hnRNP K: An HDM2 Target and Transcriptional Coactivator of p53 in Response to DNA Damage

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DOI 10.1016/j.cell.2005.09.032

SUMMARY

In response to DNA damage, mammalian cells trigger the p53-dependent transcriptional induction of factors that regulate DNA repair, cell-cycle progression, or cell survival. Through differential proteomics, we identify heterogeneous nuclear ribonucleoprotein K (hnRNP K) as being rapidly induced by DNA damage in a manner that requires the DNA-damage signaling kinases ATM or ATR. Induction of hnRNP K ensues through the inhibition of its ubiquitin-dependent proteasomal degradation mediated by the ubiquitin E3 ligase HDM2/MDM2. Strikingly, hnRNP K depletion abrogates transcriptional induction of p53 target genes and causes defects in DNA-damage-induced cell-cycle-checkpoint arrests. Furthermore, in response to DNA damage, p53 and hnRNP K are recruited to the promoters of p53-responsive genes in a mutually dependent manner. These findings establish hnRNP K as a new HDM2 target and show that, by serving as a cofactor for p53, hnRNP K plays key roles in coordinating transcriptional responses to DNA damage.

INTRODUCTION

The eukaryotic DNA-damage response (DDR) has evolved to optimize cell survival following damage to the genome. Key DDR regulators are the phosphatidylinositol 3-kinase-like kinases (PIKKs) ataxia telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which are activated following DNA damage and then phosphorylate downstream targets (Abraham, 2004; Shiloh et al., 2004). Targets of ATM and ATR include transcription factor p53 and the checkpoint kinases CHK1 and CHK2; these in turn regulate the activities of downstream effector proteins controlling DNA repair, cell-cycle progression, or the initiation of apoptotic or senescence programs.

In addition to being an important target of ATM and ATR, p53 is the most frequently mutated protein known in human cancers, with loss of p53 function thought to contribute to tumorigenesis by fostering genome instability and the consequent acquisition of additional oncogenic mutations (Vousden and Prives, 2005). A fundamental property of p53 is that its levels and transcriptional activity are markedly induced by DNA damage and a range of other cellular stresses. Under normal conditions, p53 activity is maintained at low, basal levels through the actions of HDM2 (originally identified in mice as the murine double minute 2 protein, MDM2). HDM2 acts as a ubiquitin E3 ligase to transfer ubiquitin moieties onto p53, thus promoting p53 degradation via the ubiquitin-dependent proteasomal system (Haupt et al., 1997; Kubbutat et al., 1997). In response to DNA damage, negative regulation of p53 by HDM2 is lifted, leading to stabilization of transcriptionally competent p53. This control is brought about in part by ATM- and ATR-mediated phosphorylation of both p53 and HDM2, which impairs the interaction between the two proteins (Perry, 2004).

A major consequence of p53 activation following DNA damage is the induction of cell-cycle arrest at the G1/S or G2/M transition stages. This is achieved primarily through p53-induced expression of target genes that encode factors such as p21[WAF/CIP], a negative regulator of cyclin-dependent kinases (CDKs) that induces G1/S arrest (Bartek and Lukas, 2001), and proteins such as GADD45, 14-3-3σ, and Reprimo that are needed for an efficient G2/M arrest following DNA damage (Taylor and Stark, 2001). The importance of these transcriptional responses is highlighted by the fact that over 90% of known tumor-derived p53 mutations occur in its DNA binding domain (Hainaut and Hollstein, 2000). The
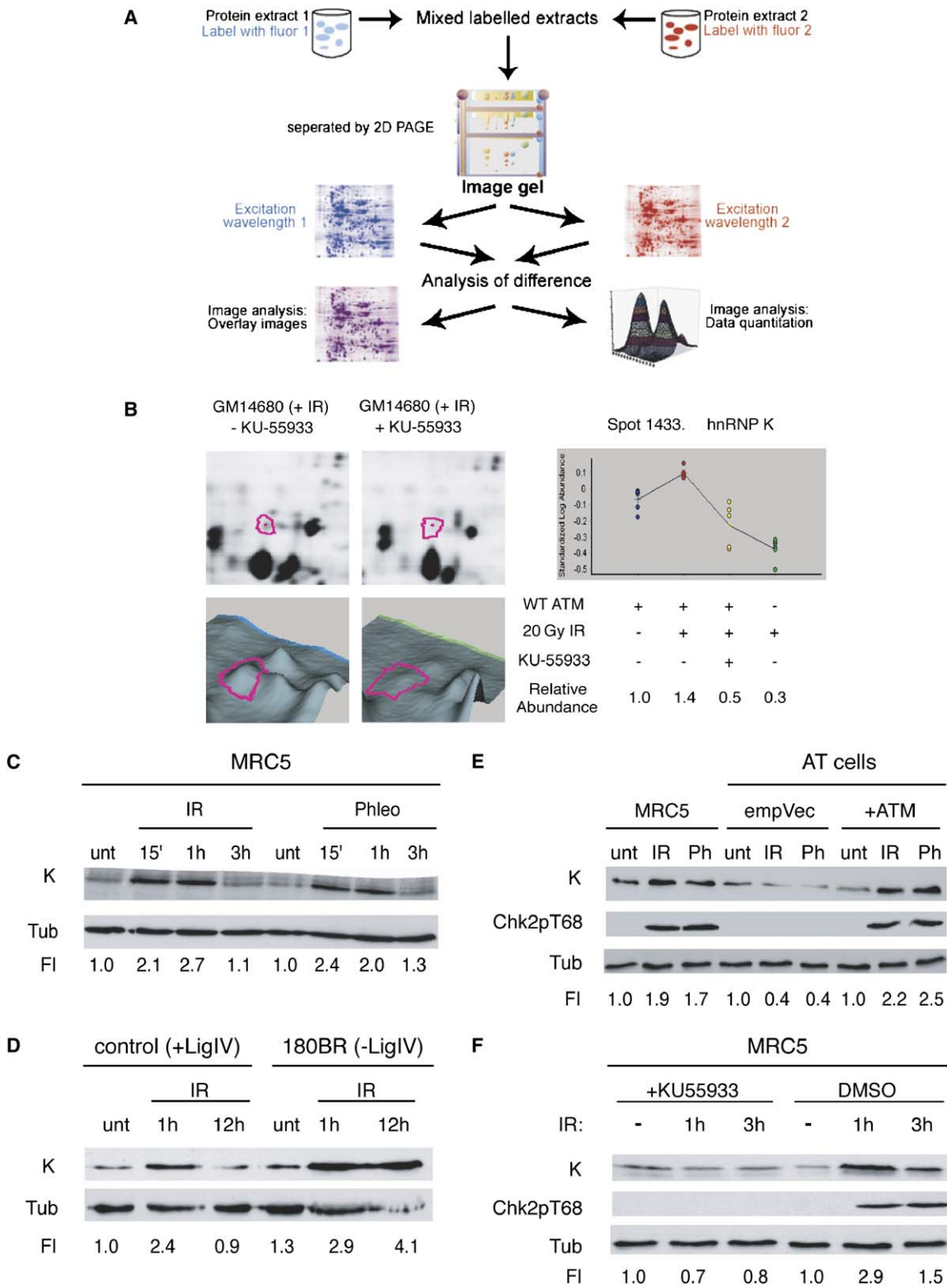
Figure 1. Induction of hnRNP K by IR Is ATM Dependent

(A) In 2D DIGE, spectrally distinct fluorescent Cy dyes are used to label proteins of different cell lysates before mixing and running these on the same 2D gel. Differences in levels of individual protein spots between lysates are determined after fluorescent imaging and analysis.

(B) Profile of a nuclear protein upregulated in response to IR in an ATM-dependent manner and later identified by mass spectrometry as hnRNP K. Left panel: relative abundance of this highlighted hnRNP K spot in lysates from GM14680 cells (wild-type ATM) obtained 3 hr after 20 Gy of IR exposure in either the

pivotal role of p53 in cellular stress responses is reflected by the complex regulatory mechanisms that control its activity; these include the existence of many forms of p53 posttranslational modification, the regulation of p53 DNA binding activity by other transcription factors, and the cooperation of p53 with transcriptional coactivators that modify chromatin structure and/or facilitate transcription-complex formation (Coutts and La Thangue, 2005).

