas the *p53*/^{32}P-labeled *p21-p53RE* binding was blocked completely at only a 25-fold molar excess of the unlabeled $Ppkr_{-81/-29}$ competitor (Fig. S4*B*). In the EMSA with nuclear extracts and $Ppkr_{-81/-29}$ oligonucleotides, *p53*-mediated band shift and anti-*p53* antibody-mediated supershift also

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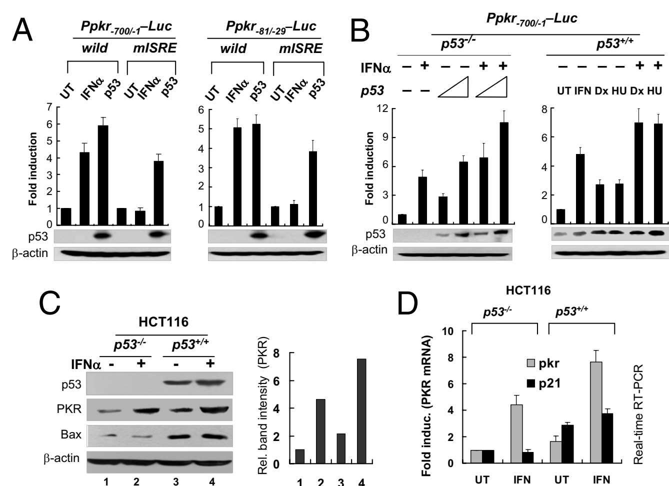

Fig. 3. *p53* activates *PKR* promoter independently of *ISRE.* (*A*) Activity of the *PKR* promoters harboring mutant *ISRE* (*mISRE*) was assessed by luciferase assays in untreated (UT), *p53*-transfected (3 μg *pcDNA3-p53*) or IFNα-treated (1,000U/ml for 24 h) HCT116 $p53^{-/-}$ cells. Results are expressed as means ± SEM (n = 3). (*B*) Additive effects of *p53* and IFN-α on the activity of the *PKR* promoter, accessed in *p53*-transfected HCT116 ($p53^{-/-}$) cells (*Left*), or in genotoxin-treated (0.5 μM doxorubicin [Dx], 1 mM hydroxyurea [HU] for 12 h) HCT116 ($p53^{+/+}$) cells in the presence (+) or absence (−) of IFN-α (*Right*). HU, hydroxyurea; UT, untreated Results are reported as means ± SEM (n = 3). (*C*, *D*) Additive effects of *p53* and type 1 IFN on the expression of *PKR* at protein (*C*) and mRNA (*D*) levels in HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells were assessed by Western blotting and real-time RT-PCR, respectively. Results are reported as means ± SEM (n = 3). UT, untreated.

were observed in doxorubicin-treated HCT116 $p53^{+/+}$ cells (Fig. 2*C*). In the luciferase reporter assay, the $Ppkr_{-81/-29}$ promoter was sufficiently active to induce luciferase activity, but the promoter encompassing mutant *pkr-p53RE* was not active (Fig. 2*D*). These combined data indicate that *p53* activates the *PKR* promoter via direct, specific, and high-affinity binding to the *p53RE*.

To ascertain whether *p53* binds to the *pkr-p53RE* under physiological conditions, we conducted a ChIP assay. As shown in Fig. 2*E*, *Ppkr*-specific ChIP bands were detected clearly only when *p53*-expressing samples (*p53*-transfected $p53^{-/-}$ cells or genotoxin-treated $p53^{+/+}$ cells) were precipitated by anti-*p53* antibody. The ChIP band was notably thicker in the samples obtained from doxorubicin-treated $p53^{+/+}$cells than in those from untreated control (Fig. 2*E*, *Bottom*). These data suggest that *p53* binds directly to the *pkr-p53RE* and activates the *PKR* promoter, resulting in the induction of *PKR* expression in response to DNA damage.

