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Role for Krüppel-Like Factor 4 in Determining the Outcome of p53 Response to DNA Damage

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Abstract

Cells are incessantly exposed to many sources of genotoxic stress. A critical unresolved issue is how the resulting activation of the p53 tumor suppressor can lead to either cell cycle arrest or apoptosis depending on the extent of DNA damage. The present study shows that the level of Krüppel-like factor 4 (KLF4) expression is inversely correlated with the extent of DNA damage. KLF4 is activated by p53 following cytostatic, mild DNA damage, whereas it is strongly repressed via enhanced turnover of mRNA on severe DNA damage that irreversibly drives cells to apoptosis. Blocking the repression of KLF4 on severe DNA damage suppresses p53-mediated apoptosis, whereas ablation of the KLF4 induction on mild DNA damage shifts the p53 response from cell cycle arrest to cell death. Our results suggest that coordinate regulation of KLF4 expression depending on the extent of DNA damage may be an important mechanism that dictates the life and death decisions of p53. [Cancer Res 2009;69(21):8284–92]

Introduction

Eukaryotic cells harbor a complex network of signaling pathways that are activated on DNA damage to maintain genetic integrity (1). The key molecular component that acts in response to DNA damage is the tumor suppressor p53. DNA damage leads to the stabilization of p53 and the activation of pathways that arrest cell cycle progression, allowing DNA repair if the damage is not severe, or trigger apoptosis if the damage is severe, irreparable (2–4). Whereas cell cycle arrest depends on the ability of p53 to induce the transcription of target genes such as *p21* (5) and *SFN* (6), apoptosis depends on induction of a distinct class of target genes such as *Bax* (7), *Puma* (8), and *Noxa* (9). What remains unclear is precisely how p53 "knows" which genes to turn on or off to achieve the desirable outcome.

Much effort has been invested in understanding the selectivity of the p53 response. A plethora of partner proteins have been implicated in modulating the selection of p53 targets. The cellular environment and the relative abundance of these partners under different conditions could obviously tip the life-or-death balance of p53 activity (4, 10–15). Recently, the zinc finger protein Hzf was found to be involved in opposite arms of the p53 response, providing an important insight into the mechanisms that dictate the life and death decisions of p53 (16).

Krüppel-like factor 4 (KLF4) is a zinc finger protein of the KLF family, which is involved in the regulation of cell proliferation and differentiation (17–20). KLF4 is of particular interest because it shows dramatically different functions depending on cell context. As a transcription factor, KLF4 can activate genes that are inhibitors of proliferation (21, 22) but repress genes that promote proliferation (20, 23). As such, KLF4 functions as a tumor suppressor by inhibiting cellular proliferation. However, the available evidence indicates that KLF4 also shows oncogenic properties. Overexpression of KLF4 in E1A-immortalized rat kidney cells results in hyperplasia and dysplasia (24). Moreover, KLF4 could override Ras^{V12}-induced senescence and induce transformation in primary fibroblasts (25). Recently, it was found that KLF4, in combination with three other transcription factors, could transform mouse fibroblasts into a state resembling embryonic stem cells (26–28).

Given the profound effect of KLF4 on physiologic and pathologic processes, it is not surprising that the expression of KLF4 is tightly regulated. While studying KLF4 activation in response to DNA damage, we found that although cytostatic DNA damage is associated with the previously characterized increment of KLF4 expression (21, 29), apoptotic DNA damage strongly represses KLF4 expression. Further study revealed post-transcriptional repression of KLF4 via enhanced turnover of mRNA. Blocking the repression of KLF4 on apoptotic DNA damage suppresses p53-mediated apoptosis, whereas ablation of the KLF4 induction on cytostatic DNA damage shifts p53 response from cell repair to cell death. Our results suggest that tight regulation of KLF4 expression according to the extent of DNA damage may be an important mechanism that dictates the life and death decisions of p53 in response to genotoxic stress.

Materials and Methods

Cell culture, transfection, and RNA interference. HCT116, HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HeLa, H1299, and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ were kindly provided by Dr. B. Vogelstein. Subconfluent cells were treated with Adriamycin, etoposide, or UV-C irradiation at indicated doses. Cell viability and cell cycle profiles were done as described (30). Small interfering RNAs (siRNA; Genechem) targeting the HuR (#1 AAGAGGCAATTACCAGTTTCA and #2 AACGACTCAATTGTCCCGATA) and a control siRNA (AATTCTCCGAACGTGTCACGT) were used at 25 nmol/L. siRNAs (Genechem) targeting the *KLF4* (GATCAAGCAGGAGGCGGTCTC) were used at 40 nmol/L. Stable silencing of *KLF4* was achieved using the short hairpin RNA–based vector with the target sequence (GGACGGCTGTGGATGGAAA; ref. 25). Transfections were done by using HiperFect transfection reagent (Qiagen) for siRNAs and Lipofectamine 2000 (Invitrogen) for plasmids according to the manufacturer's recommendations.

Plasmid construction. We have subcloned the full-length or 3′-untranslated region (UTR)–deleted *KLF4* cDNA into pTRE vector (Clontech). DNA fragments of KLF4 cDNA coding region were amplified by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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PCR and subcloned into pCDE-HA vector. For generation of the *KLF4* 3′-UTR reporters, the 3′-UTR of *KLF4* was PCR-amplified from pTRE-KLF4 and subcloned into pISO.

Western blotting. Cell lysates were size-fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The following antibodies were used to detect specific proteins: KLF4, p53, p21, Bax, β-actin (Santa Cruz Biotechnology), and poly(ADP-ribose) polymerase (PARP; Cell Signaling).

RNA isolation and PCR analysis. Total RNA was isolated by using Trizol reagent (Invitrogen) and conventional reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (RT-qPCR) were done using One-Step RT-PCR kit (Qiagen) and SYBR Green PCR Master Mix (Applied Biosystems) respectively. Primer sequences are available upon request.

Immunoprecipitation and RNA immunoprecipitation. Immunoprecipitation of endogenous RNA-protein complexes was done as described (31). The RNA isolated from immunoprecipitation material was reverse-transcribed by using random hexamers or oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) and used for conventional RT-PCR and RT-qPCR analysis. Primer sequences are available upon request.

Reporter assay. Luciferase assay was done by using the Dual-Luciferase Reporter Assay System (Promega) as described (32). The difference in transfection efficiency across samples was normalized by cotransfecting pRL-CMV. Each experiment was done in triplicate and repeated at least three times.

Chromatin immunoprecipitation experiments. Chromatin immunoprecipitation analysis was done, as described previously (33), with anti-p53 (DO-1/pAb-421) or control IgG. Primer sequences are available upon request.

