Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation

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Summary

Nucleophosmin (NPM, B23) is an abundant nucleolar phosphoprotein involved in ribosome biogenesis, and interacts with tumor suppressor proteins p53 and Rb. Here we show that NPM is a UV damage response protein that undergoes nucleoplasmic redistribution and regulates p53 and HDM2 levels and their interaction. By utilizing RNAi approaches and analyses of endogenous and ectopically expressed proteins, we demonstrate that NPM binds HDM2 and acts as a negative regulator of p53-HDM2 interaction. Viral stress, enforced by expression of Kaposi's sarcoma virus K cyclin, causes NPM redistribution, K cyclin-NPM association, and p53 stabilization by dissociation of HDM2-p53 complexes. The results demonstrate novel associations of HDM2 and K cyclin with NPM and implicate NPM as a crucial controller of p53 through inhibition of HDM2.

Introduction

Nucleophosmin (NPM, B23, numatrin, NO38) is a nucleolar phosphoprotein constantly shuttling between the nucleolus and cytoplasm (Borer et al., 1989). It functions as a ribosomal assembly and transport protein (Spector et al., 1984; Yung et al., 1985; Borer et al., 1989), binds to proteins containing nuclear localization signals (NoLS) for their import (Szebeni et al., 1995), acts as a molecular chaperone (Szebeni and Olson, 1999; Okuwaki et al., 2001), and is essential in centrosome duplication (Okuda et al., 2000). Nucleolar translocation of NPM occurs in mitosis as the nucleolus disappears in the prophase (Ochs et al., 1983; Zatsepina et al., 1997; Dundr et al., 2000). This is coupled with phosphorylation of NPM by CDK1/cyclin B during mitosis (Peter et al., 1990). NPM binds to centrosomes, and its interaction is dissociated by its phosphorylation on threonine 199 by CDK2/cyclin E, which initiates centrosomal duplication (Okuda et al., 2000; Tokuyama et al., 2001; Tarapore et al., 2002). Phosphorylation of NPM by CDK2/cyclin E also decreases its RNA binding activity (Okuwaki et al., 2002).

NPM is redistributed from the nucleolus in response to cytotoxic drugs and genotoxic stress like inhibition of RNA polymerase I, DNA intercalating agents, and UV damage (Wu and Yung, 2002; Wu et al., 2002a; Yang et al., 2002; Rubbi and Milner, 2003). There is, however, no evidence that phosphorylation events are involved in the NPM stress responses. NPM exists in two heavily phosphorylated splicing variants, NPM1 and NPM1.2 (B23.1 and B23.2, respectively), which form multimers (Chang and Olson, 1989; Umekawa et al., 1993). NPM1 is mostly nucleolar, while NPM1.2 is present in cells in low levels and is detected both in cytoplasm and nucleoplasm (Wang et al., 1993; Okuwaki et al., 2002). The differences in their localization patterns are due to the absence of C-terminal 35 amino acids in NPM1.2, which mediate the RNA binding and RNase activities of NPM1 (Wang et al., 1994; Okuwaki et al., 2002). The main function of NPM1 is in ribosome biogenesis, while the function of NPM1.2 is not well understood (Savkur and Olson, 1998; Herrera et al., 1995). NPM binds several cellular and viral proteins, including nucleolin, which is another nucleolar phosphoprotein (Li et al., 1996), human immunodeficiency virus (HIV) Rev and Tat proteins (Fankhauser et al., 1991; Li, 1997), hepatitis delta virus delta antigens (Huang et al., 2001), retinoblastoma protein (Takemura et al., 1999), and recently, p53 (Colombo et al., 2002). NPM association with p53 regulates the stability and activity of p53, although the mechanism is unresolved (Colombo et al., 2002). In addition, noting its function in ribosome biogenesis, NPM associates with a large RNA-ribonucleoprotein com-

SIGNIFICANCE

The turnover rate of p53, mediated through the proteasome pathway and effected by HDM2, is the major route controlling p53 levels and is specifically abrogated in DNA-damaged cells. We identify here a major cascade of UV damage-provoked changes initiated by the subcellular redistribution of NPM and its enhanced association with HDM2 leading to the release of p53 from HDM2-mediated negative pressure. We demonstrate that a similar NPM-mediated set of events controls p53 in viral stress induced by KSHV K cyclin expression. The results underline the significance of NPM in the control of p53 and warrant further studies of its relevance in human tumors.

plex containing nucleolin, fibrillarin, and several ribosomal proteins (Pinol-Roma, 1999).

Human MDM2 (HDM2) is a nucleoplasmic and nucleolar RING-finger protein interacting with several tumor suppressor proteins, including p53, retinoblastoma protein, p14ARF (hereafter ARF for *a*lternative *r*eading *f*rame), and promyelocytic leukemia protein (PML) (Barak and Oren, 1992; Momand et al., 1992; Haines et al., 1994; Xiao et al., 1995; Kamijo et al., 1998; Wei et al., 2003; Kurki et al., 2003). HDM2 controls the levels of p53 by acting as an E3 ubiquitin ligase initiating p53 proteasomal degradation, and possesses autoubiquitin activity (Honda et al., 1997; Honda and Yasuda, 2000; Fang et al., 2000). The potent activity of p53 as inducer of cellular apoptosis and cell cycle arrest demands tight control for its function. The major mode for provoking p53 action is DNA damage, which leads to the release of interaction between HDM2 and p53, increasing p53 stability and activity (Vousden and Lu, 2002). p53 activity is also induced by cellular oncogenes, such as Ras and Myc (Sherr, 2001), and viral proteins like viral cyclin (K cyclin) expressed by Kaposi's sarcoma-associated herpesvirus (KSHV) (Verschuren et al., 2002). Ras and Myc increase the levels of ARF, which binds HDM2 and translocates it to the nucleoli, relieving the negative regulation of p53 by HDM2 (Weber et al., 1999, 2000; Lohrum et al., 2000). The mechanism of action by which K cyclin stabilizes and activates p53 is, however, unresolved.