_p53_ Activates the _PKR_ Promoter Independently of _ISRE_. To determine whether *p53*-mediated *PKR* expression is dependent on the *ISRE*, we conducted a reporter assay with *PKR* promoter harboring a mutant *ISRE* (*mISRE*) (Fig. S4*A*). Transient expression of *p53* induced substantial amounts of luciferase activity in the reporter assay with the *mISRE*-harboring *PKR* promoter (*Ppkr-Luc*), whereas IFNα treatment showed no luciferase activity (Fig. 3*A*). These results indicate that *Ppkr-p53RE* is not associated with *ISRE*. However, *p53*, expressed by transfection of *pCDNA-p53* (Fig. 3*B*, *Left*) or physiologically induced by genotoxin-treatment (Fig. 3*B*, *Right*), and IFN-α showed mutual additive effects on *Ppkr*-mediated reporter expression levels. In accordance with the results from the reporter assay, endogenous *PKR* expression itself also was additively enhanced by both *p53* and IFN-α, rather determined by protein levels (Fig. 3*C* and Fig. S5) and mRNA levels (Fig. 3*D*) of each alone. These data indicate that *PKR* can be induced not only by IFN but also by *p53* noncompetitively.

PKR Plays an Important Role in the _p53_-Mediated Inhibition of Translation Under Conditions of DNA Damage. When the cells were treated with genotoxins, the *PKR* in the cytoplasm was largely phosphorylated in the immunohistochemistry (Fig. 4*A*). Genotoxin treatment enhanced *PKR* activation and eukaryotic initiation factor-2α (eIF2α) phosphorylation at Ser-51 in a dose-dependent manner in $p53^{+/+}$ cells, whereas no enhancement was observed in isogenic $p53^{-/-}$ cells (Fig. 4*B*) or in $p53^{KD}$ (*si-p53*) cells (Fig. S6), suggesting that *p53* also is involved in *PKR* activation and eIF2α phosphorylation in response to DNA damage stresses.

Type I IFN-mediated inhibition of translation has been well defined with regard to *PKR*-mediated eIF2α phosphorylation (23). However, DNA damage/*p53*-mediated inhibition of translation has not yet been demonstrated clearly. When cells were treated with doxorubicin, inhibition of translation was observed clearly by the pulse–chase metabolic labeling assay in $p53^{+/+}$ HCT116 cells, but only minor inhibitions were detected in $p53^{-/-}$ HCT116 cells (Fig. 4*C*, *Left*). On the other hand, genotoxin-mediated inhibition of translation in $p53^{+/+}$ HCT116 cells was markedly obliterated by *PKR*-knockdown (*sh-PKR*) (Fig. 4*C*, *Middle*), as was observed in the isogenic $p53^{-/-}$ cells (Fig. 4*C*, *Left*) and other $p53^{KD}$ (*si-p53*) RKO cells (Fig. S7*A*). However, additional *PKR*-knockdown (*sh-PKR*) to the HCT116 $p53^{-/-}$ cells did not increase the resistance of $p53^{-/-}$ cells to the genotoxin-mediated inhibition of translation (Fig. 4*C*, *Right*). These results were supported further by the data from *PKR*-knockout ($PKR^{-/-}$) MEF cells. Translation was markedly inhibited by etoposide in $p53^{+/+}$ MEF cells, whereas no inhibition was observed in $p53^{-/-}$ MEF cells (Fig. 4*D*, *Left*). The difference between $p53^{+/+}$ and $p53^{-/-}$ MEF cells in genotoxin-mediated inhibition of translation also was observed between $PKR^{+/+}$ and $PKR^{-/-}$ MEF ($p53^{+/+}$) cells (Fig. 4*D*, *Right*). Genotoxin-mediated inhibition of translation also was obliterated in eIF2α constitutively active mutant ($eIF2\alpha^{CA}$) (Fig. 4*E*). Taken together, our present findings suggest that inhibition of translation mediated by DNA damage is associated with a *p53*/*PKR*/eIF2α-phosphorylation pathway.