Results

KLF4 is differentially expressed in cells exposed to cytostatic or apoptotic doses of DNA-damaging agents. To investigate the role of KLF4 in p53-mediated DNA damage response, we first assessed the cell sensitivity to Adriamycin in wild-type p53-carrying HCT116 cells (Fig. 1*A*). Forty-eight hours after the treatment, 99% of the cells survived at a dose of 0.5 μmol/L, whereas ~50% cells died after exposure to 5 μmol/L Adriamycin for the same length of time. Cell cycle profiling confirmed apoptotic death at the 5 μmol/L dose, whereas the cells showed cell cycle arrest at the 0.5 μmol/L dose (data not shown). We analyzed the expression of KLF4, p53, and p53-related markers of DNA damage response. As expected, the expression of p53 was induced by Adriamycin at both cytostatic and apoptotic doses. By contrast, p21, a key mediator of p53-dependent cell cycle arrest, was induced by Adriamycin only at the cytostatic dose. Additionally, cleaved PARP, a marker of apoptosis, was only detected in cells treated with the apoptotic doses of drug. Consistent with previous findings that KLF4 expression is transcriptionally activated by p53 on DNA damage agents such as methyl methanesulfonate and irradiation (21, 29), the expression of KLF4 was increased in response to Adriamycin at the cytostatic dose. However, surprisingly, the expression of KLF4 was strongly repressed on apoptotic-dose Adriamycin treatment (Fig. 1*B*). Moreover, Z-VAD-FMK, a pan-caspase inhibitor, did not prevent 5 μmol/L Adriamycin-induced repression of KLF4 (Fig. 1*C*). Comparable repression of KLF4 was detected in $p53^{+/+}$

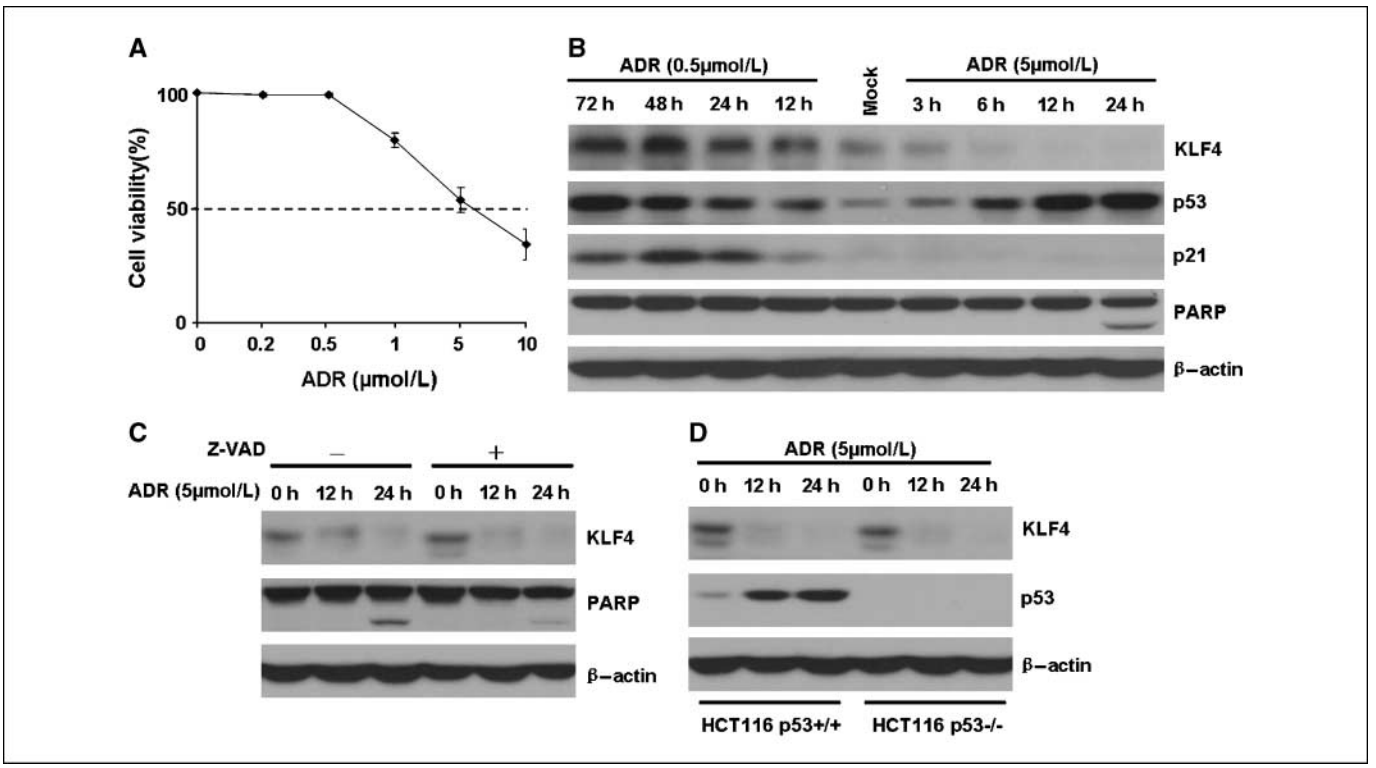

Figure 1. KLF4 expression is different in cells exposed to cytostatic or apoptotic doses of Adriamycin. *A,* percentage of cell viability of HCT116 cells treated with Adriamycin (*ADR*) at the indicated doses and measured 48 h post-treatment by trypan blue exclusion test. Mean ± SD of triplicate experiments. *B,* HCT116 cells were treated with cytostatic (0.5 μmol/L) or apoptotic (5 μmol/L) doses of Adriamycin for the indicated time, and Western blots were done for the indicated proteins. *C,* HCT116 cells were treated with apoptotic (5 μmol/L) doses of Adriamycin alone or in combination with Z-VAD (50 μmol/L) for the indicated time. KLF4 and PARP were detected by Western blots. *D,* HCT116 $p53^{+/+}$ and $p53^{-/-}$ derivatives were treated with apoptotic (5 μmol/L) doses of Adriamycin for the indicated time, and Western blots were done for the indicated proteins.