Here we demonstrate that cellular stress by UV damage causes NPM nucleoplasmic redistribution and its interaction with HDM2, and that this interaction leads to the stabilization of p53. We show that NPM also regulates the basal levels of p53 in nonstressed cells through its interaction with HDM2. We find that viral K cyclin utilizes a NPM-mediated pathway for p53 stabilization, affecting NPM localization and its interactions with HDM2 and p53. The results show novel associations between NPM, HDM2, and K cyclin, and add a novel mechanism into the regulation of p53 stability in response to DNA damage and viral stress.

Results

NPM is translocated from nucleoli in response to UV damage

NPM has been described as a UV damage-inducible protein (Wu and Yung, 2002). In our studies of stress responses of cellular proteins we noticed a pronounced rapid shift of NPM from nucleoli to the nucleoplasm following treatment of SaOS-2 osteosarcoma cells with UVC radiation (Figure 1A). The translocation was evident by a decrease in the number of NPM-positive nucleoli and an increase in the nucleoplasmic staining intensity within 3 hr after the damage (Figures 1A and 1B), and was observed in a number of cell lines, both wild-type and null for p53 (U2OS and SaOS-2), and in human mortal fibroblasts (WS-1). A relocalization of NPM following UV damage was also recently reported by Rubbi and Milner (2003). The relocalization of NPM did not result from complete disruption of the nucleolar structure, as nucleoli were clearly discernible under phase contrast (Kurki et al., 2003) and stained with a RNA dye (Syto 12 Green) indicating the presence of ribosomal RNA (Figure 1C). The UVC-induced relocalization of NPM to the nucleoplasmic fraction was detected as an increase in the NP-40 soluble fraction and a decrease of NP-40 insoluble fraction of the cells (Figure 1D). There was also a 1.7-fold increase in total NPM as described earlier by Wu and Yung (2002). The UV-induced effects on NPM were time- and dose-dependent, being more pronounced with higher doses of UV ($\geq$10 J/m^2, results not shown). Similar UV-induced translocations of other nucleolar proteins, nucleolin, and Ki-67 were also observed (data not shown).

HDM2 interacts with NPM through its amino- and carboxytermini

As HDM2 is a damage-responsive protein, binds ribosomal proteins L5 and L11 (Marechal et al., 1994; Lohrum et al., 2003; Zhang et al., 2003), and localizes to nucleoli during proteasomal stress (Klibanov et al., 2001; Xirodimas et al., 2001; Latonen et al., 2003), we wanted to address a possible interaction between NPM and HDM2 following inhibition of the proteasome. In unstressed cells, HDM2 has a diffuse nucleoplasmic staining, while MG132-treatment of p53 null SaOS-2 cells caused a partial translocation of HDM2 to the nucleoli and its colocalization with NPM (Figure 2A). In order to verify a possible association between these proteins, control and MG132 treated cells were lysed with NP-40 buffer, and the soluble proteins were precipitated with a HDM2 antibody mix and analyzed by Western blotting. Downregulation of the proteasome led to an increase in HDM2 levels and to an association between HDM2 and NPM, whereas the total levels of NPM were unchanged (Figure 2B). The results demonstrate a significant relocalization and increased association of HDM2 with NPM in a p53-independent manner.

To test which functional domains of HDM2 are required for NPM interaction, GST-HDM2 deletion constructs were used in pulldown assays with in vitro translated ^{35}S-NPM (Figure 2C). The pulldown assays demonstrated that NPM bound to HDM2-constructs with amino acid sequences 1–110 containing the p53 interaction domain and to the HDM2 C terminus harboring the RING domain, whereas no specific binding was observed to region 108–280 containing the nuclear localization and export signals and the acidic domain (Figure 2C). The results indicate that in vitro translated NPM interacts with both N- and C termini of HDM2. Noting that HDM2 binds to p53 through its N terminus and that NPM has been shown to associate with p53 (Colombo et al., 2002), we wanted to establish whether NPM affects the complex formation between HDM2 and p53 and to address a possible competition between these proteins. We therefore performed an in vitro interaction and competition experiment by adding to the binding reactions increasing amounts of in vitro translated p53 and HDM2 and cellular lysate of *p53*$^{-/-}$*Mdm2*$^{-/-}$ murine embryo fibroblasts as a source of NPM. The presence of low amounts of p53 in the binding reactions increased the association of HDM2-NPM, suggesting that p53 promotes the formation of a trimeric complex (Figure 2D). Higher amounts of p53, however, decreased the HDM2-NPM association, indicating competition between their binding sites (Figure 2D). As p53 binds HDM2 amino acids 19–108, the above results are indicative that p53 and NPM compete for the binding of HDM2 N terminus.

UV damage-induced NPM-HDM2 complex formation precedes p53 stabilization

Given that NPM is a DNA damage-responsive protein and that it affected HDM2-p53 interaction in vitro, we studied in more detail the UV-induced effects on HDM2-NPM complex formation and its impact on p53. WS-1 human fibroblasts and U2OS