It was reported that, upon activation, mammalian target of rapamycin (mTOR) C1 increases the phosphorylation levels of p70S6 kinase and eIF4E-binding protein 1 (4EBP1), leading to an enhancement of translation (24), whereas, *p53* inhibits the activity of mTOR by activating the AMP-activated protein kinase (AMPK) and tuberous sclerosis (TSC)1/TSC2 pathway (25). We examined whether genotoxin-induced inhibition of translation is associated with the *p53*-mediated mTORC1 inhibitory pathway. As shown in Fig. S7*B*, *PKR*-knockdown of $p53^{+/+}$ cells abrogated the genotoxin-induced inhibition of translation (from 23% to 92%), as shown in $p53^{-/-}$ cells (96%), whereas treatment of $p53^{+/+}$ cells with AMPK inhibitor to block the *p53*-mTORC1 inhibitory pathway attenuated genotoxin-induced inhibition of translation only weakly (from 23% to 33%). These data suggest that the *p53*/AMPK/mTORC1 inhibitory pathway is a minor route compared with the *p53-PKR* pathway in genotoxin-induced inhibition of translation.

PKR Plays an Important Role in the _p53_-Mediated Cell Apoptosis Under Conditions of DNA Damage. On the other hand, it has been well documented that *p53* plays a crucial role in genotoxin/UV-mediated apoptosis and that the *p53*-knockout/knockdown ($p53^{KO/KD}$) cells are resistant to apoptosis mediated by DNA damage (26). The underlying mechanism remains uncertain, however. In the present study, the resistance of $p53^{KO/KD}$ cells to apoptosis mediated by DNA damage also was observed in the PKR^{KD}(*sh-PKR*) HCT116 $p53^{+/+}$ cells in the analyses of early and later-stage apoptosis, by examining annexin V-expressing cells and subG1 populations, respectively (Figs. 4*F* and S8*A*). However, additional *PKR*-knockdown in HCT116 $p53^{-/-}$ cells did not show further resistance to the apoptosis mediated by stress resulting from DNA damage (Fig. 4*F*). These data suggest that *PKR* plays an important role in genotoxin-induced *p53*-mediated cell apoptosis.

Although genotoxin/*p53*-mediated apoptosis has been well established with regard to anti-cancer activity, it remains unclear