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is an evolutionarily conserved factor found in the nucleus and cytoplasm that was initially discovered as a component of hnRNP complexes (Matunis et al., 1992). Since then, work has implicated hnRNP K in processes including chromatin remodeling and transcription as well as mRNA splicing, export, and translation (Bomsztyk et al., 2004). The involvement of hnRNP K in these events appears to reflect its ability to interact with a range of molecular partners, including DNA, RNA, protein kinases, and proteins involved in chromatin remodeling (Bomsztyk et al., 1997, 2004). Perhaps the most characterized function of hnRNP K is its role in transcription. For example, it has been reported to associate with the κB enhancer motif (Ostrowski et al., 1994); to enhance the expression of the *c-myc*, *EGR*, and *BRCA1* genes (Michelotti et al., 1996; Ostrowski et al., 2003; Thakur et al., 2003); to activate or repress RNA polymerase II transcription in a context-dependent manner (Lee et al., 1996; Michelotti et al., 1996; Tomonaga and Levens, 1995, 1996); and to stimulate transcription by purified RNA polymerase II in vitro (Gaillard et al., 1994). Although the available data suggest that these effects reflect the ability of hnRNP K to bind with high affinity to single-stranded DNA (ssDNA; Tomonaga and Levens, 1995, 1996), the precise mechanisms by which hnRNP K regulates transcription and the ways in which hnRNP K is itself regulated remain largely obscure.

Here, by studying PIKK-dependent proteomic changes that occur in human cells in response to DNA damage, we identify hnRNP K as a protein that is upregulated in response to DNA damage in an ATM- and ATR-dependent manner. Furthermore, we show that hnRNP K is stabilized following DNA damage through the inhibition of its HDM2-mediated ubiquitin-dependent degradation. Finally, by investigating the functional consequences of hnRNP K depletion from cells, we establish that it is crucial for DNA-damage-induced cell-cycle-checkpoint arrest and serves as a transcriptional cofactor for p53.

RESULTS

Proteomic Analysis Identifies hnRNP K as a Candidate for ATM-Dependent Regulation

To analyze the DDR, we used the differential proteomic technology of two-dimensional difference gel electrophoresis (2D DIGE; Unlu et al., 1997). 2D DIGE overcomes a major hurdle encountered with traditional 2D gel methods—the difficulty in matching protein spots from multiple samples run on different gels—by labeling proteins from different samples with spectrally distinct fluorescent cyanine (Cy) dyes, mixing the samples, and then running them on the same gel before overlaying the separate fluorescent images (Figure 1A; Gharbi et al., 2002). In a 2D DIGE analysis of changes in nuclear proteins in response to ionizing radiation (IR), we used as controls both a potent and specific small molecule inhibitor of the ATM kinase (KU-55933; Hickson et al., 2004) and A-T cells that fail to express functional ATM protein. Figure 1B illustrates the profile of a protein species that was identified by mass spectrometry as hnRNP K. In GM14680 lymphoblastoid cells that contain functional ATM, this protein species was induced following IR treatment; however, in the same cells exposed to the ATM inhibitor KU-55933, or in ATM-deficient GM01526 cells, the amount of the protein following IR treatment was 3- to 4-fold lower, suggesting that hnRNP K is regulated by ATM kinase activity.

To confirm and further characterize hnRNP K induction in response to DNA damage, we carried out immunoblot analyses with a monoclonal antibody directed against it (Matunis et al., 1992). This revealed that hnRNP K levels increased 2- to 3-fold within 15 min after exposure of MRC5 fibroblast cells to IR or following their acute treatment with the radiomimetic drug phleomycin (Figure 1C). In each case, hnRNP K returned to near basal levels within 3 hr of treatment. The initial identification of hnRNP K by 2D DIGE analysis at the 3 hr time point presumably reflects the high sensitivity of this method. This transient increase in hnRNP K was also observed by indirect immunofluorescence analysis of irradiated cells (data not shown). Importantly, in contrast to control cells where hnRNP K induction was transient, hnRNP K induction was still observed 12 hr after IR treatment of 180BR cells that bear a mutation in the gene for DNA ligase IV (Figure 1D). Since 180BR cells are specifically defective in repairing DNA double-strand breaks (DSBs; Riballo et al., 1999), these data reveal that it is DSBs and not other

presence or absence of KU-55933. Right panel: abundance of the hnRNP K spot relative to the mean level observed in untreated GM14680 cells (blue circles), in the same cells treated with IR (red circles) or with IR and KU-55933 (yellow circles), or in IR-treated GM01526 cells that lack functional ATM (green circles). All lysates treated with IR were obtained 3 hr postirradiation.

(C) MRC5 cells were untreated (unt) or treated with 20 Gy of IR or 100 μg/ml of phleomycin (Phleo) and incubated for the times indicated. Immunoblot analysis was with an hnRNP K monoclonal antibody (K) or an anti-tubulin antibody (Tub).

(D) hnRNP K was assessed up to 12 hr following IR (10 Gy) in wild-type fibroblasts (control) or DSB-repair-defective 180BR cells.

(E) hnRNP K in extracts of normal fibroblast cells (MRC5) or ATM-defective AT fibroblasts stably transfected with either empty vector (empVec) or an ATM-expressing construct (+ATM). Cells were untreated (unt) or treated with 15 Gy IR or 100 μg/ml of phleomycin (Ph) 1 hr before harvesting and assessing hnRNP K levels (K). An anti-tubulin antibody (Tub) was used as a control, and a CHK2 phosphospecific antibody (Chk2pT68) was used to confirm ATM activity.

(F) hnRNP K was assessed following IR (15 Gy) at the times indicated in either the presence or absence of 10 μM KU-55933, as indicated. Given below (C)–(F) is the fold of increase (FI) in hnRNP K following DNA damage relative to the relevant untreated sample.

IR-induced lesions that trigger increased hnRNP K levels. Furthermore, while treatment with IR or phleomycin led to hnRNP K induction in ATM-positive MRC5 cells and in A-T cells complemented with the wild-type *ATM* gene, this was not the case for ATM-defective cells (Figure 1E). Moreover, hnRNP K induction following IR or phleomycin treatment required ATM kinase activity as it was prevented by the ATM inhibitor KU-55933 (Figure 1F). Taken together, these data reveal that hnRNP K levels are rapidly induced by DSBs in a manner that requires the kinase activity of ATM.

hnRNP K Is Induced by UV in a ATR-Dependent Manner

To determine whether hnRNP K induction is specific to DNA damage or is a more general response to stress, we exposed cells to ultraviolet (UV) light, hypotonic conditions, hypertonic conditions, or heat shock. Of these treatments, only UV resulted in an appreciable and reproducible induction of hnRNP K levels, suggesting that hnRNP K is specifically induced by DNA damage (Figure 2A). Indeed, while UV-mediated elevation of hnRNP K was transient in repair-proficient MRC5 fibroblasts, its induction was much more protracted in xeroderma pigmentosum group A (XPA) cells (Ichikawa et al., 2000) that are defective in nucleotide-excision repair of UV-induced DNA lesions (Figure 2B). In contrast to induction of hnRNP K by IR, its UV-mediated induction still took place in A-T cells (data not shown) and in cells treated with the ATM inhibitor KU-55933 (Figure 2C). However, UV-mediated induction of hnRNP K was prevented by wortmannin or caffeine, which inhibit both ATM and ATR (Sarkaria et al., 1998), suggesting that it might rely on ATR. Indeed, while hnRNP K was induced by UV in cells treated with a control siRNA (si-GFP), induction did not take place in cells that had been siRNA depleted of ATR (si-ATR; Figure 2D). Thus, hnRNP K induction following UV exposure is ATR dependent.

hnRNP K Induction after DNA Damage Reflects Inhibition of Its Ubiquitin-Mediated Degradation

While characterizing hnRNP K levels in cells exposed to cycloheximide, a potent inhibitor of mRNA translation, we found that both IR and UV led to a marked increase in hnRNP K half-life in comparison to unirradiated controls (Figure 3A and data not shown). Thus, hnRNP K induction must occur at least in part through its posttranslational stabilization, possibly through inhibition of its degradation. As the major pathway for regulated protein degradation in eukaryotic cells is the ubiquitin-dependent proteasomal pathway, we used the proteasome inhibitor MG132. As shown in Figure 3B, when cells were not treated with a DNA-damaging agent, MG132 led to significantly higher hnRNP K levels. Moreover, in the presence of MG132, levels of hnRNP K were not further increased upon IR or UV treatment (Figure 3B and data not shown). These data suggest that, as for p53, hnRNP K levels are controlled by proteasome-mediated degradation and that, following DNA damage, this process is disrupted, leading to hnRNP K induction.