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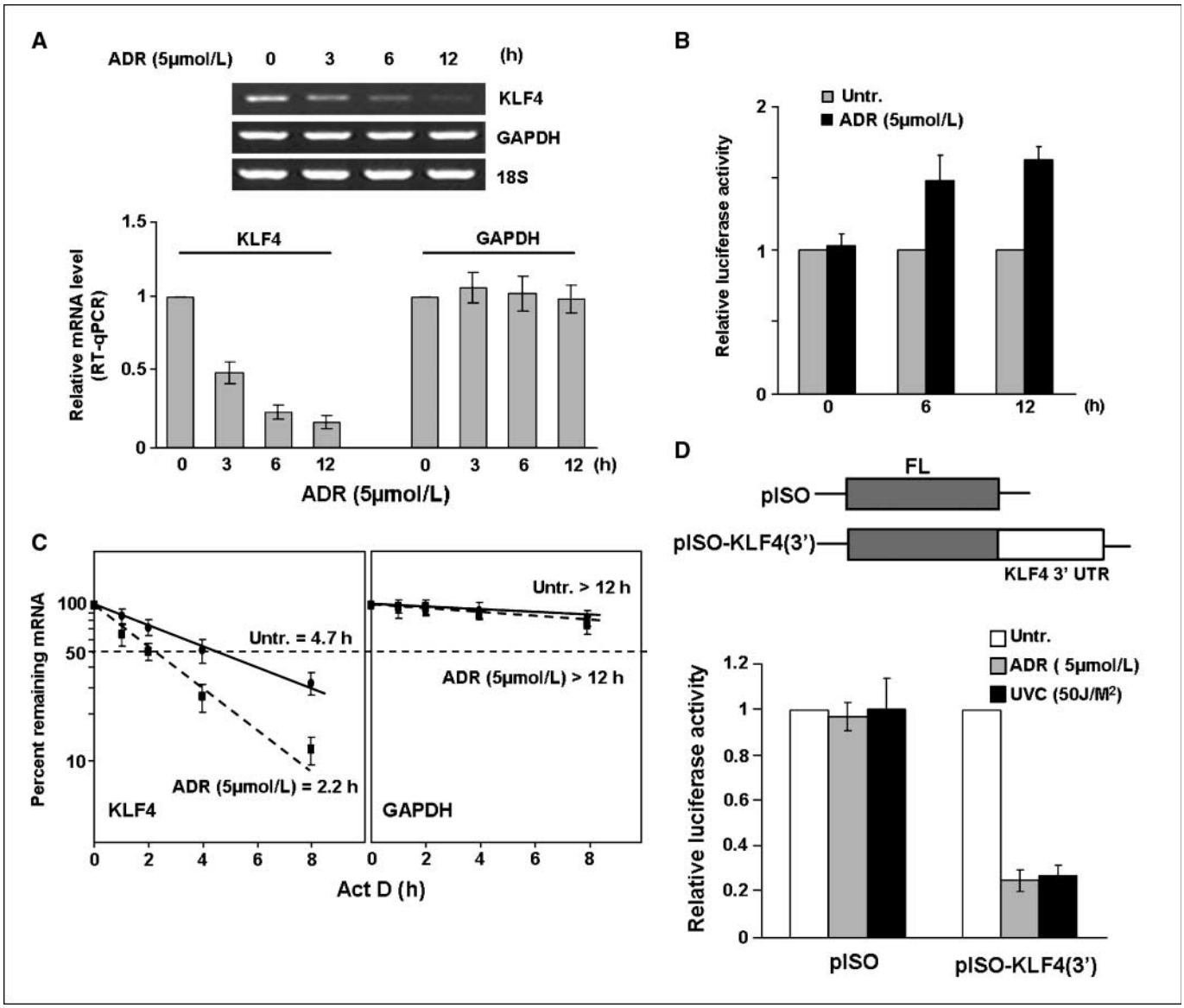

Figure 2. Decreased *KLF4* mRNA stability after apoptotic doses of Adriamycin treatment. *A,* HeLa cells were left untreated or treated with 5 μmol/L Adriamycin for the indicated time. Levels of *KLF4* and *GAPDH* mRNA and loading control 18S rRNA were monitored by conventional RT-PCR (*top*) and RT-qPCR (*bottom*). Mean ± SD of triplicate experiments. *B,* HCT116 cells were transfected with *KLF4* promoter reporter construct. Twenty-four hours after transfection, cells were left untreated or treated with 5 μmol/L Adriamycin for the indicated time. Luciferase activity was determined. Data are relative luciferase value (mean ± SD of triplicate experiments) compared with untreated cells, which were set to 1. *C,* HeLa cells were untreated or treated with 5 μmol/L Adriamycin for 1 h followed by actinomycin D (*Act D*; 5 mg/mL) treatment for the indicated time. *KLF4* (*left*) and *GAPDH* (*right*) mRNA levels were measured by RT-qPCR, normalized to 18S rRNA levels, and plotted on a logarithmic scale to calculate the time required for each mRNA to reach one-half of its initial abundance. Mean ± SD of triplicate experiments. *D,* schemes of reporter construct bearing *KLF4* 3′-UTR fused to the firefly luciferase (*FL*) coding region (*top*). HeLa cells were transfected with *KLF4* 3′-UTR reporter construct. Twenty-four hours after transfection, cells were left untreated or treated with Adriamycin (5 μmol/L) or UV-C (50 J/M^2) irradiation for 16 h. Luciferase activity was then determined. Data are relative luciferase value (mean ± SD of triplicate experiments) compared with untreated cells, which were set to 1 (*bottom*).

and p53$^{-/-}$ HCT116 cells (Fig. 1*D*), suggesting that the repression of KLF4 on apoptotic-dose treatment is p53-independent. Repression of KLF4 was also observed in LoVo and HeLa cells treated with apoptotic doses of Adriamycin or UV-C irradiation (data not shown).

Apoptotic DNA damage results in enhanced *KLF4* mRNA turnover. The protein stability of KLF4 was not affected by apoptotic doses of Adriamycin treatment (Supplementary Fig. S1). However, a significant decrease in *KLF4* mRNA levels was triggered by apoptotic doses of Adriamycin treatment (Fig. 2*A*). Apoptotic doses of Adriamycin treatment triggered a modest activation rather than inhibition of *KLF4* promoter (Fig. 2*B*), suggesting that changes in transcriptional regulation are unlikely to account for the marked loss of *KLF4* mRNA after apoptotic doses of Adriamycin exposure. We then measured *KLF4* mRNA half-life by incubating cells with actinomycin D to block *de novo* gene transcription. As shown in Fig. 2*C*, apoptotic doses of Adriamycin treatment caused a significant decrease in the stability of *KLF4* mRNA, suggesting that the depression of KLF4 by the apoptotic doses of Adriamycin is due to, at least in part, the enhanced mRNA turnover. Furthermore, removal of the 3′-UTR completely ablates the enhanced turnover triggered by apoptotic doses of treatment

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(Supplementary Fig. S2), suggesting that 3′-UTR, not the coding region or 5′-UTR, confers this regulation. To directly investigate the role for *KLF4* 3′-UTR in this regulation, we constructed a luciferase reporter in which *KLF4* 3′-UTR was fused to the coding region of firefly luciferase (Fig. 2*D, top*). Luciferase activities from the empty vector (pISO) remained unchanged at apoptotic doses of Adriamycin or UV-C, whereas pISO-KLF4(3′), in which the *KLF4* 3′-UTR was fused to the firefly luciferase coding region, showed a significant decrease in luciferase activity following treatment with apoptotic doses of Adriamycin or UV-C (Fig. 2*D, bottom*), suggesting that the 3′-UTR of *KLF4* mRNA is both necessary and sufficient to confer the enhanced turnover of *KLF4* mRNA in response to apoptotic doses of DNA damage agents.

HuR binds to KLF4 mRNA and stabilizes it. To further determine the *cis*-acting factors on 3′-UTR of *KLF4* mRNA that confer the enhanced turnover of *KLF4* mRNA on apoptotic DNA damage, we searched for putative RNA regulatory elements in the human *KLF4* 3′-UTR. Remarkably, we identified five putative HuR binding sites (34), suggesting that *KLF4* mRNA might be a direct target of HuR (Supplementary Fig. S3). We then tested if the *KLF4* mRNA is associated with HuR by performing RNA immunoprecipitation assays using anti-HuR antibody (or control IgG). As shown in Fig. 3*A, KLF4* mRNA was immunoprecipitated by anti-HuR antibody but not by the control IgG. Comparable result was observed for *cyclin D1* mRNA, which is a well-established HuR target (31) and served as a positive control. Notably, *GAPDH* transcript, which served as a negative control (35), was not immunoprecipitated by anti-HuR Ab. Because AUF1, another RNA-binding protein that specifically influence mRNA turnover, shares many target mRNAs with HuR (31), we further extended these studies to AUF1. However, unlike HuR, AUF1 did not appear to bind the *KLF4* mRNA directly under this experiment condition (data not shown).