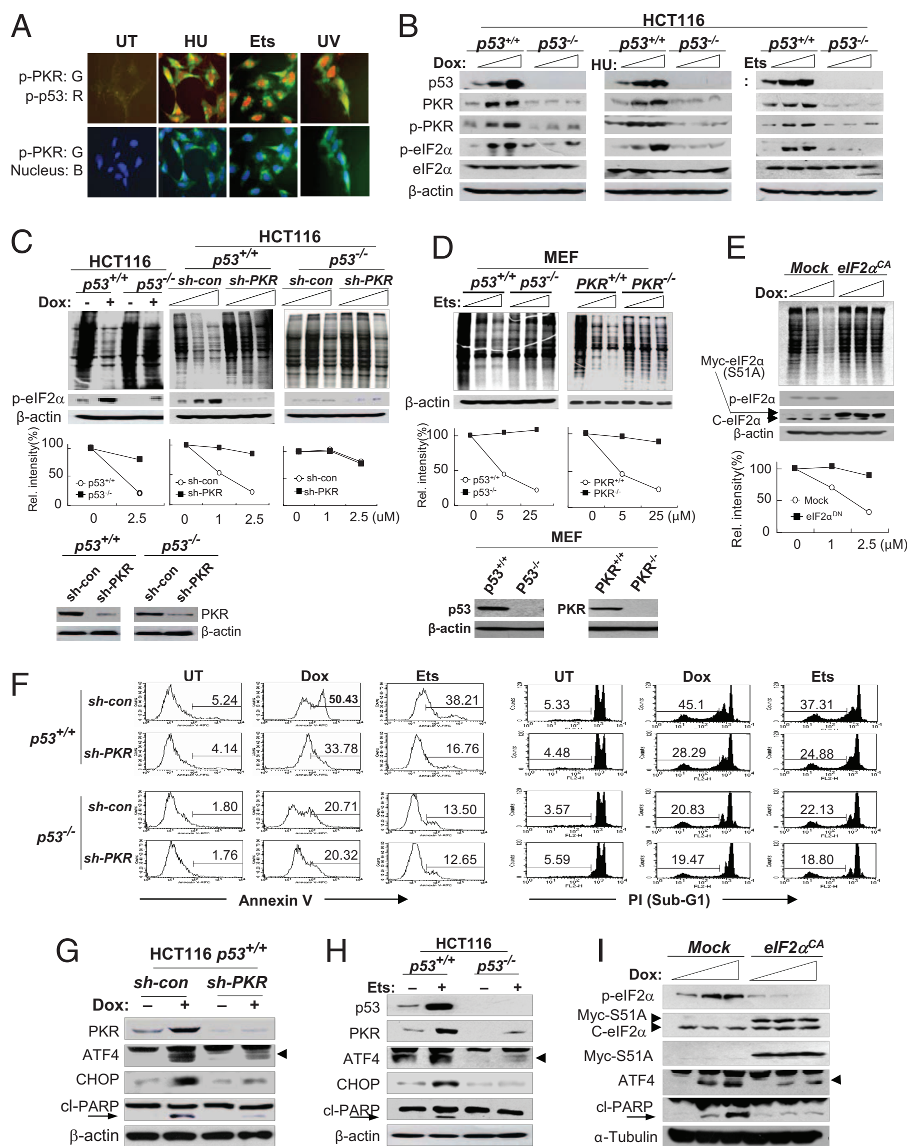

Fig. 4. *PKR* plays a role in the *p53*-mediated inhibition of translation and apoptosis. (*A*) Genotoxin-treated (5 μM etoposide [Ets] and 1 mM hydroxyurea [HU] for 12 h) RKO cells were examined by immunocytochemistry with phospho-*PKR* and phospho-*p53* antibodies and each isotype control (control data not shown). (*B*) Phospho-*PKR* and phospho-eIF2α were assessed in HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells following genotoxin-treatment (0–1 μM doxorubicin [Dox], 0–5 μM etoposide [Ets], and 0–2 mM hydroxyurea [HU] for 12 h). (*C, D*) A metabolic labeling assay performed with $p53^{+/+}$, $p53^{-/-}$, $PKR^{+/+}$, and PKR^{KD} isogenic HCT116 cells and $p53^{+/+}$, $p53^{-/-}$, $PKR^{+/+}$, and $PKR^{-/-}$ MEF cells after genotoxin treatment with increasing concentrations for 12 h (*Top*). Relative band intensities are represented by histograms (*Middle*). *PKR*-knockdown, *p53*-knockout, and *PKR* knockout were confirmed (*Bottom*). Dox, doxorubicin; Ets, etoposide. (*E*) A metabolic labeling assay was performed with normal and eIF2α constitutively active cells ($eIF2\alpha^{CA}$ by permanent expression of eIF2α/S51/A) after doxorubicin (Dox) treatment with increasing concentrations for 12 h. Expression of cellular (c-eIF2α) and mutant eIF2α (Myc-S51A) was confirmed. (*F*) Cell apoptosis was assessed by flow cytometry in untreated (UT) PKR^{KD} (*sh-PKR*) HCT116 ($p53^{+/+}$ and $p53^{-/-}$) cells and after genotoxin treatment. Early-stage apoptosis was assessed by annexin V staining of genotoxin-treated (0.5 μM doxorubicin [Dox] and 5 μM etoposide [Ets] for 36 h) cells (*Left*). Later-stage apoptosis was assessed by measuring subG1 cells after propidium iodide (PI) staining of genotoxin-treated (0.5 μM doxorubicin [Dox] and 5 μM etoposide [Ets] for 48 h) cells, as described in *Materials and Methods*. (*G, H*) *PKR*-associated proapoptotic molecules and cleaved PARP were assessed in PKR^{KD} (*sh-PKR*) isogenic HCT116 ($p53^{+/+}$ and $p53^{-/-}$) cells after genotoxin treatment (0.5 μM doxorubicin [Dox] and 5 μM etoposide [Ets]) for 12 h. (*I*) Phospho-eIF2α, ATF4, and cleaved PARP were determined in normal and $eIF2\alpha^{CA}$ cells after genotoxin treatment with increasing concentrations for 12 h. Dox, doxorubicin.

whether genotoxin-mediated apoptosis is functionally associated with the inhibition of translation. In the kinetic studies, genotoxin-mediated inhibition of translation was observed in the relatively early stage, and cell apoptosis occurred in the later stage in response to doxorubicin in *PKR*-competent (*sh-con*) cells, but inhibition of translation was delayed significantly, in parallel with cell apoptosis, under the same genotoxic condition in PKR^{KD}(*sh-PKR*) cells (Fig. S8 *B* and *C*). These data suggest that even though *PKR* plays a role in genotoxin-mediated inhibition of translation and apoptosis, the genotoxin-mediated apoptosis is, to some extent, functionally associated with but temporally dissociated from *PKR*-mediated inhibition of translation.