To test the above model, we incubated U2OS cells in the presence or absence of MG132, mock treated or treated the
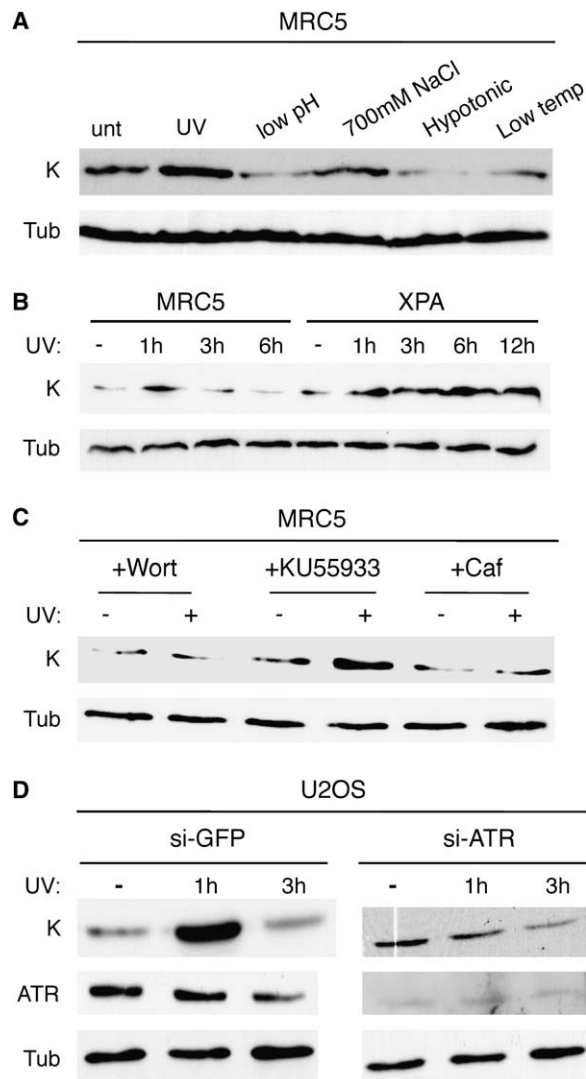

Figure 2. Induction of hnRNP K in Response to UV Is ATR Dependent

(A) MRC5 fibroblasts were exposed to different stress factors, including UV, and, 1 hr after exposure, hnRNP K (K) was evaluated by Western immunoblot analysis with anti-hnRNP K antibody. Tubulin (Tub) was assessed as a loading control.

(B) Effect of UV (25 J/m^2) treatment on hnRNP K in normal fibroblasts or XPA cells.

(C) hnRNP K levels in MRC5 cells 1 hr after UV (25 J/m^2) exposure in the presence of wortmannin (10 μM; +Wort), caffeine (5 mM; +Caf), or KU-55933 (10 μM).

(D) Levels of hnRNP K (K), ATR, and tubulin (Tub) were evaluated in extracts from U2OS cells treated with control siRNA (si-GFP) or ATR siRNA (si-ATR) with or without subsequent UV exposure.

cells with IR, and prepared protein extracts from them. We then immunoprecipitated hnRNP K and analyzed the immunoprecipitates by immunoblotting with an antibody directed against hnRNP K. Notably, when cells had been treated with MG132, in addition to there being a band corresponding to unmodified hnRNP K, we also detected a series of additional, more slowly migrating forms of the protein (Figure 3C).
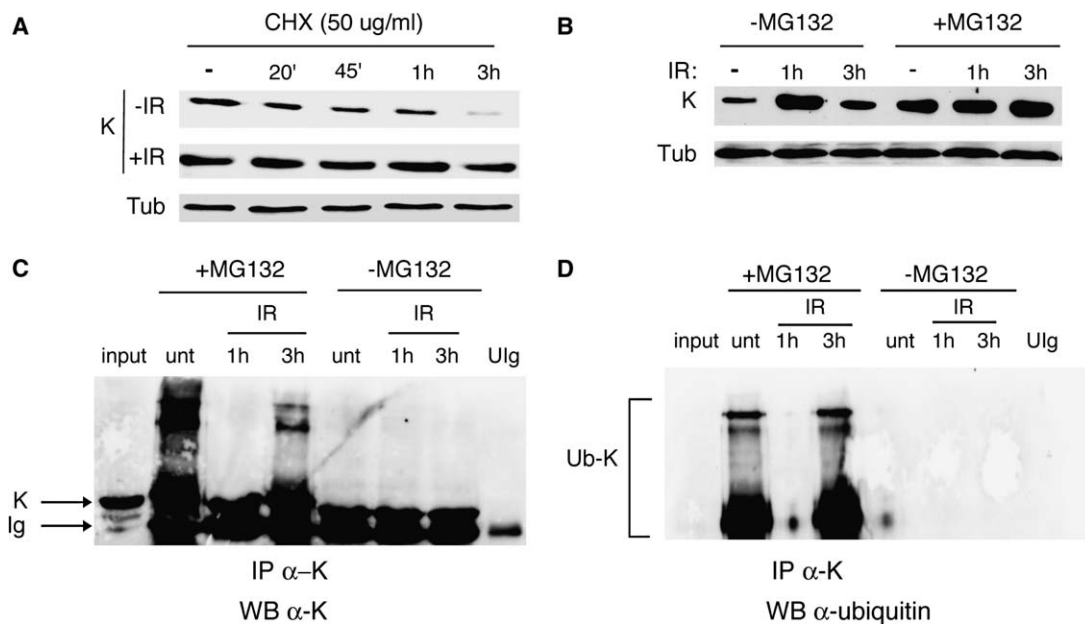
Figure 3. hnRNP K Stabilization following DNA Damage Reflects Inhibition of Its Proteasomal Degradation

(A) U2OS cells were pretreated with cycloheximide (CHX), mock (−) treated or treated with IR (20 Gy), and incubated in the presence of cycloheximide for the times indicated before determining hnRNP K (K) or tubulin (Tub) levels by Western blotting (WB).

(B) U2OS cells were treated with MG132 (30 μM) for 3 hr prior to IR (20 Gy), and, after incubation for the times indicated, hnRNP K levels (K) were evaluated by WB.

(C) Extracts from U2OS cells, either with or without MG132 or IR (20 Gy) treatment, were analyzed by immunoprecipitation (IP) with an anti-hnRNP K antibody followed by WB with the same antibody. Bands representing hnRNP K (K) and the immunoglobulin light chain (Ig) are indicated. The input lane contains 5% of the material used in the IP, and an unrelated antibody (UIg) was used as a specificity control.

(D) Immunoprecipitates from experiments in (C) were subjected to WB analysis with an anti-ubiquitin antibody.

These additional bands were not observed in extracts from cells that had not been MG132 treated, suggesting that they might correspond to hnRNP K ubiquitin conjugates (Figure 3C). Indeed, the more slowly migrating forms of hnRNP K were recognized in parallel immunoblots probed with an antibody directed against ubiquitin (Figure 3D). Most strikingly, while the ubiquitin-modified forms of hnRNP K were observed in unirradiated cells, they were virtually undetectable 1 hr after IR treatment but reappeared 3 hr postirradiation (Figures 3C and 3D). These results imply that stabilization of hnRNP K following DNA damage reflects a transient inhibition of its ubiquitin-dependent proteasomal degradation.

HDM2 Is a Negative Regulator of hnRNP K Protein Levels

Protein ubiquitylation results from a tightly regulated enzymatic cascade, and, in many cases, the last step of the cascade—the transfer of the ubiquitin moiety onto a specific target—is mediated by an E3 ubiquitin ligase protein (Pickart and Eddins, 2004). An E3 ligase that has been strongly linked to the DDR is human HDM2 (the ortholog of mouse MDM2). HDM2/MDM2 negatively regulates p53 in undamaged cells by directly binding p53 and blocking its transcriptional activity and also by stimulating p53 nuclear export and proteasomal degradation (Iwakuma and Lozano, 2003). This negative regulation is transiently alleviated following DNA damage,

and, in the case of IR, it is ATM dependent. Given the similar profiles and PIKK requirements of hnRNP K and p53 induction following IR, we speculated that HDM2 might also interact with and regulate hnRNP K. Indeed, HDM2 was coimmunoprecipitated with hnRNP K from extracts derived from unirradiated cells. Moreover, this coimmunoprecipitation rapidly diminished following the irradiation of cells but was reestablished 3 hr postirradiation (Figures 4A and 4B). To further analyze the interaction between hnRNP K and HDM2, we used Nutlin, a recently discovered antagonist of the HDM2-p53 interaction (Vassilev et al., 2004). While Nutlin enhanced p53 levels equivalent to those generated by IR, it did not cause detectable changes in hnRNP K levels (see Figure S1A in the Supplemental Data available with this article online). These data imply that the HDM2-hnRNP K interaction does not require p53 and suggest that the molecular nature of the p53-HDM2 interaction is likely to be distinct from that of the HDM2-hnRNP K interaction.