To assess the functional consequences of the interactions of HuR with *KLF4* mRNA, HuR levels were reduced by RNA interference. HuR depletion, induced by either of the two different siRNAs, dramatically reduced KLF4 protein as well as mRNA levels (Fig. 3*B* and *C*). We further examined the effect of HuR depletion on the heterologous reporter bearing the *KLF4* 3′-UTR. As shown in Fig. 3*D*, the luciferase activity of the pISO-KLF4(3′), not the empty pISO, decreased dramatically after HuR silencing, supporting the notion that HuR stabilizes *KLF4* mRNA via its 3′-UTR.

Apoptotic DNA damage dissociates *KLF4* mRNA from HuR. To explore if the decreased *KLF4* mRNA stability by apoptotic doses of Adriamycin treatment was linked to changes in its association with HuR, the abundance of these complexes was tested by RNA immunoprecipitation analysis. The amount of *KLF4* mRNA

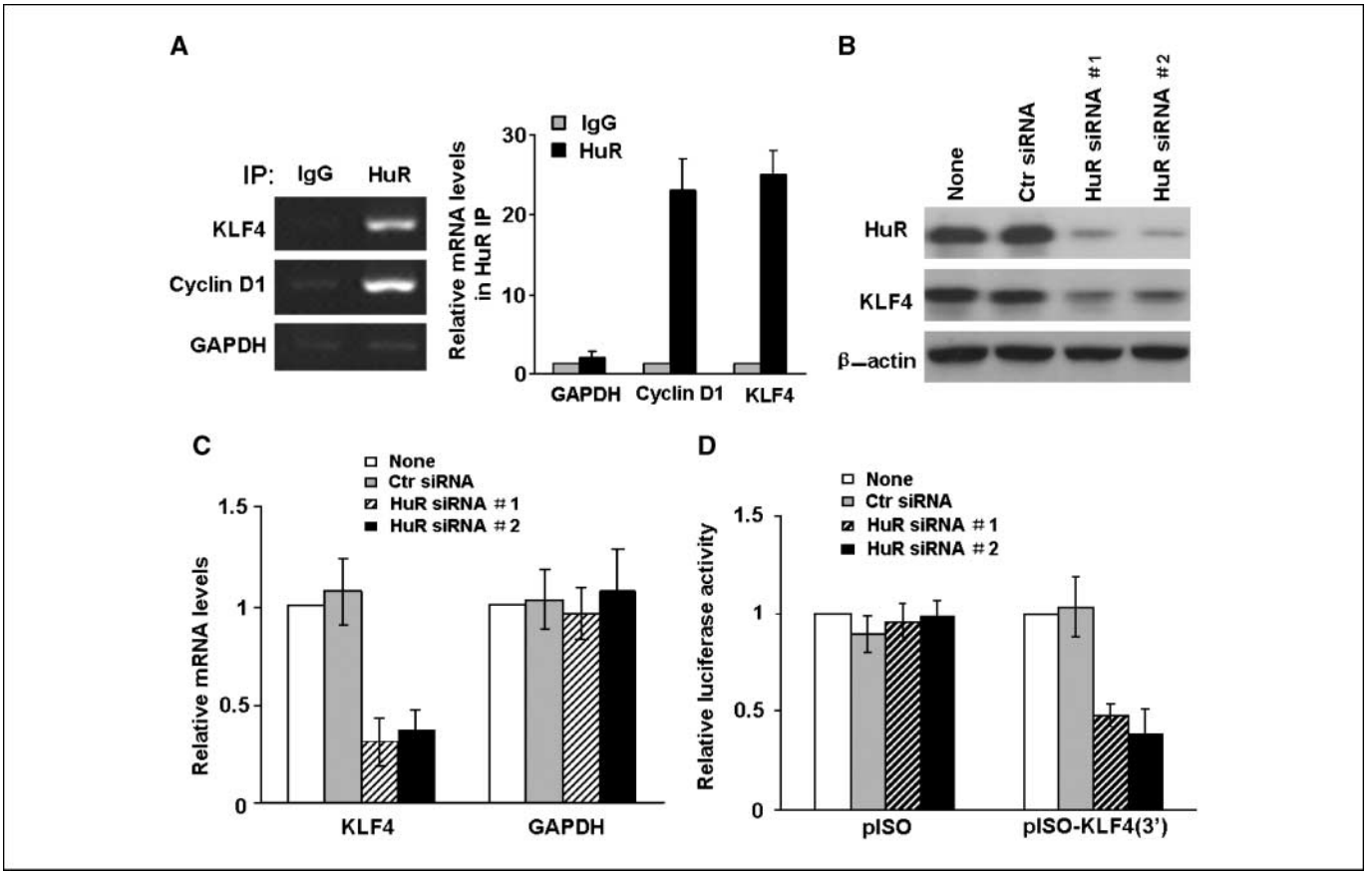

Figure 3. HuR interacts with *KLF4* mRNA and stabilizes it. *A,* RNA immunoprecipitation was carried out with the anti-HuR antibody or the control IgG. The amount of *KLF4* mRNA in each immunoprecipitation was assessed by RT-PCR (*left*) and RT-qPCR (*right*; fold enrichment in HuR immunoprecipitation compared with IgG immunoprecipitation; mean ± SD of triplicate experiments). *GAPDH* served as a negative control, whereas *cyclin D1* served as a positive control. *B,* HeLa cells were transfected with either scrambled or HuR siRNAs as indicated. Forty-eight hours after transfection, Western blots were done for the indicated proteins. *C,* HeLa cells were transfected as described in *B,* and RNA was analyzed by RT-qPCR. Mean ± SD of triplicate experiments. *D,* HeLa cells were cotransfected with pISO or pISO-KLF4(3′) reporters and indicated siRNAs. Forty-eight hours after transfection, luciferase activity was determined. Data are relative luciferase value (mean ± SD of triplicate experiments) compared with control cells, which were set to 1.

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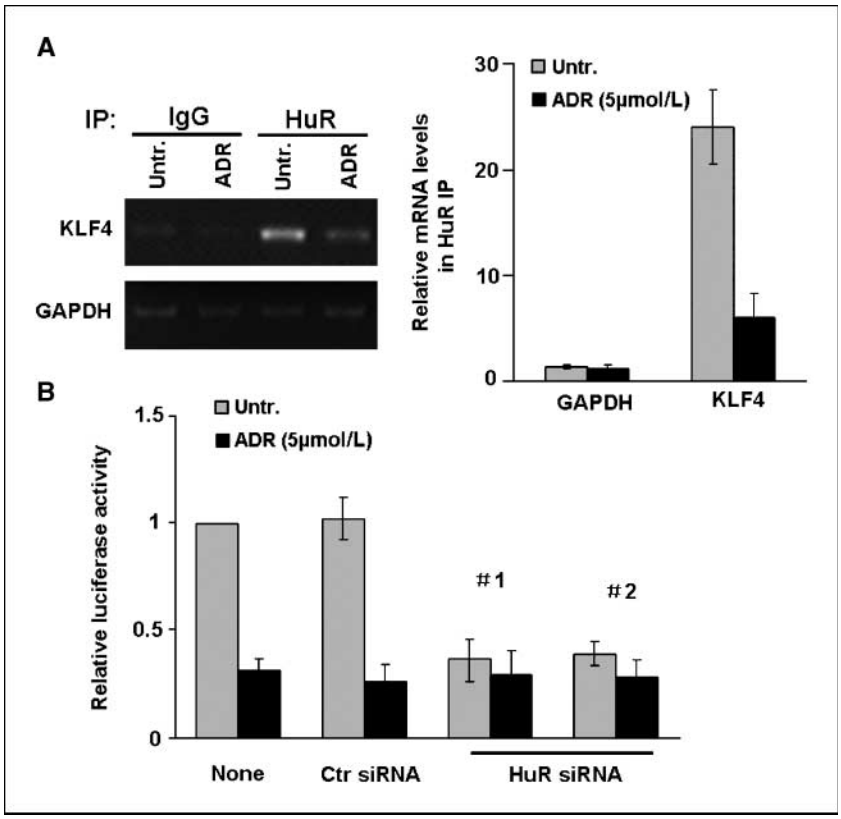