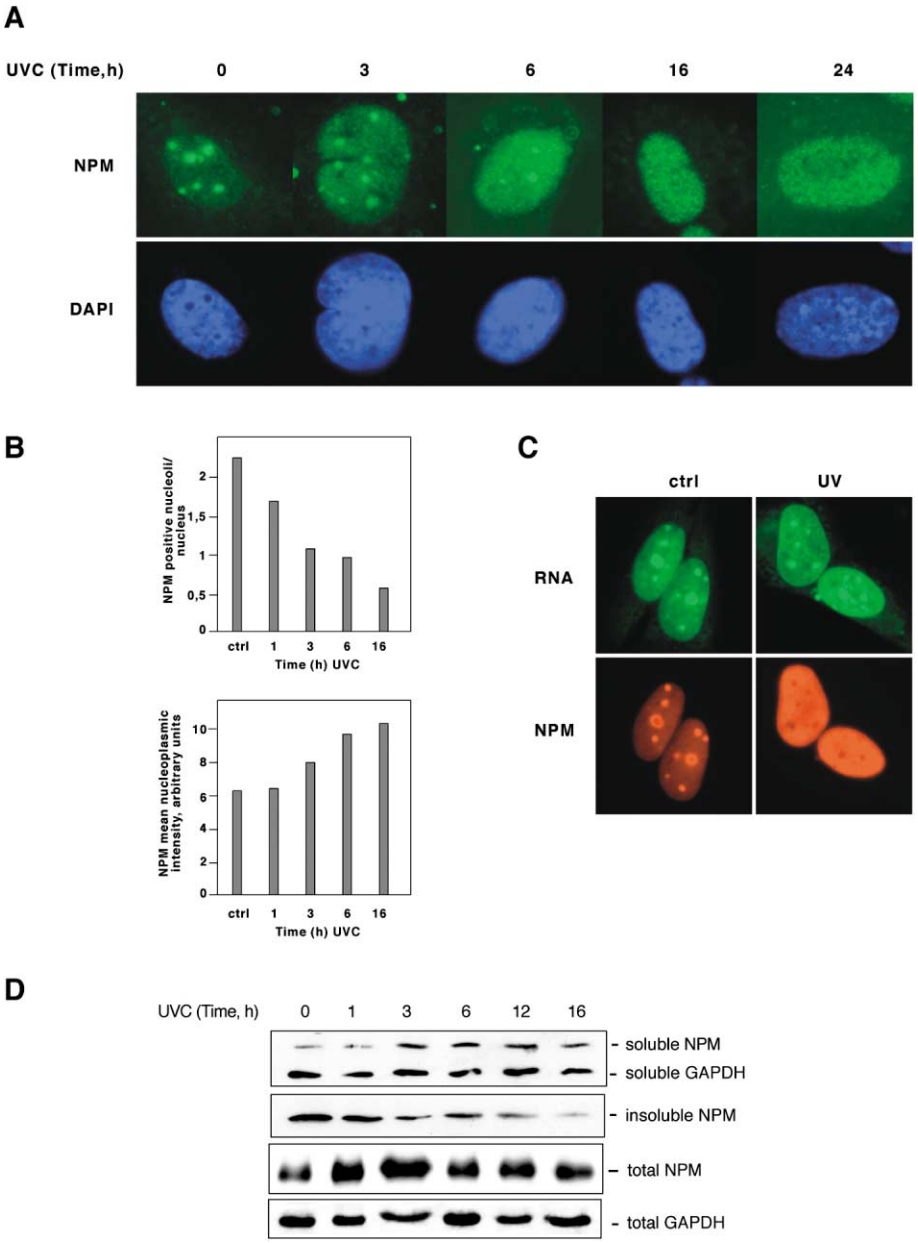

Figure 1. UV radiation causes nucleoplasmic redistribution of NPM

A: p53 null SaOS-2 osteosarcoma cells were radiated with UVC (35 J/m^2), incubated for the indicated periods of time, fixed, and stained for NPM. DNA was stained with DAPI.

B: The number of NPM-positive nucleoli and the mean nucleoplasmic staining intensity of cells treated as in **A** was analyzed from 100 cells per time point.

C: Detection of ribosomal RNA. SaOS-2 cells treated with UVC and incubated for 16 hr were stained with Syto 12 Green and for NPM.

D: Analysis of NPM levels in different cellular fractions. SaOS-2 cells were treated as in **A**. To extract soluble proteins, cells were lysed with 0.5% NP-40 lysis buffer, centrifuged, and the supernatant was collected. Alternatively, the pellet containing insoluble proteins was dissolved to Laemmli buffer and boiled. Total cellular extracts were obtained by lysing the cells in 9 M urea buffer. Protein concentrations were determined, equal amounts of proteins were loaded to gels, and NPM was detected by Western blotting. GAPDH was included as a protein loading control.

osteosarcoma cells (not shown) were radiated with UVC, and the levels of total NPM and its interaction with HDM2 were assessed at several time points (Figure 3). Similarly to SaOS-2 cells, UV stress caused a rearrangment of NPM localization from nucleoli to the nucleoplasm and to the outer perinucleolar region in WS-1 and U2OS cells (Figure 3A and data not shown). Coimmunoprecipitation analyses of the endogenous proteins demonstrated that HDM2-NPM complex was increased within 1–3 hr after the UV damage in both WS-1 and U2OS cells (Figure 3B and data not shown). At this time, p53 starts to accumulate and is fully stabilized by 6–12 hr (Figure 4D and Kurki et al., 2003). Additionally, HDM2-NPM interaction was promoted in UVC-treated *p53*$^{-/-}$*Mdm2*$^{-/-}$ murine embryo fibroblasts transfected with HDM2 expression vectors, indicating that the in vivo regulated HDM2-NPM interaction is independent of p53 (Figure 3C). The results demonstrate a transient increase in association of NPM and HDM2 in response to UV radiation and raise the possibility that these events affect early p53 stabilization.

NPM increases HDM2 levels and affects p53 sumoylation

NPM has previously been shown to interact with p53 and affect its activity and stability in response to cellular stress (Colombo et al., 2002). Given the notable translocation of NPM to the nucleoplasm following UV stress and its interaction with HDM2, we wanted to test whether the overexpression of NPM affects HDM2 or p53 levels or localization. In accordance with Colombo et al. (2002), ectopic expression of NPM was found to increase both p53 and HDM2 (Figures 4A and 4B). However, we find here that NPM increases HDM2 levels in SaOS-2 cells, thus in a p53-independent manner (Figure 4C). This suggests that NPM also impinges on HDM2 by other means besides the suggested