The above results suggested that HDM2 might destabilize hnRNP K and that this destabilizing activity is abrogated in response to DNA damage. In line with this model, we found that siRNA-mediated downregulation of HDM2 in SAOS2 cells led to induction of hnRNP K protein levels (Figure 4C). Furthermore, when we analyzed a panel of cancer cell lines displaying markedly different levels of HDM2—previously attributed to the presence or absence of functional p53 (Ramos et al., 2001)—there was an inverse correlation
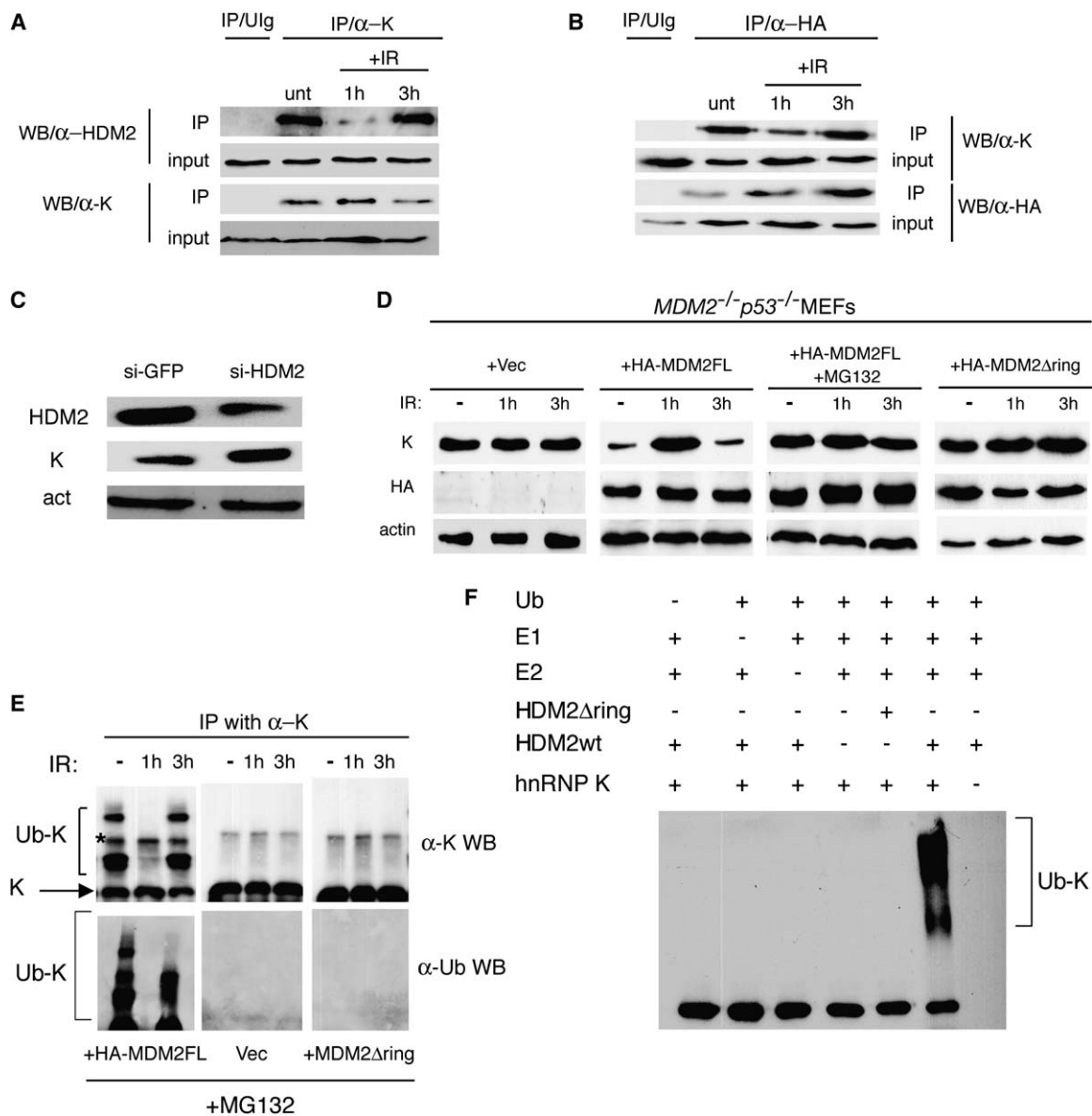
Figure 4. HDM2 Mediates Ubiquitin-Dependent Degradation of hnRNP K

(A) Lysates from U2OS cells, mock treated or treated with IR (20 Gy) and incubated for the times indicated, were subjected to IP with hnRNP K antibody (IP/α-K) and analyzed by Western blotting (WB) with an HDM2 (α-HDM2) or an hnRNP K (α-K) antibody. An unrelated antibody was used as a specificity control (IP/UIg).

(B) U2OS cells were transfected with an HA-tagged HDM2 expression vector and subjected to IP with HA antibody (IP/α-HA) after being mock treated or IR treated and incubated for the indicated times. Immunoprecipitates were analyzed by WB with hnRNP K (α-K) antibody or HA antibody (α-HA).

(C) SAOS2 cells were treated with control siRNA (si-GFP) or HDM2 siRNA (si-HDM2), and, 48 hr after treatment, extracts were subjected to WB with anti-HDM2 (HDM2) or hnRNP K (K) antibodies. Actin (act) antibody was used as a control.

(D) *Mdm2*-defective MEFs were transfected with empty vector (+Vec) or an HA-MDM2 vector expressing either full-length MDM2 (+HA-MDM2FL) or RING-finger-deleted MDM2 (+HA-MDM2Δring). Cells were then mock treated or treated with IR (20 Gy) and incubated for the indicated times. Cells transfected with the full-length MDM2 expression vector were also either mock treated or treated with MG132. Lysates were then prepared and subjected to WB with hnRNP K antibody (K), HA antibody (HA) for detection of HA-MDM2, or actin antibody.

(E) *Mdm2*-defective MEFs complemented as described in (D) were mock treated or treated with IR (20 Gy) and incubated for the indicated times before subjecting to IP with hnRNP K antibody followed by WB with hnRNP K antibody (α-K) or ubiquitin antibody (α-Ub). Ubiquitin-modified forms of hnRNP K (Ub-K) and hnRNP K (K) are indicated. The asterisk indicates a nonspecific band.

(F) HDM2-mediated in vitro ubiquitylation of hnRNP K (see Experimental Procedures).

between the levels of HDM2 and those of hnRNP K (Figure S1B). Indeed, induction of hnRNP K in response to DNA damage was not generally seen in cells displaying low HDM2 levels, probably attributable to mutations in p53 (data not shown). To more rigorously address the functional relationship between hnRNP K and HDM2, we used mouse

embryonic fibroblasts (MEFs) deleted for both copies of the *Mdm2* and *p53* genes (*Mdm2$^{-/-}$ p53$^{-/-}$*; Montes de Oca Luna et al., 1995). In *Mdm2$^{-/-}$ p53$^{-/-}$* cells transfected with parental vector, hnRNP K levels were not induced by IR, and the profile of hnRNP K expression corresponded to that of MDM2-competent cells in the presence of MG132, with constitutively high levels of the protein (Figure 4D; see also Figure 3B). Significantly, the introduction of MDM2 into *Mdm2$^{-/-}$ p53$^{-/-}$* cells led to a marked reduction of hnRNP K levels in unirradiated cells and restored the ability of IR to induce hnRNP K in a manner that was abolished by the MG132 (Figure 4D). Furthermore, these effects were mediated by wild-type MDM2 but not by a RING-finger-deleted version (MDM2Δring) of the protein that is deficient in E3 ligase activity (Figure 4D). Consistent with these findings, while incubation with MG132 allowed detection of ubiquitin-modified forms of hnRNP K in MDM2-negative cells transfected with wild-type *Mdm2* (Figure 4E), these ubiquitylated forms were not apparent in MG132-treated cells transfected with either the RING-deleted derivative of *Mdm2* or an empty vector (Figure 4E). To see whether HDM2 could serve as a direct E3 ligase for hnRNP K, we performed in vitro ubiquitylation assays with purified proteins. Indeed, while no ubiquitylation was observed in the presence of the HDM2Δring protein, hnRNP K ubiquitylation was clearly mediated by wild-type HDM2. Together, these results reveal that MDM2/HDM2 serves as a DNA-damage-regulated ubiquitin E3 ligase for hnRNP K.