Figure 4. Apoptotic doses of Adriamycin treatment trigger the dissociation of *KLF4* mRNA from HuR. *A,* RNA immunoprecipitation was carried out by using lysates from either untreated or 5 μmol/L Adriamycin-treated HeLa cells. The amount of *KLF4* mRNA in each immunoprecipitation was assessed by RT-PCR (*left*) and RT-qPCR (*right*; fold enrichment in HuR immunoprecipitation compared with IgG immunoprecipitation; mean ± SD of triplicate experiments), *GAPDH* served as a negative control. *B,* HeLa cells were cotransfected with pISO-KLF4(3′) reporter and indicated siRNAs. Thirty-six hours after transfection, cells were left untreated or treated with 5 μmol/L Adriamycin for additional 16 h. Luciferase activity was then determined. Data are relative luciferase value (mean ± SD of triplicate experiments) compared with untreated cells, which were set to 1.

that bound to HuR was substantially reduced by apoptotic doses of Adriamycin treatment (Fig. 4*A*), suggesting that apoptotic doses of Adriamycin treatment trigger the rapid dissociation of *KLF4* mRNA from HuR, in turn destabilizing the *KLF4* mRNA. To further confirm the role of HuR in the enhanced *KLF4* mRNA turnover triggered by apoptotic DNA damage, we tested if HuR depletion affected the apoptotic doses of Adriamycin-induced decrease in luciferase activity of the pISO-KLF4(3′) reporter. As shown in Fig. 4*B*, HuR depletion reduced the apoptotic doses of Adriamycin-triggered decrease in luciferase activity of the pISO-KLF4(3′) reporter, suggesting that apoptotic doses of Adriamycin-triggered decrease in *KLF4* mRNA stability is mediated, at least in part, by HuR. Similar results were observed with HCT116 cells (Supplementary Fig. S4).

Repression of KLF4 on apoptotic DNA damage is necessary for p53-mediated apoptosis. Our data revealed that KLF4 expression was repressed on apoptotic DNA damage. If the repression of KLF4 is required for induction of the apoptotic response, blocking the repression of KLF4 should suppress this response. To test this scenario, a vector that contained only *KLF4* coding region was used to overexpress KLF4 in HCT116 cells. HCT116 cells overexpressing KLF4 were less prone to undergo apoptotic doses of Adriamycin-induced or UV-C–induced apoptosis than vector-transfected control cells (Fig. 5*A*; Supplementary Fig. S5). Importantly, compared with HCT116 $p53^{+/+}$ cells, the protection effects of KLF4 on DNA damage–induced apoptosis were not observed in HCT116 $p53^{-/-}$ cells, suggesting that this protection is p53-dependent. Western blotting analyses of the cleavage of PARP, a marker of apoptosis, confirmed these findings (Fig. 5*B*).

Recently, KLF4 was shown to repress transcription of *p53*, resulting in resistance to apoptosis induced by DNA damage in MDA-MB-134 cells (25). However, overexpression of KLF4 only caused a low-extent decrease of endogenous p53 expression in HCT116 cells (Fig. 5*B*), suggesting that repressing transcription of p53 is probably not the only mechanism by which KLF4 confers resistance to the p53-mediated apoptosis. It was shown that KLF4 can associate with p53 and promote p53 transactivation on proarrest gene *p21* (21). Importantly, upregulation of KLF4 also inhibits p53 transactivation on the proapoptotic gene *Bax* following γ-irradiation (36). We then tested the hypothesis that repression of KLF4 on apoptotic DNA damage may contribute to the ability of p53 to preferentially transactivate proapoptotic gene *Bax* over proarrest gene *p21*. As expected, on apoptotic doses of Adriamycin treatment, concomitant with p53 induction, Bax was significantly induced, whereas p21 was not increased. However, KLF4 overexpression significantly reduced Bax induction but increased p21 induction (Fig. 5*B*). Similar results were observed with the levels of *p21* and *Bax* transcripts as measured by RT-qPCR analyses (Fig. 5*C*), whereas the induction of SFN, Puma, Noxa, and MDM2 was not significantly affected by KLF4 overexpression (Fig. 5*C*), indicating that the modulation of p53 transcriptional activity by KLF4 is promoter-specific. To further explore the mechanisms

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how KLF4 modulate p53 transactivation function on *p21* and *Bax*, we measured DNA-binding activity of p53 and KLF4 on the promoters of *p21* and *Bax*. Consistent with a previous report (36), we observed that KLF4 overexpression decreased the Adriamycin-dependent recruitment of p53 on the binding site (p53RE) of *Bax* promoter region. The recruitment of p53 on the binding site (p53RE) of *p21* promoter region was not significantly affected by KLF4 overexpression, whereas the recruitment of p53 on the proximal regulation region of *p21* was significantly increased (Supplementary Fig. S6), a situation that was consistent with previous report that KLF4 mediates the transactivating effect of p53 on p21 through the proximal promoter region of *p21* (21).

Introduction of p53 in p53-null H1299 cells can led to apoptosis (37). To further assess the effects of KLF4 overexpression on p53-induced apoptosis, H1299 cells were transfected with p53 alone or cotransfected with p53 and KLF4. As expected, introduction of p53 in H1299 cells led to apoptosis, which was lowered by coexpression of KLF4 (Fig. 5*D, left*). Western blot analysis showed that expression of KLF4 had no effect on the levels of p53 derived by a heterologous promoter (Fig. 5*D, right*).