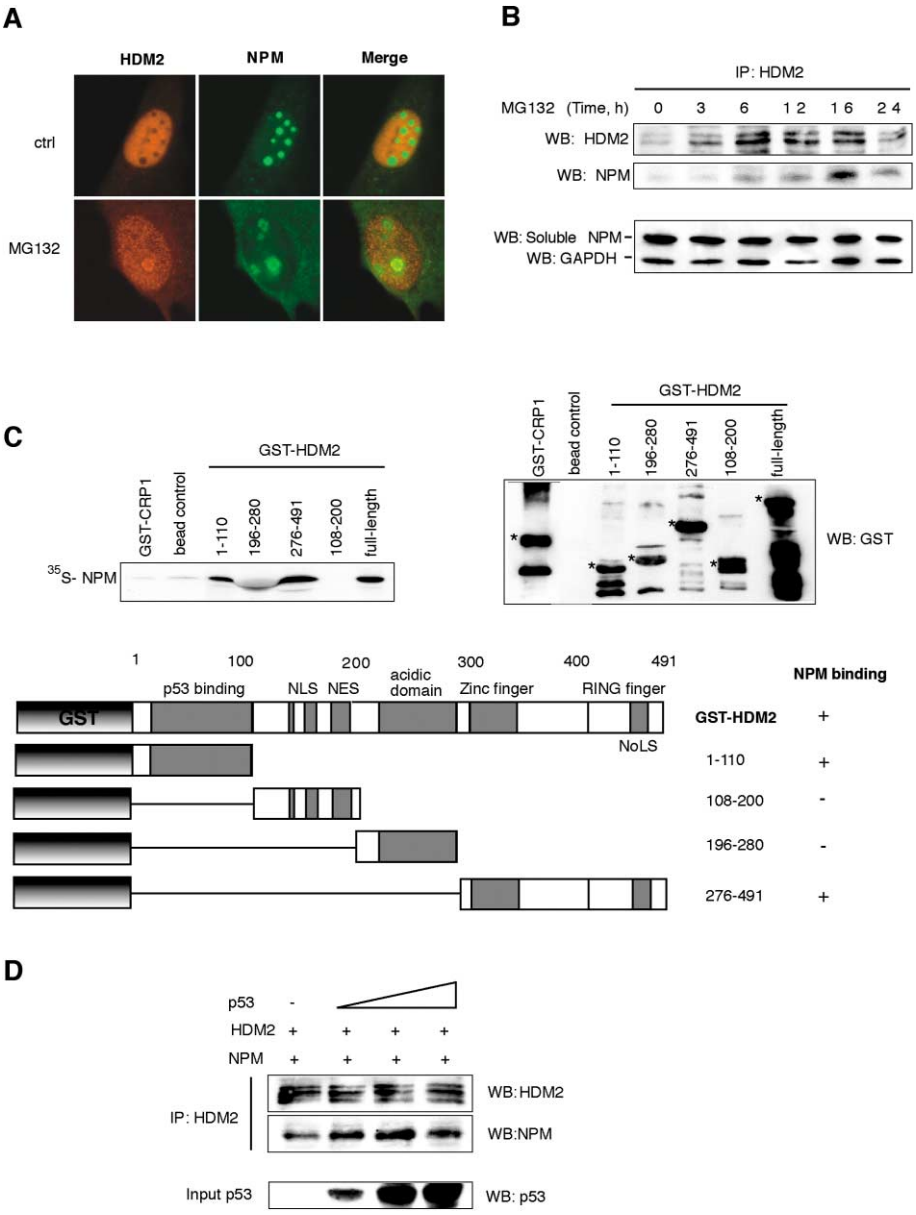

Figure 2. HDM2 interacts with NPM in vivo and in vitro

A: Inhibition of the proteasome causes HDM2 nucleolar redistribution and NPM colocalization. SaOS-2 cells were treated with MG132 (10 μM) for 6 hr or left untreated (ctrl), and immunostained for HDM2 and NPM.

B: HDM2 interacts with NPM. Cellular lysates of SaOS-2 cells, incubated with MG132 for the indicated time or left untreated, were prepared by lysing the cells with 0.5% NP-40 buffer. HDM2 and HDM2-NPM complexes were detected by coimmunoprecipitation followed by blotting for the respective proteins as indicated, or were analyzed directly from the NP-40 lysates. GAPDH was included as a protein loading control.

C: In vitro interaction analyses. GST-HDM2 deletion constructs or full-length HDM2 and irrelevant GST-vector (GST-CRP1) coupled to glutathione beads or beads alone were mixed with in vitro translated ^{35}S-NPM and incubated overnight, followed by extensive washing and analysis of the protein complexes by Western blotting for NPM. The amount of loaded GST-fusion proteins, indicated by asterisks, was controlled for by Western blotting of parallel samples for GST.

D: Low levels of p53 augment NPM-HDM2 interaction, whereas high levels of p53 decrease NPM-HDM2 interaction. Cellular lysate (100 μg) from *p53*$^{-/-}$*mdm2*$^{-/-}$ MEFs was mixed with in vitro translated HDM2 and increasing amounts of in vitro translated p53 followed by immunoprecipitation with HDM2 antibodies and blotting for NPM. The amounts of reticulosyte lysate in the reactions were balanced to equal volumes.

increase in transcriptional activity of p53 (Colombo et al., 2002). Subcellular compartmentalization could pose a means to regulate the interaction and levels of p53 and HDM2. However, we could not detect any change in the nucleoplasmic localization of p53 or HDM2 in NPM-transfected cells (data not shown). This indicates that their increase is not a consequence of, e.g., nucleolar sequestration as suggested for the ribosomal protein L11-induced effect (Lohrum et al., 2003). In contrast, overexpression of NPM led to increased complex formation between the soluble, nucleoplasmic fractions of NPM-p53 and NPM-HDM2 (Figures 4A and 4B). The fraction of NPM coprecipitating with HDM2 was significantly increased, while there was only a minor change in the amount of p53 coprecipitating with HDM2 (Figure 4B). This suggests that NPM prefers complex formation with HDM2, but is unable to dissociate the preformed p53-HDM2 complexes, at least in unstressed cells.

We have previously noted that p53 is present in UV-treated cells as a 65 kDa insoluble form shortly after UV damage prior to its stabilization (Kurki et al., 2003). Kinetically, this is also the time frame when p53 forms complexes with PML, and is indicative of a rapid p53 sumoylation event taking place in the PML-p53 complexes (Kurki et al., 2003). To test whether a SUMO modification of p53 plays a role in regulating p53-NPM interaction, we treated U2OS cells with UVC and followed p53-NPM association and their total levels during several time points (Figure 4D). We found that NPM is bound to a slower migrating 65 kDa form of p53 during 1–3 hr following UV treatment, but did not bind the 53 kDa form of p53 (Figure 4D). A similar result was obtained in UV-treated WS-1 fibroblasts (Figure 4E). To further verify the identity of the 65 kDa protein and its association with NPM, cellular lysates of UV-treated WS-1 cells were immunodepleted for NPM, followed by immunoprecipitation for p53 and its detection by Western blotting (Figure 4E). The data shows that the 65 kDa p53 form was exhausted at 1 hr time