hnRNP K Is Required for p53-Mediated Cell-Cycle-Checkpoint Responses

To probe hnRNP K function, we developed siRNA duplexes that rapidly and efficiently downregulated it in a range of cell lines (Figures 5A and 5B and data not shown). Importantly, downregulation of hnRNP K for up to 48 hr did not cause significant levels of cell death, thus allowing us to explore the effects of hnRNP K depletion on cell-cycle progression in the presence or absence of DNA damage. To address the possible role of hnRNP K in regulating cell-cycle checkpoints, we treated telomerized MRC5 fibroblast cells with control or hnRNP K siRNA oligonucleotides, treated or mock treated the cells with IR, and then analyzed them by flow cytometry. Downregulation of hnRNP K did not in itself cause significant alterations to the cell-cycle distribution (Figure 5A; see also Figure S2A); however, while cells treated with the control siRNA (si-GFP) displayed a normal G1/S checkpoint arrest following IR treatment, this response was almost completely abolished in cells depleted for hnRNP K (Figure 5A and Figure S2A). In parallel studies, we analyzed the effects of depleting hnRNP K from U2OS cells that are known to arrest mainly in G2 in response to DNA damage. While U2OS cells treated with the control GFP siRNA exhibited a clear G2/M arrest following IR, this response was curtailed by hnRNP K depletion or by siRNA depletion of p53 (Figure 5B and Figure S2B). Taken together, these data reveal that hnRNP K is required for the efficient induction of both the G1/S and G2/M cell-cycle arrests in response to IR. Significantly, when we analyzed IR-mediated cell-cycle responses in p53-deficient SAOS2 cells, we found that the IR-induced G2/M

arrest exhibited by these cells (probably mediated by Chk1 activation) took place whether or not hnRNP K was depleted (Figure S2C). These data therefore reveal that hnRNP K has selective effects on cell-cycle-checkpoint responses that are mediated by p53.

hnRNP K Is Required for p53-Dependent Transcription in Response to DNA Damage

Given the involvement of hnRNP K in cell-cycle-checkpoint events that require p53 and because of previous work linking hnRNP K to transcriptional control, we speculated that hnRNP K might facilitate the induction of p53 target genes following DNA damage. Indeed, while the p53 target proteins p21, GADD45, HDM2, and p53R2 were strongly induced by IR in p53-positive human U2OS cells that had been treated with a control siRNA (si-GFP), little or no induction was observed in hnRNP K-depleted cells (Figure 6A). Importantly, however, hnRNP K depletion did not affect IR-induced p53 stabilization or phosphorylation of p53 on Ser15 (Figure 6A), an event mediated primarily by ATM and ATR (Canman et al., 1998; Khanna et al., 1998).

To explore the mechanism behind the above observations, we carried out RT-PCR analyses on samples derived from cells that had been treated with control or hnRNP K siRNAs and then exposed to IR. We found that hnRNP K depletion prevented IR induction of the mRNAs for p21, HDM2, and 14-3-3σ, suggesting that hnRNP K exerts its effects at the transcriptional level (Figure 6B). Indeed, when we used a U2OS cell line containing a stably integrated luciferase construct under the control of the synthetic p53-responsive *PG13* promoter (Kern et al., 1992), luciferase expression was strongly induced by IR when these cells had been treated with a control siRNA, but almost no induction was apparent following hnRNP K depletion (Figure 6C). Similar effects of hnRNP K knockdown were seen in U2OS cells transiently transfected with a plasmid containing the gene coding for luciferase under the control of the p53-responsive *p21* promoter and also for p53-dependent transcription following UV treatment (Figures 6D and 6E). By contrast, downregulation of hnRNP K did not significantly affect luciferase expression directed by p53-independent promoters; these included the constitutive *SV40* early promoter, the serum-inducible *c-fos* promoter, and a synthetic promoter driven by the glucocorticoid-responsive elements (GREs; Figure 6D and Figure S3). Taken together, these data reveal that hnRNP K specifically influences DNA-damage-induced expression of p53 target genes at the transcriptional level. Furthermore, since hnRNP K is not required for IR-induced p53 phosphorylation or stabilization (Figure 6A), these results imply that hnRNP K does not regulate p53 activation per se but instead is required for p53 to promote the transcription of its target genes. Moreover, as shown in Figure 6F, we found that, when p53 is induced in the absence of DNA damage—by treating cells with Nutlin or by forced p53 over-expression—ensuing p53-dependent transcription is still impaired upon hnRNP K depletion, albeit to a lesser degree than in cells treated with IR. While these are clearly artificial situations, they nevertheless reveal that the constitutive level of hnRNP K present in cells not containing deliberately
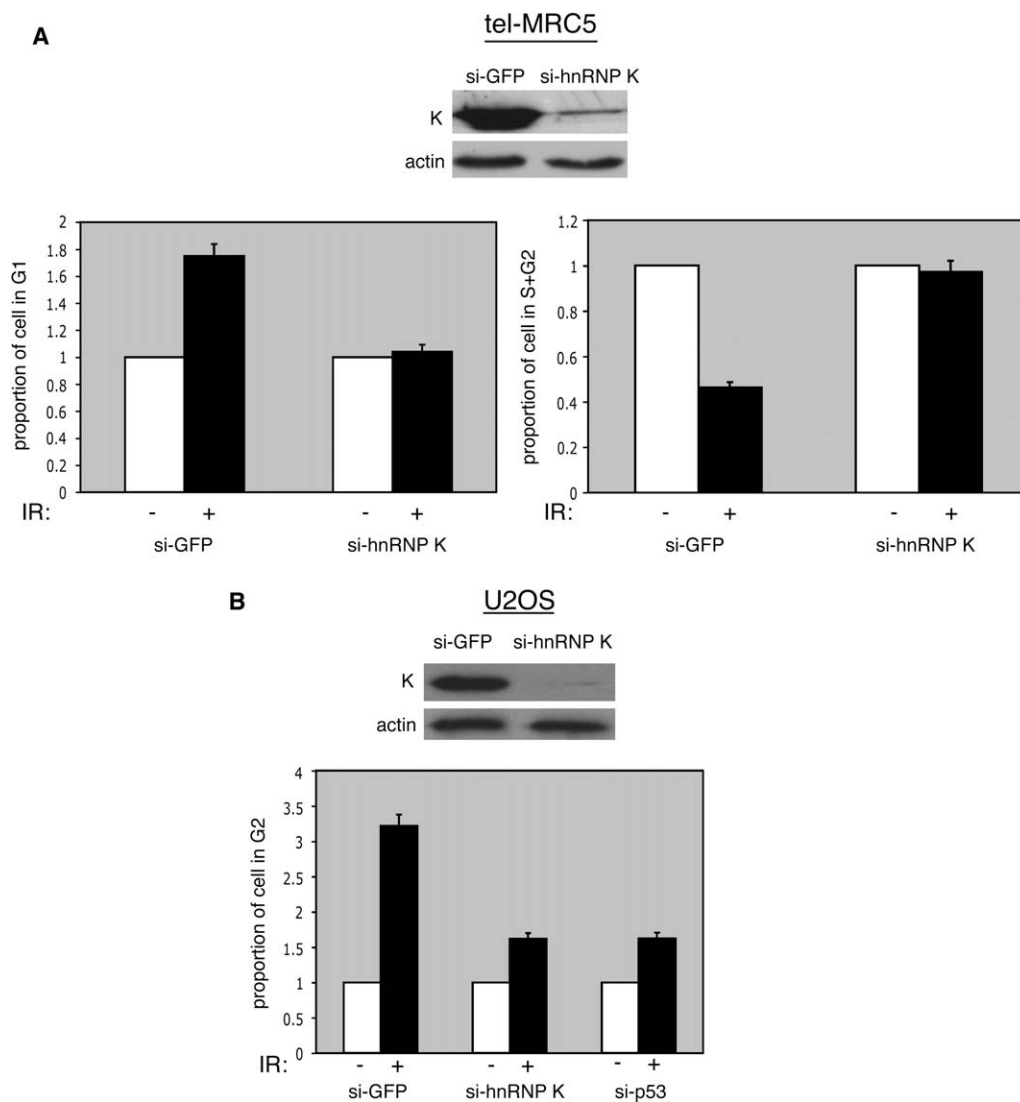
Figure 5. hnRNP K Is Required for Efficient G1 and G2 Cell-Cycle Arrest following DNA Damage

(A) MRC5 cells immortalized by ectopic expression of telomerase (tel-MRC5) were transfected with GFP siRNA or hnRNP K siRNA, mocked treated (−) or treated with 20 Gy IR (+) 48 hr later, and then analyzed by flow cytometry 24 hr later. The amount of cells accumulating in G1 or S + G2 in irradiated cells is given as a fold change relative to unirradiated cells.