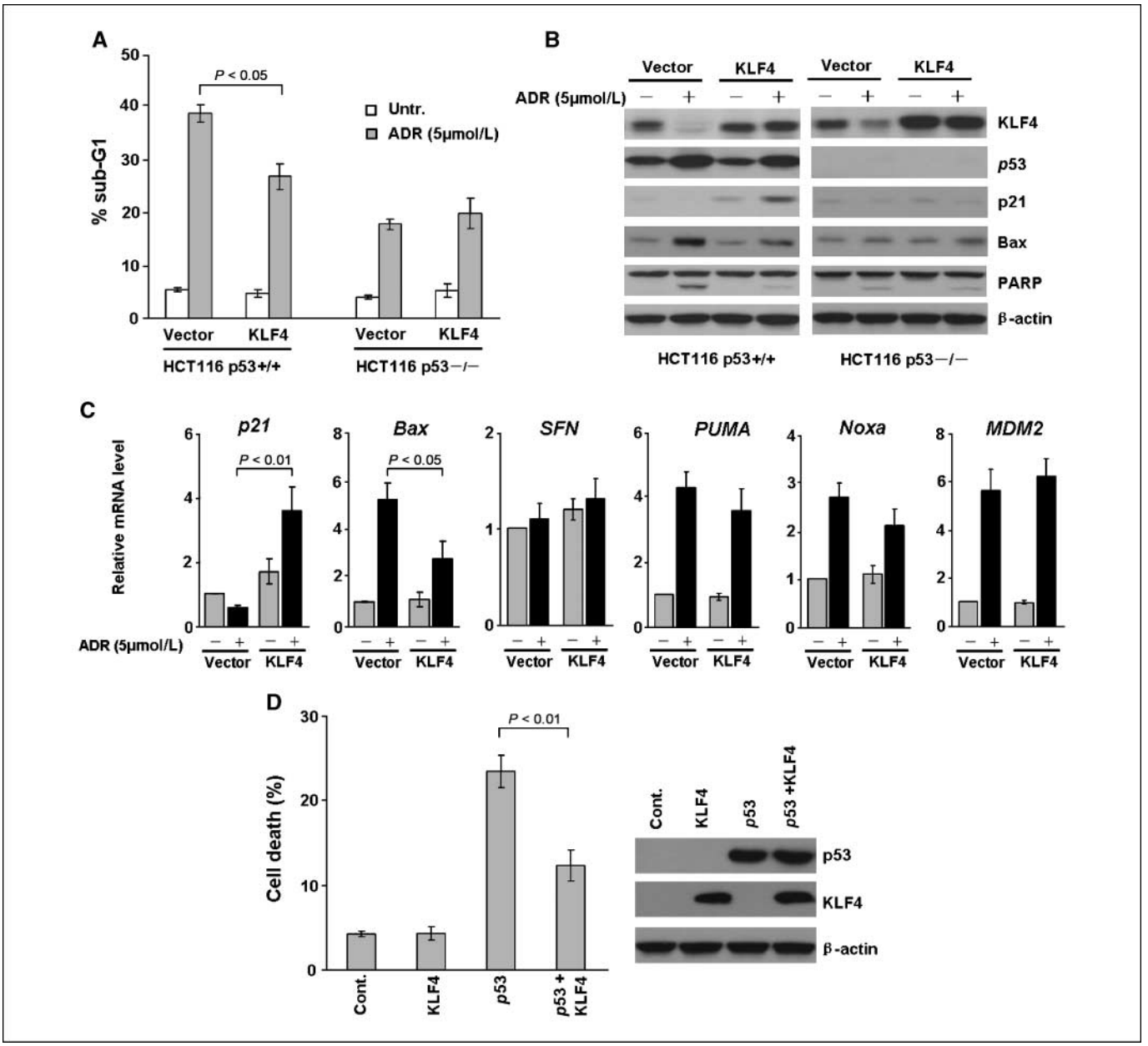

Figure 5. KLF4 inhibits p53-mediated apoptosis. *A,* HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with KLF4. Thirty-six hours after transfection, cells were left untreated or treated with 5 μmol/L Adriamycin for 24 h. DNA content was analyzed by fluorescence-activated cell sorting. Percentages of cells with sub-G_1 DNA content, indicative of apoptosis, are shown as mean ± SD of triplicate experiments. Statistical analysis was done using a paired *t* test. *B,* HCT116 cells were transfected and treated as in *A*. Western blots were done for the indicated proteins. *C,* HCT116 cells were transfected and treated as in *A*. mRNA expression levels of *p21, Bax, PUMA, Noxa*, and *MDM2* were measured by RT-qPCR. Data are fold induction (mean ± SD of triplicate experiments) over untreated HCT116/vector cells and normalized to 18S rRNA levels. Statistical analysis was done using a paired *t* test. *D,* H1299 cells were transfected with *p53* alone or cotransfected with *p53* and *KLF4*. Forty-eight hours after transfection, the percentage of cell death (mean ± SD of triplicate experiments) was measured by trypan blue exclusion test (*left*). Protein expression levels of p53 and KLF4 were assessed by immunoblotting (*right*).

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Ablation of KLF4 induction on cytostatic DNA damage shifts the p53 response from cell repair to cell death. The findings presented above suggest that blocking the repression of KLF4 on apoptotic DNA damage suppress apoptosis; we then explore whether ablation of the induction of KLF4 on cytostatic DNA damage will modify the cytostatic response. HCT116 cells stably silenced with *KLF4* short hairpin RNA or control short hairpin RNA were used to examine the effects of KLF4 ablation on the cell cycle arrest induced by cytostatic DNA damage. As expected, cytostatic doses of Adriamycin or etoposide induced cell cycle arrest, with little evidence of DNA fragmentation indicative of cell death (sub-G_1). Ablation of KLF4 did not cause obvious changes in the basal cell cycle distribution under the non-DNA damage condition. However, the KLF4-silencing cells showed a blunted G_2-M block