The phosphorylation of eIF2α at Ser-51 leads to a significant reduction in protein synthesis, concomitant with induced expression of the basic leucine zipper (bZIP) regulator, activating transcription factor 4 (ATF4), and its target gene CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), resulting in caspase activation and cell apoptosis (27). In accordance with our recent report (28), enhancements of ATF4/*CHOP*, followed by caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP) (29) were detected readily in $p53^{+/+}PKR^{+/+}$ cells but were barely detectable in the $p53^{+/+}PKR^{KD}$ (*sh-PKR*) cells under conditions of DNA damage (Fig. 4*G*). These phenomena also were observed in $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 4*H*). ATF4 enhancement was not detected in $eIF2\alpha^{CA}$ cells even after treatment with genotoxins (Fig. 4*I*), suggesting that the induction of ATF4 in response to genotoxins is dependent on the phosphorylation of eIF2α. ATF4-knockdown or *PKR*-knockdown (by si-RNA) HCT116 $p53^{+/+}$ cells were found to be resistant to genotoxin-induced apoptosis, as compared with the control (*si-con*) $p53^{+/+}$ cells, whereas additional knockdown of ATF4 or *PKR* in $p53^{-/-}$ cells did not increase the resistance of HCT116 $p53^{-/-}$ cells to genotoxin-induced apoptosis (Fig. S8*D*). These results strongly indicate that *PKR* has an important role in *p53*-mediated inhibition of translation and apoptosis under conditions of DNA damage.

PKR Contributes to the Tumor-Suppressor Function of p53. Finally, we attempted to determine whether *PKR* is involved in the tumor-suppressor function of *p53*. Once *PKR* was knocked down (*sh-PKR*), HCT116 $p53^{+/+}$ cells became small and grew rapidly, and the growth rate of the $p53^{+/+}PKR^{KD}$ cells was almost equivalent to that of $p53^{-/-}$ cells (Fig. S9 *A* and *B*). *p53* has been reported to induce G2 arrest under genotoxic stresses (9), but the underlying mechanism remains unclear. In our present studies, doxorubicin-mediated G2 arrest was clearly attenuated by *PKR*-knockdown (by *sh-PKR*) in HCT116 $p53^{+/+}$ cells (Fig. 5*A*), suggesting that *PKR* downstream