(B) U2OS cells were analyzed in a similar fashion to those in (A) but, in addition to si-GFP and si-hnRNP K, siRNA for p53 (si-p53) was used to evaluate G2 arrest in response to IR. Levels of hnRNP K knockdown in tel-MRC5 (A) or U2OS (B) cells are also shown. In both (A) and (B), the plots represent the mean of three independent experiments and error bars represent SEM.

introduced DNA damage is intrinsically competent to promote p53-dependent transcription.

hnRNP K Interacts with p53, and Both Proteins Localize to p53-Responsive Promoters in an Interdependent Manner

Given the dependence of p53 transcriptional activity on hnRNP K, we investigated the potential interaction between the proteins by immunoprecipitation. Thus, we found that p53 could be coimmunoprecipitated with hnRNP K from extracts derived from unirradiated or irradiated cells (Figure 7A). Previous work has established that hnRNP K can serve as a transcriptional activator following its recruitment to the pro-

moter regions of target genes (Ostrowski et al., 2003; Thakur et al., 2003). We therefore used antibodies against endogenous hnRNP K in chromatin immunoprecipitation (ChIP) experiments to test for the potential recruitment of hnRNP K to genes regulated by p53. As illustrated in Figure 7B, while hnRNP K was weakly associated with the *p21* and *HDM2* promoters in the absence of exogenous DNA-damaging agents, this association was rapidly and markedly enhanced upon IR exposure but then returned to near basal levels after 3 hr. By contrast, IR did not trigger detectable recruitment of hnRNP K to the *GAPDH* promoter that is not regulated in response to DNA damage (Figure S4A). Notably, in contrast with the data obtained with p53-positive U2OS cells, IR did

not bring about the recruitment of hnRNP K to the *p21* and *HDM2* promoters in p53-deficient SAOS2 cells (Figure 7B), despite the fact that IR still triggered hnRNP K stabilization in these cells (see Figure S4B). These results indicate that the increased binding of hnRNP K to the *p21* and *HDM2* promoters following IR treatment in U2OS cells does not simply reflect higher levels of the protein and furthermore reveal that hnRNP K recruitment to p53-responsive promoters requires functional p53.

In parallel with the above analysis of hnRNP K recruitment, we observed the expected recruitment of p53 to its target genes in response to IR (Figure 7B). Strikingly, when we analyzed cells that had been treated with a control siRNA or had been siRNA depleted of hnRNP K, we found that the IR-induced recruitment of p53 to the *p21* and *HDM2* promoters was severely compromised in the absence of hnRNP K (Figure 7B), despite the fact that hnRNP K depletion does not affect p53 stabilization following DNA damage in these cells (Figure 6A). These findings thereby establish that p53 and hnRNP K depend on one another for their effective recruitment to p53 target genes in response to DNA damage. Consistent with this mutual dependency, while the kinetics of p53 and hnRNP K stabilization in response to IR are somewhat different, their temporal profiles of recruitment and retention on p53-dependent promoters were very similar (see Figure S4C).

DISCUSSION

We have identified hnRNP K as a new component of the mammalian DDR and have established that this protein is crucial for cells to mount effective responses to genotoxic agents. Specifically, following its ATM-dependent induction after IR or its ATR-dependent induction after UV irradiation, hnRNP K cooperates with p53 to elicit the activation of p53 target genes and thereby trigger cell-cycle-checkpoint events. A notable feature of hnRNP K induction by DNA damage is its rapidity: it can be detected within 5 min, and full induction is reached within 30 min to 1 hr. As in many other instances where protein levels are rapidly modulated, this reflects posttranscriptional control of (hnRNP K) protein stability. Indeed, we have shown that hnRNP K is a direct target of the ubiquitin E3 ligase HDM2/MDM2, a protein previously connected with the DDR through its control of p53 activity and levels (Iwakuma and Lozano, 2003). Furthermore—and with striking parallels to the control of p53 in response to DNA damage—we have shown that hnRNP K is targeted for HDM2/MDM2-dependent proteasomal turnover in undamaged cells and that this degradation promptly ceases upon the creation of DNA damage. Additionally, and with further resonance with p53, hnRNP K forms a complex with HDM2 in undamaged cells, and this complex is rapidly dissolved following DNA damage.

Although hnRNP K is a component of the hnRNP complex, composed of mRNA binding proteins that facilitate various stages of mRNA biogenesis and maturation (Bomsztyk et al., 2004; Dreyfuss et al., 1993), biochemical studies have indicated that a significant proportion of it is not part of the hnRNP complex and presumably has other functions

(Bomsztyk et al., 1997). Indeed, hnRNP K has been linked with DNA-dependent processes including chromatin remodeling and gene transcription (Bomsztyk et al., 2004), properties that are likely to reflect its ability to bind to DNA, particularly ssDNA (Tomonaga and Levens, 1995; 1996). Because of its diverse functions, it is possible that hnRNP K influences various aspects of the DDR. However, we have found that its depletion does not prevent PIKK-dependent phosphorylation of p53 on Ser15 or the focal recruitment of DDR factors to sites of DNA damage, and, furthermore, hnRNP K does not itself detectably accumulate at such sites (A.M., unpublished data). Instead, our data indicate that the main role of hnRNP K in the DDR is as a transcription cofactor. Thus, it is specifically required for the induction of p53 target genes, acting at the level of transcription and not through downstream events such as mRNA stability. Furthermore, we have shown that hnRNP K is recruited to the promoters of p53 target genes in a p53-dependent manner, and it is required for p53 to be recruited to these promoters as measured by ChIP analysis. These findings thereby establish hnRNP K as a novel transcriptional coactivator for p53.

A key outcome of hnRNP K and p53 cooperation is the induction of the G1/S and G2/M cell-cycle checkpoints. Thus, while normal telomerized MRC5 cells arrested in G1 in response to IR, this did not take place following hnRNP K depletion. This presumably reflects an inability of hnRNP K-depleted cells to induce p21, which normally mediates G1 arrest by inhibiting cyclin-dependent kinases (Harris and Levine, 2005) and by preventing PCNA from participating in DNA replication with DNA polymerase δ (Waga et al., 1994). Notably, p53 is also required for efficient G2/M arrest in response to DNA damage in certain cell lines (Taylor and Stark, 2001); here, DNA damage is thought to trigger the p53-dependent induction of p21, GADD45, and 14-3-3σ, which are required for effective suppression of the G2/M transition (Taylor and Stark, 2001). Consistent with the fact that hnRNP K depletion abrogates induction of these proteins, we have found that depletion of hnRNP K (or p53) from U2OS cells prevented them from efficiently arresting in G2/M in response to DNA damage. We obtained similar results with other p53-positive cells, such as HCT116, that also normally arrest in G2/M following DNA damage (A.M., unpublished data). These effects of hnRNP K were p53 dependent, as its depletion did not abrogate DNA-damage-induced cell-cycle arrest in p53-deficient SAOS2 cells.