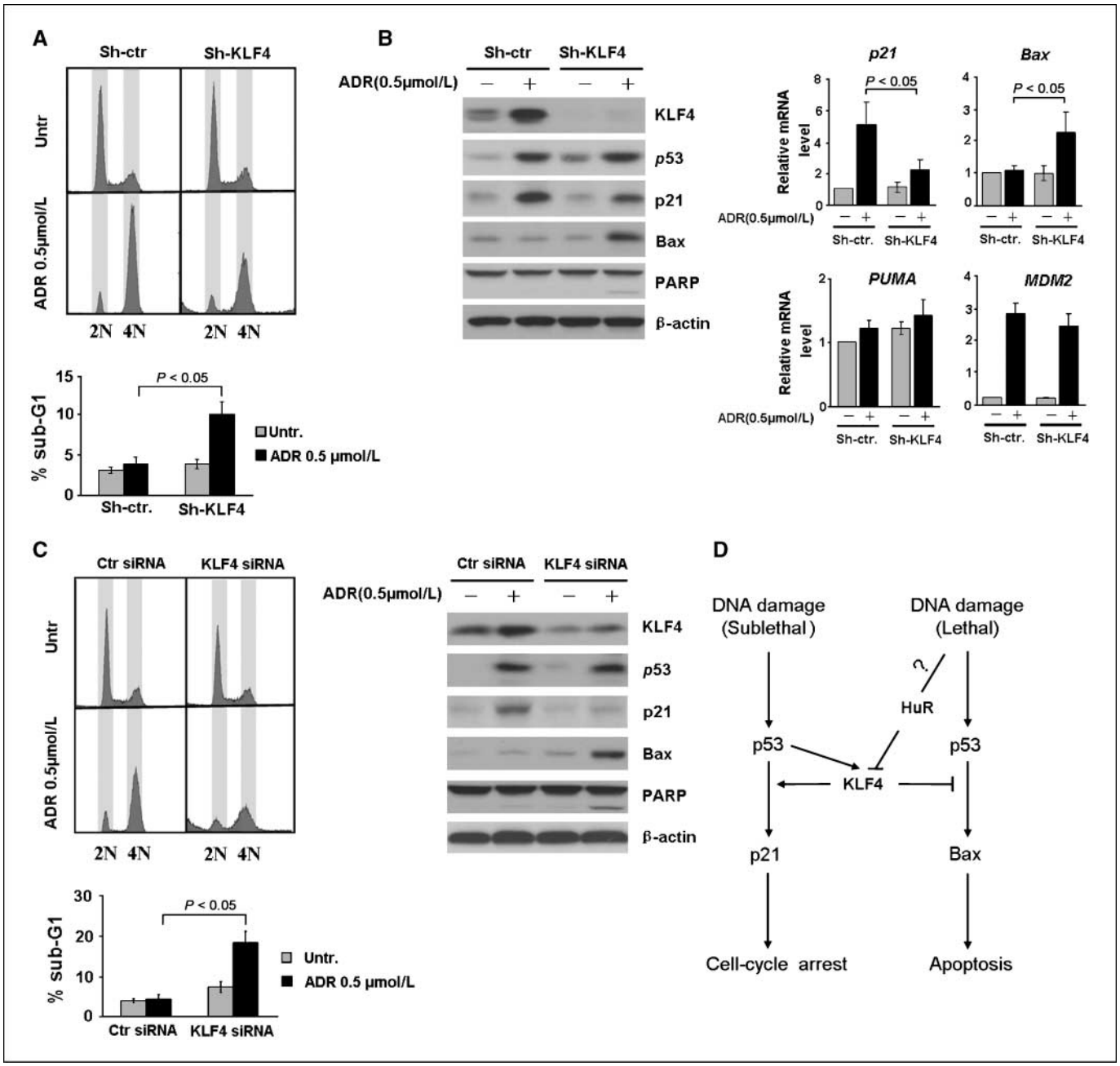

Figure 6. KLF4 silencing shifts the p53 response from cell cycle arrest to apoptosis in response to cytostatic DNA damage. *A,* HCT116 cells stably silenced with *KLF4* short hairpin RNA (*Sh-KLF4*) or control short hairpin RNA (*Sh-ctr*) were left untreated or treated with cytostatic doses of Adriamycin (0.5 μmol/L) for 48 h. DNA content was analyzed by fluorescence-activated cell sorting (*top*). The percentage of sub-G_1 DNA content was determined. Mean ± SD of triplicate experiments (*bottom*). Statistical analysis was done using a paired *t* test. *B,* HCT116 cells were silenced and treated as in *A.* Western blots were done for the indicated proteins (*left*), and mRNA expression levels of *p21, Bax, PUMA*, and *MDM2* were measured by RT-qPCR (*right*). Data are fold induction (mean ± SD of triplicate experiments) over untreated cells and normalized to 18S rRNA levels. Statistical analysis was done using a paired *t* test. *C,* MCF-7 cells transiently transfected with control siRNA and KLF4 siRNA and treated as in *A.* DNA content was analyzed by fluorescence-activated cell sorting (*left, top*). The percentage of sub-G_1 DNA content was determined. Mean ± SD of triplicate experiments (*left, bottom*). Western blots were done for the indicated proteins (*right*). *D,* model summarizing the role of KLF4 in cell response to DNA damage.

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response to cytostatic doses of Adriamycin or etoposide and entered apoptosis (Fig. 6*A*; Supplementary Fig. S7), suggesting that induction of KLF4 is required for p53-mediated cell cycle arrest, and ablation of the KLF4 induction on cytostatic DNA damage shifts the p53 response from cell repair to cell death. Consistent with this notion, we also observed that ablation of KLF4 decreased the cytostatic doses of Adriamycin-dependent induction of p21 but enhanced the induction of Bax (Fig. 6*B* and *C*) and the cleavage of PARP (Fig. 6*B*). Comparable results were observed with another clone with comparable levels of KLF4 knockdown (data not shown). Similar results were also obtained with MCF-7 and HCT116 cells transiently silenced with siRNA targeting with a different sequence in KLF4 (Fig. 6*C*; data not shown).

Discussion

Previous studies showed that KLF4 is transcriptionally activated by p53 following DNA damage and that KLF4 is required for p53-mediated induction of p21, which leads to cell cycle arrest (21). Consistent with this observation, activation of endogenous p53 by cytostatic dose of Adriamycin treatment in HCT116 cells resulted in induction of KLF4 and p21 expression. However, apoptotic-dose Adriamycin treatment and the induction of the p53-mediated apoptotic response correlated with a reduction in the expression of KLF4 and p21 (Fig. 1*B*). Repression of KLF4 occurred in cells within 12 h, before the time when the cleaved PARP was detected, and the repression of KLF4 was still observed in cells treated with the Z-VAD-FMK. Thus, it appeared to be an active rather than a passive event of apoptosis.

It is known that, in cells responding to damaging stimulation, gene expression changes profoundly affect the cellular outcome, directly influencing whether the cell survives or succumbs to the injury. Although the transcriptional mechanisms of gene regulation after genotoxic stress have been investigated extensively, the critical influence of post-transcriptional events such as mRNA turnover and translation is becoming increasingly appreciated (38–40). Interesting enough, we show that enhanced mRNA turnover rather than decreased gene transcription is involved in the repression of KLF4 in response to apoptotic DNA damage. It is well established that the post-transcriptional fate of a given mRNA is governed by the interaction of specific mRNA sequences (*cis*-elements) with specific *trans*-factors such as RNA-binding proteins or microRNAs (35, 41). Our study shows that HuR bound to the *KLF4* mRNA and affected its stability. Notably, apoptotic doses of Adriamycin treatment trigger the rapid dissociation of *KLF4* mRNA from HuR, in turn destabilizing the *KLF4* mRNA.

HuR is an ubiquitously expressed member of the ELAV family of proteins involved in different aspects of post-transcriptional regulation. In response to different types of cellular stress, HuR is mobilized from the nucleus to the cytosol, where it regulates gene expression at the post-transcriptional level (42–44). Through its post-transcriptional effects on the activity of many important regulatory genes, HuR has been proposed to play a role not only in the stress response but also in cell proliferation, differentiation, tumorigenesis, apoptosis, and immune response (42–44). Although HuR has also been implicated in the regulation of translation, the best understood functions of HuR have been attributed to its ability to increase transcript stability. Our preliminary findings that the association of HuR with *KLF4* mRNA was reduced dramatically after apoptotic doses of Adriamycin treatment might explain the enhanced turnover of *KLF4* mRNA triggered by apoptotic DNA damage, but the mechanisms responsible for this regulation remain poorly understood. The findings that HuR can undergo phosphorylation and methylation (45) or, in some cases, synergize with other RNA-binding proteins (44) indicate that HuR is a part of a complex network dedicated to post-transcriptional regulation of gene expression. It will be of interest to identify pathways that trigger the rapid dissociation of *KLF4* mRNA from HuR on apoptotic DNA damage.