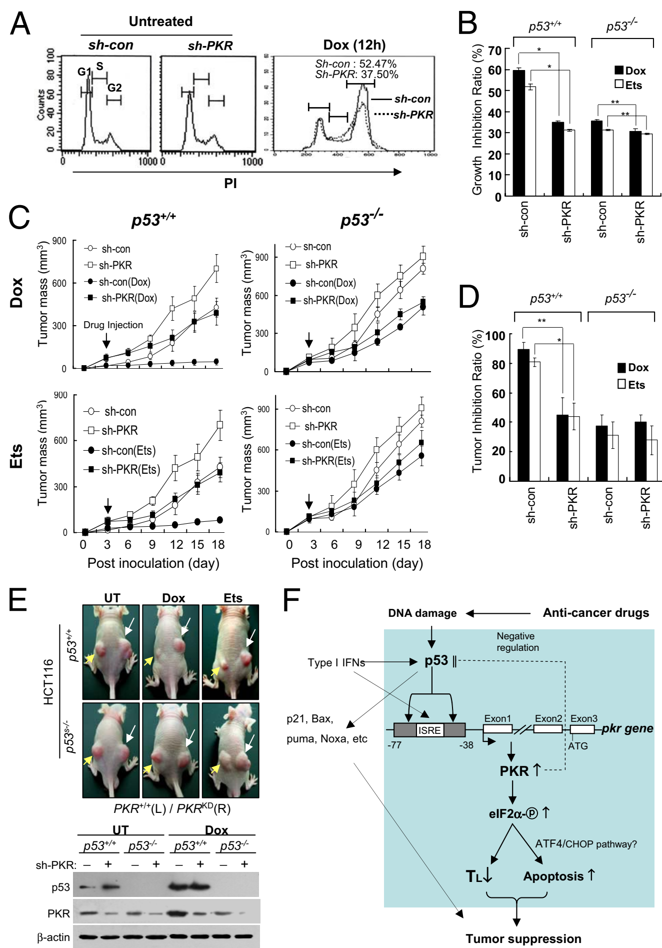

Fig. 5. *PKR* contributes to the tumor-suppressor function of *p53*. (*A*) Normal (*sh-con*) and PKR^{KD} (*sh-PKR*) HCT116 cells were treated or not treated with 0.2 μM doxorubicin (Dox) for 12 h. Each sample was subjected to cell-cycle analysis by flow cytometry with CellQuest software (Adobe) after propidium iodide staining. (*B*) *PKR*-normal (*sh-con*) and PKR^{KD} (*sh-PKR*) HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells were cultured in the presence or absence of 50 nM doxorubicin (Dox) or 1 μM etoposide (Ets), respectively, and the number of cells was recorded every day for 4 days (shown in Fig. S9*C*). Growth inhibition ratios [(1 − number of cells after drug treatment/number of cells without drug treatment) × 100] were calculated with the data obtained on day 4 in Fig. S9C. *, $P < 0.01$ and **, $P < 0.05$, as compared with the group of cells harboring control sh-RNA, respectively. Results are reported as means ± SEM ($n = 5$). (*C*) Nude mice were inoculated s.c. in the dorsal area (10^7 cells/injection, 4 mice/sample) with *sh-con* (left dorsal) and *sh-PKR* (right dorsal) HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells. Three days later, mice were treated i.p. once with doxorubicin (Dox) (2 mg kg^{-1}) or etoposide (25 mg kg^{-1}), and tumor growth was monitored for 18 days. Results are reported as means ± SEM ($n = 4$). (*D*) Inhibition ratios of tumor growth in tumor-bearing mice by doxorubicin (Dox) or etoposide (Ets) treatment were recalculated with the data on day 18 and are represented as means ± SEM ($n = 4$). *, $P < 0.01$ and **, $P < 0.02$, respectively, as compared with the mouse group bearing tumors expressing control sh-RNA. (*E*) Untreated (UT) and genotoxin-treated tumor-bearing mice were imaged on day 15 (*Top*). Yellow arrows and white arrows indicate the tumors established by inoculating *sh-con* (left dorsal) and *sh-PKR* (right dorsal) HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells, respectively. The expression of *p53* and *PKR* was examined from each tumor (*Bottom*). (*F*) *p53*-induced *PKR* expression and associated tumor-suppression mechanisms are described together with other *p53* target genes under conditions of DNA damage. Our findings described in this article are boxed.