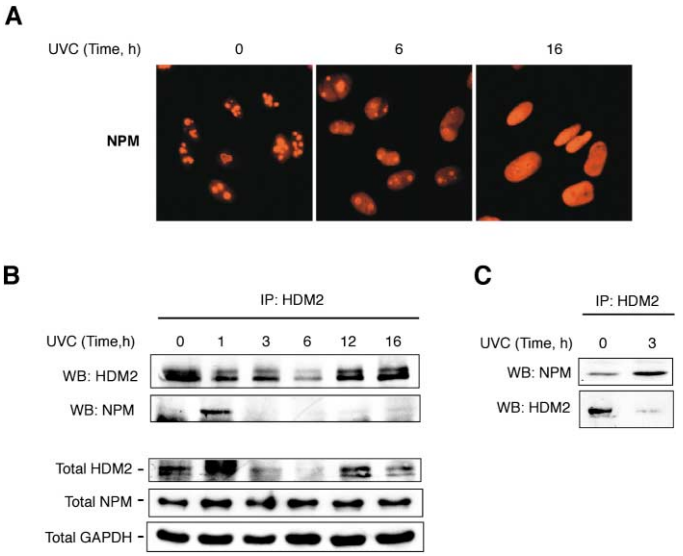

Figure 3. HDM2, p53, and NPM complexes undergo changes in response to UV radiation

A and B: WS-1 cells were radiated with UVC (35 J/m^2) and incubated for the indicated time. **A:** Localization of NPM was detected by immunostaining. **B:** Levels of HDM2 and NPM were detected by Western blotting, and HDM2-associated NPM was detected by coimmunoprecipitation. Total cellular extracts were prepared by lysing the cells with 9 M urea buffer. GAPDH was included as a loading control.

C: *p53*$^{-/-}$*mdm2*$^{-/-}$ murine embryo fibroblasts were transfected with HDM2 and were treated with UVC (35 J/m^2) 48 hr after transfection and incubated for 3 hr. HDM2 levels and HDM2-associated NPM were analyzed by coimmunoprecipitation of the cellular lysates.

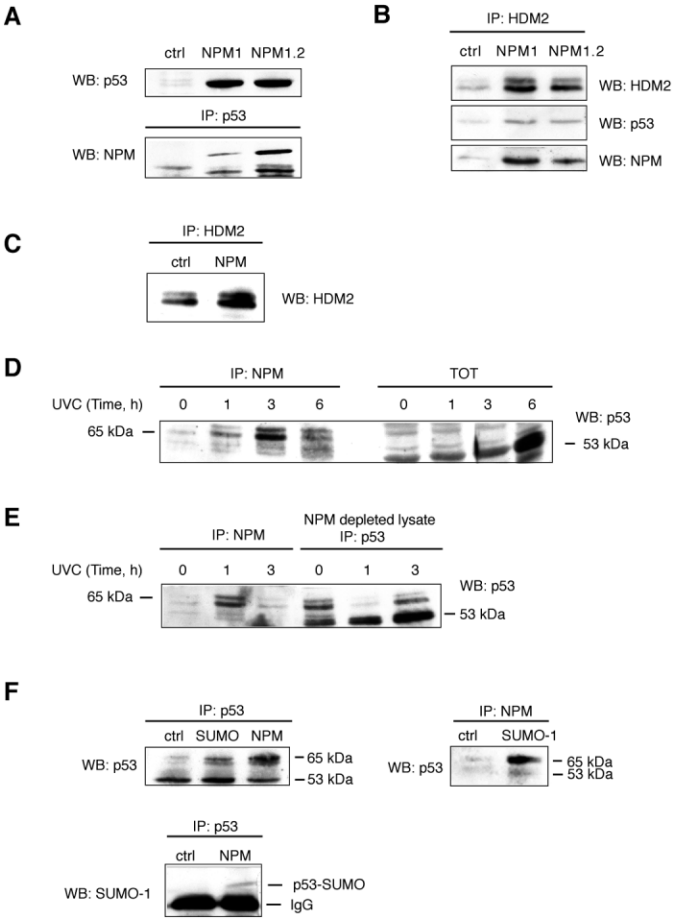

Figure 4. NPM increases the levels of both p53 and HDM2 and affects p53 sumoylation

A and B: U2OS cells were transfected with NPM1 or NPM1.2, and the levels of p53, HDM2, NPM, and their complexes were detected by Western blotting or coimmunoprecipitation experiments as indicated. Note that NPM co-precipitating with p53 has an altered electrophoretic mobility as compared to NPM present in HDM2 complexes. These could reflect differences in the phosphorylation pattern or charge of NPM.

C: NPM increases HDM2 levels in a p53-independent manner. SaOS-2 cells were transfected with either pSG5 control plasmid (ctrl) or NPM expression vector. Three days after transfection, cells were lysed and precipitated with a HDM2 antibody mix. HDM2 levels were detected by Western blotting.

D: p53 complexed with NPM has an altered mobility. U2OS cells were treated with UVC (35 J/m^2) and incubated for the indicated times. NPM-associated p53 was detected by coimmunoprecipitation and was compared to p53 present in total cellular lysate. p53 present in complex with NPM migrated as 65 kDa protein.

E: Depletion of NPM abolishes the 65 kDa form of p53. WS-1 fibroblasts were treated with UVC (35 J/m^2) and incubated for the indicated times. Cellular lysates were immunoprecipitated with a NPM antibody and either subjected to Western blotting for p53 directly (left panel), or the NPM-depleted lysates were subjected to a second round of immunoprecipitation with a mixture of p53 antibodies (right panel) followed by western blotting for p53 (FL-393).

F: NPM increases the amount of SUMO-modified p53. U2OS cells were transfected with either a NPM or SUMO-1 expression vector as indicated and incubated for 48 hr. Cellular lysates were prepared and immunoprecipitated with a p53 antibody mix or anti-NPM antibody as indicated, followed by blotting for either p53 or SUMO-1.

point following NPM depletion, whereas the 53 kDa form of p53 was not affected. Thus, the data indicates preferential and transient interaction of NPM with a 65 kDa form of p53 in UV-damaged cells.