How is it that p53 and hnRNP K cooperate at the transcriptional level? Based on our data, we surmise that hnRNP K facilitates the assembly and/or stability of *p53* promoter complexes. Significantly, hnRNP K and p53 can be coimmunoprecipitated from extracts of cells that have, or have not, been subject to DNA-damaging treatment, suggesting that they interact both prior and subsequent to their assembly onto transcriptional promoters. Previous work has established that the DNA binding activity of p53 is central to its biological functions as a tumor suppressor (Prives and Hall, 1999), and several studies have shown that p53 binding—even to its cognate sequences—is not a stable event and that p53 can dissociate from DNA in the absence
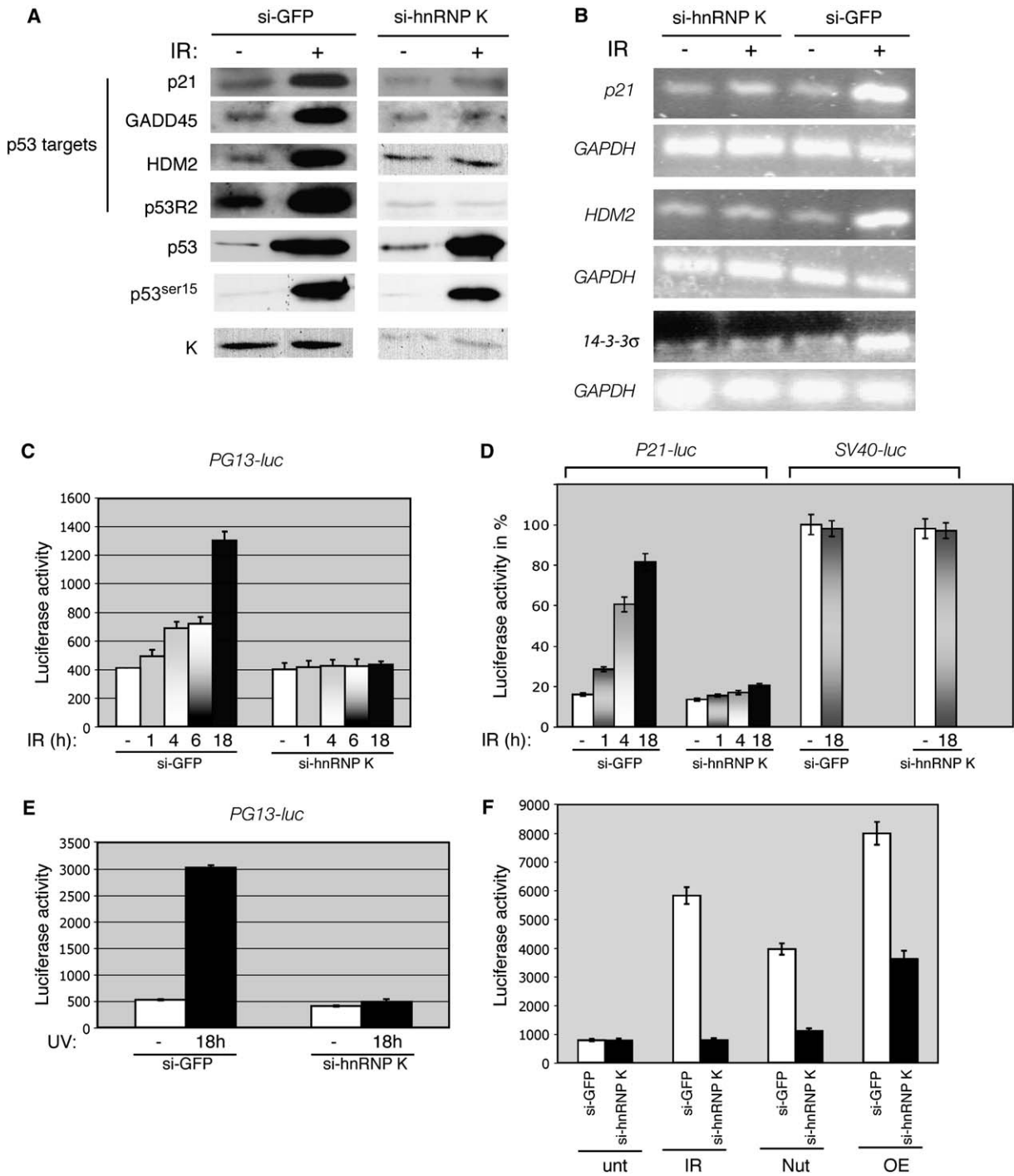
Figure 6. hnRNP K Is Required for p53-Dependent Transcription

(A) Induction of p53-dependent transcription targets was assessed in U2OS cells treated with a control siRNA (si-GFP) or hnRNP K siRNA (si–hnRNP K), and, 48 hr after siRNA transfection, cells were mock treated (−) or treated with 20 Gy IR (+); the induction of p53-dependent targets was determined by Western blot analysis 12 hr later. hnRNP K levels (K) are shown, confirming siRNA knockdown, and p53 stabilization and phosphorylation on Ser15 were determined 4 hr after IR treatment.

(B) Forty-eight hours following siRNA transfection, U2OS cells were mock treated or treated with 20 Gy IR and incubated for 6 hr before mRNA extraction. RT-PCRs were performed with primers recognizing sequences within the mRNAs for p21, HDM2, and 14-3-3σ. As a control, RT-PCR was carried out with primers specific for *GAPDH*.

(C) U2OS cells stably transfected with a luciferase reporter gene under the control of multiple p53 binding sites (*PG13-luc*) were subjected to si-GFP or si-hnRNP K treatment, and, 48 hr later, they were mock treated or exposed to IR (15 Gy). Luciferase activity was measured at the indicated times.
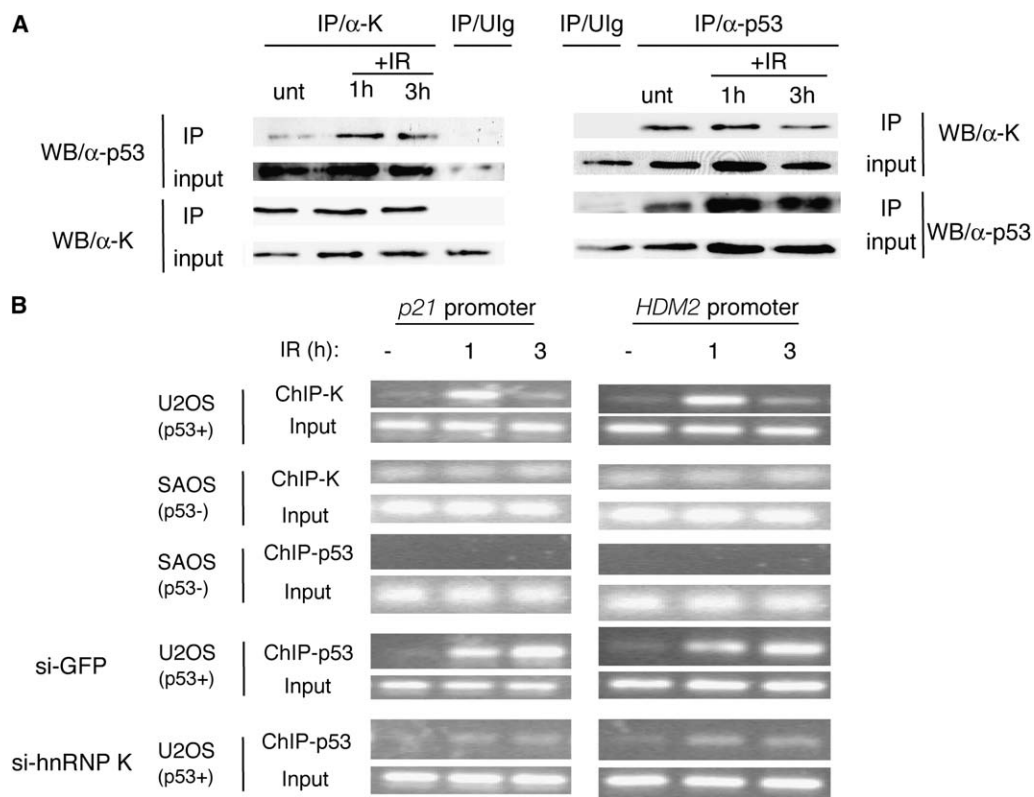
Figure 7. hnRNP K Interacts with p53, and the Two Proteins Associate with p53-Dependent Promoters in an Interdependent Manner

(A) Coimmunoprecipitation (IP) of hnRNP K with p53 was shown by using either hnRNP K antibody (IP/α-K) or p53 antibody (IP/α-p53) before or after IR treatment (15 Gy) at the times indicated. Western blot analysis was with hnRNP K antibody (WB/α-K) or p53 antibody (WB/α-p53). UIg is an unrelated antibody used as a control.

(B) U2OS (p53-positive) and SAOS2 (p53-negative) cells that had been mock treated or treated with IR (20 Gy) and incubated for the indicated times were subjected to ChIP with either hnRNP K antibody (ChIP-K) or p53 antibody (ChIP-p53). Precipitated DNA was subjected to PCR with primers covering the p53-response elements of the *p21* or *HDM2* promoters. ChIPs with anti-p53 antibody were carried out on lysates from U2OS cells obtained 48 hr after transfection with GFP siRNA or hnRNP K siRNA.

of other factors (Banerjee et al., 2004; Prives and Hall, 1999). If hnRNP K stabilizes *p53* promoter binding, it could help circumvent this instability. hnRNP K might also help p53 to search for its target sites in the genome, an event that Prives and colleagues have implicated as a key rate-limiting step in the activation of p53 target genes (McKinney et al., 2004). The recent identification of the DEAD box RNA helicase p68 as a new p53 coactivator further fuels this type of model (Bates et al., 2005). Notably, hnRNP K has been reported to display some sequence preference for its binding to ssDNA, so it is possible that it differentially affects various p53 target promoters, depending on their sequence features and propensity to form ssDNA. Whatever the case, it will be of inter-

est to see whether hnRNP K regulates transcription from all, or only a subset of, p53 target genes. It will also be interesting to determine whether the influence of hnRNP K extends to genes induced by DNA damage via p53-independent mechanisms (Elkon et al., 2005).