of *p53* plays an important role in G2 arrest under genotoxic conditions. The $p53^{+/+}PKR^{KD}$ HCT116 cells (*sh-PKR*) were profoundly resistant to the growth-inhibitory effects of anti-cancer drugs such as doxorubicin and etoposide; their resistance was equivalent to that of $p53^{-/-}$ HCT116 cells under the same conditions (Fig. 5*B* and Fig. S9 *C and D*). These results suggest that *PKR* plays an important role in the *p53*-mediated inhibition of cell growth. We then evaluated the kinetics of tumor growth and the sensitivity of *PKR*-knockdown tumors to anti-cancer drugs in vivo. When inoculated into nude mice, the $p53^{+/+}PKR^{KD}$ HCT116 cells were shown rapidly to establish solid tumors that grew faster than tumors established with control (*sh-con*) $p53^{+/+}$ HCT116 cells. Furthermore, the tumors established with PKR^{KD} cells were resistant to treatment with doxorubicin or etoposide, whereas tumors established with control (*sh-con*) cells were readily blocked by identical treatments (Fig. 5*C*, *Left 2 panels*). On the other hand, the growth rates and drug-resistance patterns of PKR^{KD} tumors were similar to those of $p53^{-/-}$ tumors (Fig. 5*C, Right 2 panels*). In $p53^{+/+}$ tumors, PKR^{KD} (*sh-PKR*) tumors were more resistant to anti-cancer drugs than *PKR*-normal (*sh-con*) tumors, whereas the resistance of $p53^{-/-}$ tumors to anti-cancer drugs was not further affected by additional *PKR* knockdown (Fig. 5*D*). $PKR^{KD}p53^{+/+}$ tumors were larger and more resistant to genotoxin treatments than $PKR^{+/+}p53^{+/+}$ tumors (Fig. 5*E*, *Top*). However in $p53^{-/-}$ tumors, tumor growth and resistance to anti-cancer drugs were not further affected by additional *PKR* knockdown (Fig. 5*E*, *Lower panels*). These results suggest that *PKR* is involved in the *p53*-mediated tumor suppression downstream of *p53*.

Discussion

Although many *p53* target genes have been described, the precise mechanisms of *p53*-mediated tumor suppression remain uncertain. In the present study, we found that *PKR* is a *p53* target gene, regardless of type I IFN or viral infection, and plays an important role in the tumor-suppressor function of *p53* at least in part through inhibition of translation and induction of cell apoptosis. *PKR* was markedly induced by *p53* without the aid of type I IFN (Fig. 1). Recently, more than 540 *p53*-binding loci and 98 *p53* target genes were revealed in the human genome by a ChIP-and-PET (paired-end ditag) coupled screening strategy (30). However, *PKR* and also other *p53* target genes, such as *DRAM* (11), *TIGAR* (31), *POMC/MSH* (12), human cell apoptosis susceptible protein (*hCAS*) (32), and others, were not listed in the report (30).

In accordance with the well-defined *p53RE* consensus sequence (22), we identified 2 *p53RE* domains (*p53RE*-D1 and *p53RE*-D2) near the *ISRE* region on the *PKR* promoter. The binding affinity of *p53* to the *p53RE* on the *PKR* promoter seemed higher than that of *p53* to *p21-p53RE* as determined by competitive EMSA and ChIP assays (Fig. 2 *C* and *E* and Fig. S4*B*). The higher affinity could be attributed to our use of a single 5′-*p53RE* of the p21 promoter (8) rather than both *p21-p53REs*, which are widely separated on the p21 promoter (33). *p53*-mediated activation of the *PKR* promoter remained largely unaffected by mutations on the *ISRE* (Fig. 3*A*), indicating that *PKR* can be induced by *p53* independently of type 1 IFN. Thus *PKR* has a dual function, protecting cells both from DNA damage and from viral infection. Recently, Munoz-Fontela et al. demonstrated that *IRF9* is a *p53* direct target gene, thus causing *p53*-dependent up-regulation of *ISRE*-dependent genes (34). That finding would explain why *p53*-mediated *PKR* promoter activity was reduced slightly by *ISRE* mutation on the *PKR* promoter (Fig. 3*A*). Although genotoxin-induced/*p53*-mediated inhibition of translation has been reported as a tumor-suppressor function of *p53* (35), the precise mechanism remains uncertain.

Our data demonstrate that DNA damage induces *p53* expression, followed by the expression and activation of *PKR*, resulting in phosphor-eIF2α–mediated inhibition of translation and the induction of cell apoptosis (Fig. 4 and Figs. S6–S8). In other words, the genotoxin-mediated inhibition of translation is associated with a *p53*/*PKR*/eIF2α pathway. These results suggest that *p53*-induced *PKR* plays an important role in maintaining cell homeostasis by controlling the inefficient energy consumption of translation under conditions of DNA damage. In addition, well-addressed *p53*-mediated G2 arrest (9) was clearly attenuated by *PKR* knockdown