As the 65 kDa p53 comigrates with a SUMO-modified p53 (Rodriguez et al., 1999; Kurki et al., 2003), we tested whether the overexpression of NPM affects p53 sumoylation. For this purpose we transfected NPM or, as a comparison, SUMO-1, into U2OS cells followed by detection of p53 by immunoprecipitation and Western blotting (Figure 4F). The results demonstrate that either ectopic expression of NPM or SUMO-1 alters p53 migration to a 65 kDa form (Figure 4F). Conversely, immunoprecipitation of NPM from cells ectopically expressing SUMO-1 followed by detection of p53 confirmed that NPM interacts with the 65 kDa p53 form. Lastly, NPM was transfected into U2OS cells followed by immunoprecipitation for p53 and Western blotting for SUMO-1 (Figure 4F). Ectopic expression of NPM was found to increase SUMO modification of endogenous p53. Based on these results, we conclude that NPM associates with p53, but prefers the p53 SUMO-modified form.

Depletion of NPM increases HDM2-p53 complex formation

To test whether the NPM-mediated regulation of p53 occurred through HDM2, we employed siRNA knockdown approaches. We transfected U2OS cells with RNAi duplexes targeting NPM and analyzed the ensuing changes in NPM, HDM2, p53, and

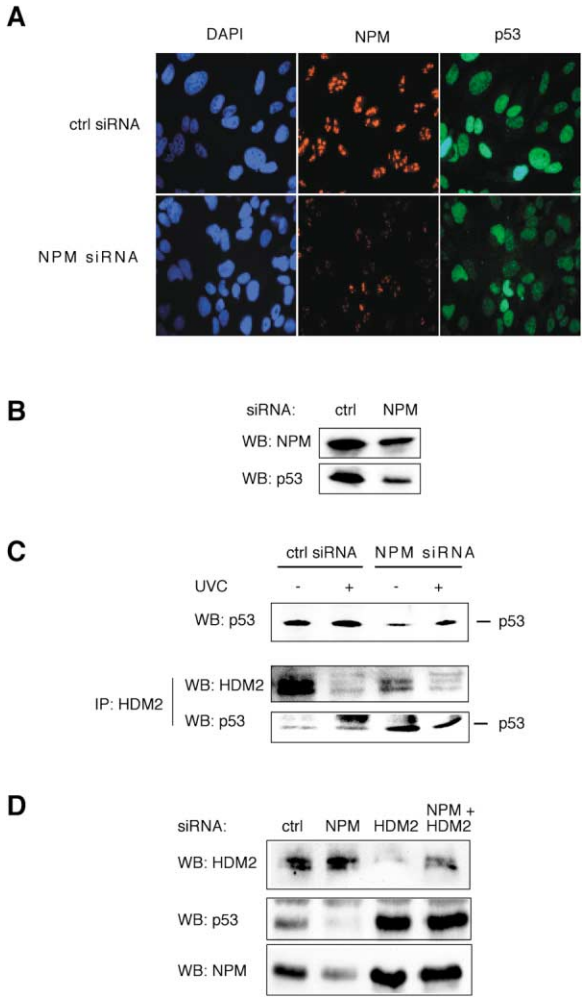

Figure 5. NPM is a negative regulator of p53-HDM2 interaction

A and B: U2OS cells were transfected with NPM or control siRNA duplexes and incubated for 3 days followed by detection of NPM and p53 by immunostaining or Western blotting.

C: Depletion of NPM decreases both basal and UV damage-induced levels of p53 and increases p53-HDM2 complex formation. NPM siRNA was carried out as in **A**. After 3 days, the cells were treated with UVC as indicated and were incubated for 3 hr. p53 and HDM2 or their complexes were analyzed by Western blotting and by coimmunoprecipitation.

D: Depletion of HDM2 rescues the negative regulation of p53 by NPM. U2OS cells were transfected with HDM2 or NPM siRNA duplexes and were incubated for one and three days, respectively, followed by detection of NPM, HDM2, or p53 by Western blotting. For depletion of both NPM and HDM2, the cells were first transfected with NPM RNAi duplexes and incubated for two days followed by transfection with HDM2 RNAi duplexes. Similar results were obtained with two sets of HDM2 RNAi duplexes.

their complexes (Figure 5). A decrease in NPM was verified by immunofluorescence and by Western blotting (by 40%) (Figures 5A and 5B). We found that depletion of NPM significantly decreased both stress-induced levels of p53 (Colombo et al., 2002) and p53 basal levels, suggesting that NPM acts both in the maintenance and stress-induced stabilization of p53 (Figures 5B and 5C). Further, the depletion of NPM led to a decrease in HDM2 (by 50%) and, remarkably, to an increase in HDM2-p53 complex formation (Figure 5C). The results suggest that NPM controls HDM2-p53 interaction and inhibits HDM2-p53 association.

To test whether the effect of NPM siRNA on p53 levels was balanced by depletion of HDM2, we proceeded to knock down either NPM, HDM2, or both by exposing U2OS cells to respective RNAi duplexes. Transfection of the cells with HDM2 RNAi duplexes led to a decrease in HDM2 and an increase in p53 levels as expected (Figure 5D). When the cells were transfected with duplexes targeting both NPM and HDM2, the decrease in p53 levels by NPM RNAi was negated, suggesting that depletion of HDM2 could effectively abrogate the decrease in p53 levels induced by NPM siRNA (Figure 5D). Surprisingly, HDM2 siRNA significantly increased the levels of NPM, suggesting that it acts either directly or indirectly as a negative regulator of NPM.