In addition to interacting with one another, it seems likely that hnRNP K and p53 will also interact with other transcription proteins, thus further stabilizing the promoter/p53/hnRNP K complex and facilitating additional steps of transcription-complex assembly together with transcriptional initiation itself. Significantly, several factors that interact with p53 and stimulate its DNA binding ability have also been reported to bind hnRNP K; these include high-mobility group 1

(D) U2OS cells transiently transfected with luciferase reporter vector under the control of a p53-dependent promoter (*p21-luc*) or p53-independent promoter (*SV40-luc*) were analyzed for their luciferase activity as above. Luciferase activity is given as a percentage relative to activity found in unirradiated *SV40*-luc-transfected cells treated with si-GFP.

(E) U2OS cells stably transfected with *PG13*-luc were subjected to UV (25 J/m^2) and incubated for the times indicated before determining luciferase activity.

(F) U2OS (*PG13-luc*) cells were subjected to si-GFP or si-hnRNP K and mock treated or treated with IR (20 Gy) or the MDM2 antagonist Nutlin (10 μm) for 24 hr or were transiently transfected with a p53 overexpression vector (OE). Luciferase activity was then measured. In (C)–(F), the plots represent the mean of at least three independent experiments ± SEM.

protein (HMBG1; Dintilhac and Bernues, 2002; Jayaraman et al., 1998), the Y box binding protein (YB-1; Okamoto et al., 2000; Shnyreva et al., 2000), and the TATA-box binding protein (TBP; Farmer et al., 1996; Michelotti et al., 1996). It is thus tempting to speculate that the linking of hnRNP K and p53 within a cooperative multiprotein complex would yield a higher degree of stability, promoter discrimination, and regulatory control than could be achieved by a more simple system involving fewer noncooperative components. It is noteworthy that hnRNP K is modified in response to various stimuli (Mandal et al., 2001; Ostrowski et al., 2001) and has been reported to interact with a range of other proteins (Bomsztyk et al., 1997). Perhaps these control hnRNP K activity in ways that allows DNA-damage-induced transcriptional events to be fine tuned by other signals.

Mutations in DDR components are strongly linked with cancer, and ongoing genome instability appears to be a hallmark of cancerous cells (Kastan and Bartek, 2004). It will hence be of interest to see whether hnRNP K mutations are associated with certain cancers. Given its widespread functions, however, we speculate that total loss of hnRNP K will be lethal to a cell, so if cancer-causing hnRNP K mutations do occur, then these will probably be more subtle—for instance, only affecting its DNA-damage-specific functions. Finally, it is noteworthy that hnRNP K overexpression has been linked to a range of cancers and has been associated with resistance to the DSB-inducing agent etoposide (Pino et al., 2003; Urbani et al., 2005). Considering these features together with its key role in p53-dependent transcription, hnRNP K may represent an attractive target for anticancer therapies.

EXPERIMENTAL PROCEDURES

Cell Culture, Expression Vectors, Transfection, and Flow Cytometry

Standard conditions and procedures were used for culturing mammalian cells. Transfections were done with calcium phosphate, and cells were harvested 48 hr afterwards. U2OS, SAOS2, HCT116, MRC5, and telomerized MRC5 cell lines were from Cancer Research UK. $Mdm2^{-/-}$ $p53^{-/-}$ MEF cells were from G. Lozano (Anderson Cancer Center, Houston), AT cells were from Y. Shiloh (Tel Aviv University, Israel), HA-MDM2 constructs were obtained from H. Lu (Oregon Health and Science University, Portland, OR), and GST-HDM2 was from M. Oren (Weizmann Institute, Israel). For flow cytometry, cells were washed with PBS, trypsinized, and fixed at 4ºC with 70% ethanol. Cells were then washed with PBS and incubated in PBS containing 30 mg/ml of RNase and stained 30 min at 37ºC with propidium iodide in the dark. DNA content was analyzed by flow cytometry.

Irradiation and Cell Extracts

Cells were treated with γ irradiation or UV at 50%–70% confluency (in 10 ml of medium in a 10 cm Petri dish for IR and 5 ml for UV). After recovery, cells were lysed in SDS sample buffer without bromophenol blue and equal amounts of protein analyzed by Western blotting.

Antibodies, Western Immunoblotting, and Immunoprecipitation

The monoclonal antibody recognizing hnRNP K was from G. Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia). p53, R2p53, Hdm2, pChk2 (T68), and anti-ubiquitin antibodies were from Cell Signaling; HA antibody was from Covance; β-tubulin and α-actin antibodies were from Abcam; and p21, Gadd45α, and ATR antibodies were from Santa Cruz Biotechnology. Cell extracts were resolved by 10% SDS-

PAGE, transferred onto nitrocellulose, and blotted by standard procedures. For immunoprecipitation, cells were washed with PBS and lysed on ice in RIPA buffer supplemented with protease inhibitors. Lysates were sheared by pipetting repeatedly through a needle and cleared by centrifugation. Extracts were precleared with 50 μl of Protein A Sepharose (Sterilin) for 2 hr at 4ºC and incubated for 2 hr with antibody and then overnight with 50 μl of Protein A Sepharose. Beads were washed with RIPA buffer three times, and bound proteins were recovered by boiling in SDS sample buffer.

In Vitro Ubiquitylation

Ubiquitylation was done as previously (Leng et al., 2003). Briefly, the reaction was performed in 30 μl of 50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, pure hnRNP K (500 ng), GST-HDM2 (or GST-HDM2Δring) (500 ng), rabbit E1 (200 ng), ubcH5b as E2 (200 ng), and ubiquitin (10 μg). After incubation at 30ºC for 1.5 hr, the reactions were stopped with 2× SDS loading buffer, heated to 95ºC for 5 min, and analyzed by Western blotting.

siRNA Design and Transfection

RNA duplexes of 21 nucleotides targeting the human hnRNP K, HDM2, p53, or ATR mRNAs were designed, chemically synthesized, and supplied in the 2′-deprotected and desalted form by Dharmacon (Lafayette, CO). Oligonucleotide sequences are in Table S1 (see Supplemental Data). In each case, the sequence was subjected to a BLAST search to ensure that the siRNA was specific to the targeted gene. U2OS cells were grown to 20%–50% confluency, and oligofectamin-mediated transient transfection of siRNA was done in 60 mm plates. siRNA (75 mM) and 7 μl of oligofectamin were mixed and incubated for 20 min at room temperature and added to each plate in DMEM containing 5% serum. After 24 hr, the medium was changed to DMEM supplemented with 10% FBS, and cells were left in culture for an additional 24 hr to bring about downregulation. GFP siRNAs were used as a control.

RT-PCR and Luciferase Expression Analyses

Total RNA was isolated with TRIzol (Invitrogen) from U2OS cells transfected with siRNA against hnRNP K or GFP and irradiated or left untreated. RT-PCR was done as described (Zeng et al., 2002). PCR products were analyzed on an agarose gel followed by ethidium-bromide staining. Primers used for PCR of *p21*, *HDM2*, and *GAPDH* were described previously (Zeng et al., 2002). For luciferase expression, U2OS cells stably transfected with an artificial p53 binding site repeat (PG13; 13 copies of GGACGGACCTGACCGGACC; Kern et al., 1992) cloned upstream of the luciferase coding sequence in a pGL3 basic vector (Promega) or transiently transfected by luciferase expression vectors under either the *p21* promoter or *SV40* early promoter were mock treated or treated with IR or UV and allowed to recover. Cells were then harvested, and luciferase activity was measured according to the manufacturer's protocol (Promega).

Chromatin Immunoprecipitation

ChIP was done with U2OS or SAOS2 cells as described (Espinosa et al., 2003). Primers used for PCR of *p21*, *HDM2*, and *GAPDH* were described previously (McKinney et al., 2004; Zeng et al., 2002).

Supplemental Data

Supplemental Data include one table and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/123/6/1065/DC1/.

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