in genotoxin-treated $p53^{+/+}$ cells (Fig. 5*A*), and similar attenuation patterns were detected regularly in isogenic $p53^{-/-}$ cells in response to doxorubicin/etoposide (data not shown). These data suggest that *PKR* probably is involved in the inhibition of G2/M transitions downstream of *p53*. It has been demonstrated that the cells lacking *p53* target genes, such as *Puma* or *Noxa*, are resistant to apoptosis induced by DNA damage (7). Interestingly, similar patterns of resistance to apoptosis induced by DNA damage were detected in PKR^{KD} human cells (Fig. 4). However, the resistance of $p53^{+/+}$ PKR^{KD} cells to apoptosis induced by DNA damage was not as strong as that of $p53^{-/-}$ cells (Fig. 4*F*), suggesting that, in addition to *PKR*, other pro-apoptotic *p53* target genes may be involved in *p53*-mediated apoptosis in conditions of DNA damage. In supporting experiments, additional *Puma*-knockdown (by *si-RNA*) further attenuated the doxorubicin-mediated apoptosis of PKR^{KD} (*sh-PKR*) HCT116 cells (Fig. S10).

Recently, the Koromilas group reported that *PKR* promotes the proteasomal degradation of *p53* in association with glycogen synthase kinase-3 (GSK-3β) and Mdm-2, independently of translational control (19). Given this background information, we examined *p53* levels in the presence or absence of *PKR* and found that *p53* levels were slightly increased in $p53^{+/+}PKR^{KD}$ HCT116 and RKO cells, as compared with $p53^{+/+}PKR^{+/+}$ cells, both under normal conditions and under genotoxic stress (Fig. S11*A*). The stability of *p53* was slightly enhanced in PKR^{KD} cells, as compared with *PKR*-competent cells (Fig. S11*B*). The treatment of cells with MG132, a proteasomal inhibitor, and Nutlin3, a Mdm2 inhibitor, abrogated the *PKR*-mediated down-regulation of *p53*, and the enhanced *p53* augmented the expression of *PKR* and other *p53* target genes only in $p53^{wt}$ cells (Figs. S11*C* and S12). These results indicate that *p53*-induced *PKR* conversely plays a role in the feedback down-regulation of *p53*. The *PKR*-mediated negative feedback of *p53* seems to be a kind of homeostatic control of *p53*-mediated *PKR* enhancements. However, the *PKR*-mediated down-regulation of *p53* may not be strong enough to obliterate the overexpression of *p53* and down-stream tumor-suppressor functions under genotoxic conditions.

PKR^{KD} cells grew faster than normal cells in vitro and in vivo. PKR^{KD} human colon cancer cells and derived tumors proved to be resistant to anti-cancer drugs as shown in $p53^{-/-}$ cells and derived tumors (Fig. 5). In our recent tissue microarray analysis, we found that the *PKR* level was lower in many human *p53*-negtive (undetectable) tumor tissues than in normal tissues (data not shown). These results indicate that the tumor-suppressor functions of *p53* are, to some degree, attributable to the functions of *p53*-induced *PKR*. Our findings suggest that *PKR* downstream of *p53* may protect cells from tumorigenesis under conditions of DNA damage and facilitate $p53^{+/+}$ tumors that are, in part, susceptible to anti-cancer drugs.

Based on our combined findings, we report that *PKR*, induced by *p53* in response to the stress of DNA damage, plays an important role in the tumor-suppressor function of *p53*, at least in part through the activation of intracellular networks summarized in Fig. 5*F*.

Materials and Methods

Animals, Cells, and Virus. Information about animals, cells, and virus used for the present study is given in *SI Materials and Methods*.

Additional Materials and Methods. DNA damage stresses and reagents, Western blot analysis, recombinant plasmids and mutagenesis, recombinant *p53* protein, real-time quantitative RT-PCR analysis, immunocytochemistry, luciferase assays, EMSA, oligonucleotide probes for EMSA, *DNase* I footprinting, ChIP assay, construction of genetically modified cell lines, and related references are provided in *SI Materials and Methods*.

Statistical Analysis. Statistical analysis was performed using Student's *t* test with GraphPad Instat Software. $P < 0.05$ was considered statistically significant.

Supporting data are available in the *SI Materials and Methods*.

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