The fact that KLF4 protein level is inversely correlated with the extent of genotoxic stress indicates that KLF4 may play a role in controlling the switch in p53 response. In concert with this notion, blocking the repression of KLF4 on apoptotic DNA damage suppresses p53-mediated apoptosis (Fig. 5*A*), whereas ablation of the KLF4 induction on cytostatic DNA damage shifts the p53 response from cell repair to cell death (Fig. 6). What is the mechanism by which KLF4 controls the switch in p53 response? One of the possibilities is that it functions through modulating p53 transactivation. The demonstration that KLF4 associates with p53 has indicated that KLF4 could directly affect the p53 transactivation function (21). Furthermore, it is reported that KLF4 is required for p53-mediated induction of p21 in response to DNA damage, which leads to cell cycle arrest (21), and that KLF4 exerts the inhibitory effect on the ability of p53 to activate the *Bax* promoter following γ-irradiation (36). In line with these previous results, we show that blocking the repression of KLF4 on apoptotic DNA damage resulted in significantly reduced Bax induction, whereas the induction of p21 was enhanced in response to apoptotic DNA damage (Fig. 5*B* and *C*), suggesting that repression of KLF4 on apoptotic DNA damage may contribute to the ability of p53 to preferentially transactivate proapoptotic gene *Bax* over proarrest gene *p21*. Although our preliminary investigations reveal that KLF4 has no effect on the accumulation of SFN, PUMA, or Noxa, we cannot, however, formally exclude the possibility that KLF4 controls the switch in p53 response through modulating additional proarrest and proapoptotic p53 targets.

In summary, the finding presented here shows that KLF4 expression is tightly regulated depending on the extent of DNA damage, which may be an important mechanism that dictates the life and death decisions of p53, and supports a model as sketched in Fig. 6*D*. In response to cytostatic reparable DNA damage, KLF4 is activated by p53, which promotes transactivation of proarrest p53 target gene *p21*, resulting in cell cycle arrest. Apoptotic irreparable DNA damage leads to the repression of KLF4 via enhanced *KLF4* mRNA turnover, which then allows p53 to activate proapoptotic target gene *Bax*, resulting in apoptotic death. This may have important implications in chemoresistance of tumor retaining wild-type p53 and for the development of overcoming strategies, as most chemotherapeutic strategies are aimed at triggering the apoptosis of tumor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2001;27:247–54.
2. Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature 2000;408:433–9.
3. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594–604.
4. Aylon Y, Oren M. Living with p53, dying of p53. Cell 2007;130:597–600.
5. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75:817–25.
6. Hermeking H, Lengauer C, Polyak K, et al. 14-3-3σ is a p53-regulated inhibitor of G_2/M progression. Mol Cell 1997;1:3–11.
7. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293–9.
8. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683–94.
9. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 2003;302:1036–8.
10. Samuels-Lev Y, O'Connor DJ, Bergamaschi D, et al. ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell 2001;8:781–94.
11. Tanaka T, Ohkubo S, Tatsuno I, Prives C. hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. Cell 2007;130:638–50.
12. Wei X, Xu H, Kufe D. Human mucin 1 oncoprotein represses transcription of the p53 tumor suppressor gene. Cancer Res 2007;67:1853–8.
13. Bernal JA, Luna R, Espina A, et al. Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. Nat Genet 2002;32: 306–11.
14. Zheng H, You H, Zhou XZ, et al. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. Nature 2002;419:849–53.
15. Zacchi P, Gostissa M, Uchida T, et al. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature 2002;419:853–7.
16. Das S, Raj L, Zhao B, et al. Hzf determines cell survival upon genotoxic stress by modulating p53 transactivation. Cell 2007;130:624–37.
17. Shields JM, Christy RJ, Yang VW. Identification and characterization of a gene encoding a gut-enriched Kruppel-like factor expressed during growth arrest. J Biol Chem 1996;271:20009–17.
18. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet 1999;22:356–60.
19. Chen X, Johns DC, Geiman DE, et al. Kruppel-like factor 4 (gut-enriched Kruppel-like factor) inhibits cell proliferation by blocking G_1/S progression of the cell cycle. J Biol Chem 2001;276:30423–8.
20. Chen X, Whitney EM, Gao SY, Yang VW. Transcriptional profiling of Kruppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. J Mol Biol 2003;326:665–77.
21. Zhang W, Geiman DE, Shields JM, et al. The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the $p21^{WAF1/Cip1}$ promoter. J Biol Chem 2000;275:18391–8.
22. Wei D, Kanai M, Jia Z, Le X, Xie K. Kruppel-like factor 4 induces $p27^{Kip1}$ expression in and suppresses the growth and metastasis of human pancreatic cancer cells. Cancer Res 2008;68:4631–9.
23. Shie JL, Chen ZY, Fu M, Pestell RG, Tseng CC. Gut-enriched Kruppel-like factor represses cyclin D1 promoter activity through Sp1 motif. Nucleic Acids Res 2000;28:2969–76.
24. Foster KW, Ren S, Louro ID, et al. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth Differ 1999; 10:423–34.
25. Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol 2005;7:1074–82.
26. Wernig M, Meissner A, Foreman R, et al. *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007;448:318–24.
27. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.
28. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature 2007;448:313–7.
29. Yoon HS, Chen X, Yang VW. Kruppel-like factor 4 mediates p53-dependent G_1/S cell cycle arrest in response to DNA damage. J Biol Chem 2003;278:2101–5.
30. Rinaldo C, Prodosmo A, Mancini F, et al. MDM2-regulated degradation of HIPK2 prevents $p53Ser^{46}$ phosphorylation and DNA damage-induced apoptosis. Mol Cell 2007;25:739–50.
31. Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M. Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. EMBO J 2004;23:3092–102.
32. Shi B, Liang J, Yang X, et al. Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. Mol Cell Biol 2007;27:5105–19.
33. Kaeser MD, Iggo RD. Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity *in vivo*. Proc Natl Acad Sci U S A 2002;99:95–100.
34. Lopez de Silanes I, Zhan M, Lal A, Yang X, Gorospe M. Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci U S A 2004;101:2987–92.
35. Abdelmohsen K, Pullmann R, Jr., Lal A, et al. Phosphorylation of HuR by Chk2 regulates SIRT1 expression. Mol Cell 2007;25:543–57.
36. Ghaleb AM, Katz JP, Kaestner KH, Du JX, Yang VW. Kruppel-like factor 4 exhibits antiapoptotic activity following γ-radiation-induced DNA damage. Oncogene 2007;26:2365–73.
37. Luo J, Nikolaev AY, Imai S, et al. Negative control of p53 by Sir2α promotes cell survival under stress. Cell 2001;107:137–48.
38. Moore MJ. From birth to death: the complex lives of eukaryotic mRNAs. Science 2005;309:1514–8.
39. Keene JD, Tenenbaum SA. Eukaryotic mRNPs may represent posttranscriptional operons. Mol Cell 2002;9:1161–7.
40. Garcia-Martinez J, Aranda A, Perez-Ortin JE. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. Mol Cell 2004;15:303–13.
41. Wilusz CJ, Wilusz J. Bringing the role of mRNA decay in the control of gene expression into focus. Trends Genet 2004;20:491–7.
42. Lal A, Kawai T, Yang X, Mazan-Mamczarz K, Gorospe M. Antiapoptotic function of RNA-binding protein HuR effected through prothymosin α. EMBO J 2005;24:1852–62.
43. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci 2001;58:266–77.
44. Katsanou V, Papadaki O, Milatos S, et al. HuR as a negative posttranscriptional modulator in inflammation. Mol Cell 2005;19:777–89.
45. Li H, Park S, Kilburn B, et al. Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase. J Biol Chem 2002;277:44623–30.

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