K cyclin binds NPM and affects its localization and interactions with HDM2 and p53

KSHV-encoded K cyclin, a viral cyclin D homolog, induces the stabilization of p53 and sensitizes mouse embryo fibroblasts to p53-dependent growth arrest and apoptosis (Verschuren et al., 2002). As NPM interacts with other viral proteins (HIV Rev and Tat), we wanted to address the possibility that p53 stabilization in K cyclin-expressing cells is mediated by NPM. U2OS cells were transfected with a K cyclin expression vector, and its effects on NPM, HDM2, and p53 were analyzed (Figure 6). Expression of K cyclin caused a major translocation of NPM from nucleoli to the nucleoplasm (Figure 6A). However, cyclin D2, the closest mammalian homolog, was without any effect on NPM localization (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/5/465/DC1). In K cyclin-expressing cells, the total levels of NPM were unaltered, suggesting that the increase in the NPM nucleoplasmic fraction is strictly due to its relocalization (Figures 6A and 6B). As demonstrated by coimmunoprecipitation analyses, K cyclin and NPM were found to form complexes (Figure 6C). As shown by Verschuren et al. (2002), the total level of p53 was increased in K cyclin-expressing cells (Figure 6D). In analogy to cells overexpressing NPM, K cyclin increased the levels of HDM2 and the interactions of HDM2 and NPM (Figure 6E) and p53-NPM (Figure 6D). However, less p53 was found to coprecipitate with HDM2 and correlated with the stabilization of p53 in K cyclin-expressing cells (Figure 6E). The results suggest that NPM interferes with the ability of HDM2 to mediate p53 degradation also in response to viral stress and reveal a novel mechanism by which p53 is stabilized following expression of an oncogenic viral protein.

Discussion

HDM2 and NPM interaction or colocalization increased consequent to divergent cellular stresses, like UV damage, proteasome inhibition, and expression of apoptosis-inducing viral K cyclin. UV radiation increased the level of NPM and affected its subcellular localization, causing alterations in p53, HDM2, and NPM complex formation and p53 stabilization (Figure 7). Our data indicates that p53 stabilization is augmented by the rapid UVC-induced nucleoplasmic translocation of NPM and an increase in nucleoplasmic p53-NPM and HDM2-NPM complexes. It is notable that the interaction between NPM and HDM2 or p53 was transient, taking place during 1–3 hr after the damage. Based on the data, we believe that the dynamic reorganization of the complexes allows early p53 stabilization and possibly

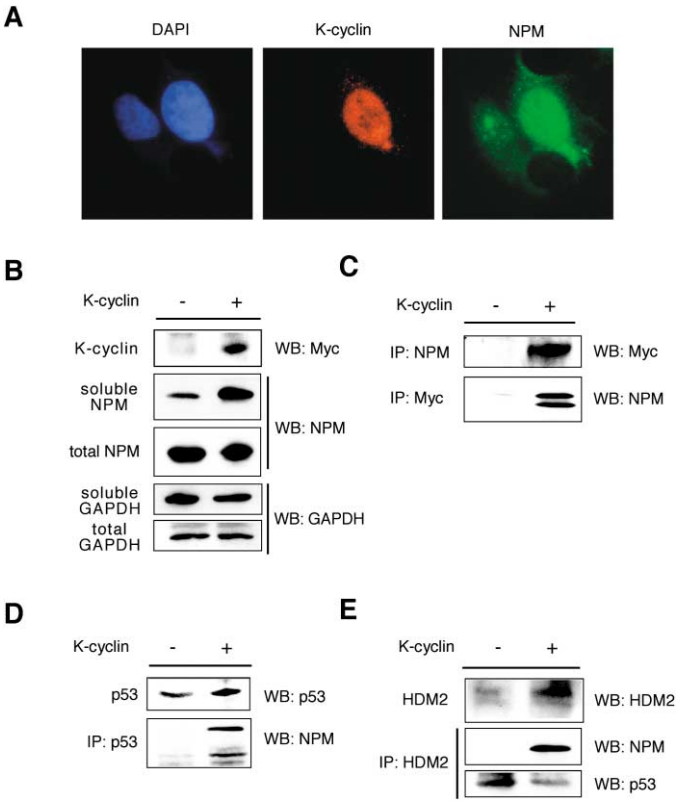

Figure 6. Viral K cyclin binds NPM, causes NPM redistribution, and affects p53-HDM2 complex formation

A: K cyclin redistributes NPM. U2OS cells were transfected with Myc-tagged K cyclin expression vector, and the cells were incubated for 48 hr followed by immunostaining for both NPM and K cyclin (anti-Myc).

B and C: K cyclin associates with NPM. U2OS cells were transfected with K cyclin expression vector as above. Total or soluble fractions of NPM and GAPDH as a loading control were analyzed as in Figure 1, or were analyzed for K cyclin associated NPM.

D and E: K cyclin increases p53 and HDM2 association with NPM and dissociates p53-HDM2 complexes. U2OS cells were transfected with K cyclin, and cellular lysates were analyzed for levels and interactions of p53 and HDM2.

assists in its modifications, like sumoylation commencing the subsequent stabilization effects. We find no evidence favoring subcellular trafficking of p53 to the nucleolus following NPM overexpression (Colombo et al., 2002), but cannot exclude its

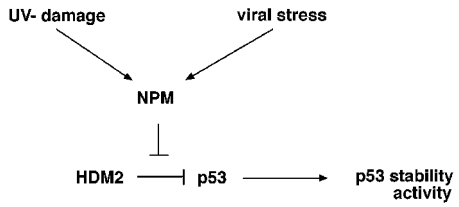

Figure 7. A model for NPM-mediated stabilization of p53 in DNA damage and viral stress

Both viral stress (K cyclin and adenovirus 5 expression [Matthews, 2001]) and DNA-damaging agents (UV radiation, cytotoxic drugs) cause nucleoplasmic translocation of NPM. Increased nucleoplasmic NPM associates with both p53 and HDM2 and prevents HDM2-mediated degradation of p53.

existence as the protein interactions and their mobility may be highly dynamic.

The continuous accumulation of nucleoplasmic NPM could be indicative of its other, possibly DNA damage repair-associated, functions. Several kinds of DNA-damaging agents, like doxorubicin, camptothecin, and actinomycin D, cause NPM translocation (Yung et al., 1985; Chan et al., 1987; Bor et al., 1992; Chan, 1992), suggesting that its relocalization closely associates with inhibition of RNA synthesis as also indicated in a recent study by Rubbi and Milner (2003). A common denominator for NPM translocation is ribosomal stress, caused either by direct inhibition of RNA polymerase I (actinomycin D) or by stalling of RNA polymerase II by bulky UV damage-caused lesions (Ljungman et al., 1999). Though NPM is a phosphoprotein, we have found no evidence that common stress-activated phosphorylation cascades (p38SAPK, PI3-K related kinases) would affect its localization (data not shown). Nucleolar proteins are highly mobile (Phair and Misteli, 2000), and other nucleolar proteins, like nucleolin, fibrillarin, and Hrad17, demonstrate similar DNA damage-provoked translocations (Chang et al., 1999; Daniely et al., 2002; Rubbi and Milner, 2003). Taken together, the above studies indicate that nucleolar proteins, perhaps signaled through alterations in ribosome biogenesis evoked by DNA damage and viral stress, are highly sensitive stress response proteins and participate in the subsequent cellular damage responses.

NPM bound to HDM2 N- and C termini, whereas it did not interact with the central domain. Thus, its binding could affect p53 stabilization by blocking the HDM2 RING domain with the E3 ligase activity. This effect may be augmented by binding of NPM to the HDM2 N terminus and thus hinder p53 interaction. Accordingly, we found that high levels of p53 competed for the interaction of NPM and HDM2 and that depletion of NPM increased HDM2-p53 complex formation. NPM acts as a molecular chaperone, with the tendency to bind denatured substrates, which exposes hydrophobic regions in the NPM-substrate complex, thus reducing protein aggregation (Szebeni and Olson, 1999). This property could be especially important in the highly crowded environment of the nucleolus and could be further employed in the nucleoplasmic compartment following UV damage of the cells. The chaperone activity of NPM could further assist p53 folding and stability. It is possible that there also exists selectivity, e.g., regulated by posttranslational modifications of NPM, HDM2, or p53, which determines the sites of interaction and types of complexes formed.

The multiple interactions of NPM with cellular proteins suggest that it can act as a platform for protein interactions, affect their stabilization, or allow protein modifications to take place. Thus, NPM could stabilize the complex formation between p53, HDM2, and other proteins needed for their posttranslational modifications in response to cellular stress. This is supported by the fact that NPM promotes p53 SUMO modification and that in response to UV radiation, a SUMO-modified p53 is found in complex with NPM prior to p53 stabilization. Whether NPM is essential for SUMO conjugation of p53 in cellular stress needs to be studied further. NPM could in this way have a dual function in regulation of p53 activity: by inhibiting p53 degradation through its interactions with HDM2, and by assembling complexes that are needed for p53 modifications augmenting its activation and stabilization.

Here we demonstrate by using both siRNA and overexpres-

sion approaches that NPM affects p53 stabilization through inhibition of its negative regulator, HDM2. Ectopic NPM increased the levels of both p53 and HDM2, whereas depletion of NPM by siRNA decreased both the basal and stress-induced levels of p53 and HDM2 and increased their interaction. The results indicate that NPM is required for the stabilization of p53 and that this is overcome by simultaneous depletion of HDM2 and NPM in UV-damaged cells. Moreover, though the amount of nucleoplasmic NPM was very low in the untreated cells, it was able to maintain the basal p53 levels as shown by NPM siRNA. In a similar manner, depletion of NPM by NPM antisense transfection has been shown to regulate the levels of PCNA both in control and UV-treated cells (Wu et al., 2002a). NPM antisense transfection also potentiates UV-induced cell death (Wu et al., 2002a, 2002b). NPM interacts with histones H3 and H4 and has the propensity to cause chromosome decondensation (Okuwaki et al., 2001), and could in this manner direct damage repair-related proteins to the sites of damage. Analogous to NPM, ribosomal L11 protein has been connected with p53 stabilization through inhibition of HDM2 (Lohrum et al., 2003; Zhang et al., 2003). This event is, however, promoted through nucleolar sequestration of HDM2 by L11 following inhibition of RNA polymerase I, and resembles mechanistically the nucleolar compartmentalization of HDM2 enforced by ARF in response to oncogenic stress (Sherr and Weber, 2000).

Viral K cyclin caused a pronounced translocation of NPM to the nucleoplasm and led to NPM interaction with K cyclin. NPM binds other viral proteins, like the human immunodeficiency virus proteins Tat (Li, 1997) and Rev (Szebeni and Olson, 1999). In fact, the nucleolus and its components may be targeted by several viruses, independent of their type or mechanism of replication (Hiscox, 2002), and therefore, NPM could be a more general mediator of viral stress responses. K cyclin expression led to NPM interaction with p53, HDM2, and K cyclin, and decreased p53-HDM2 complex formation. We therefore suggest that NPM-mediated inhibition of HDM2 is responsible for p53 stabilization in K cyclin-expressing cells. Based on the different electrophoretic migration patterns of NPM coprecipitating with K cyclin, p53, and HDM2, it is plausible that these represent differentially modified forms of NPM. Considering additionally that K cyclin is apoptotic (Ojala et al., 1999; Verschuren et al., 2002), that adenovirus infection inhibits the synthesis and processing of rRNA (Castiglia and Flint, 1983) and causes nucleoplasmic redistribution of NPM (Matthews, 2001), and that adenoviruses selectively kill cells with defective p53 function (McCormick, 2001), one should note that several oncogenic viruses may affect the p53 pathway by causing alterations in the nucleolar functions.

We propose that NPM is a major factor regulating the levels of p53 through HDM2 and possibly also the modification of p53. After exposure of cells to DNA damage or viral insult, NPM is translocated from the nucleoli to the nucleoplasm, where it binds HDM2, thereby inhibiting p53 degradation by affecting p53-HDM2 complex formation (Figure 7). How stress signal pathways are needed to modulate the interactions between HDM2, NPM, and p53 needs further studies. However, it seems clear that highly controlled regulation of NPM is essential and reflects the fate of p53 to either become stabilized or degraded. We find it highly significant that both oncogenic viruses and genomic damage employ a common nucleolar protein for this purpose. The control enforced by NPM may consequently be lost in tumors, or alternatively reflected by increased NPM expression like in melanoma (Bernard et al., 2003) or altered NPM-fusion products in promyelocytic leukemia (Alcalay et al., 2001). Clearly, further studies of expression and regulation of NPM in human tumors is warranted.

Experimental procedures

Plasmids

In order to generate GST-HDM2 constructs, regions encoding HDM2 amino acids 1–110, 108–200, 196–280, and 276–491 were amplified by PCR, using appropriate oligonucleotide primers, and a full-length cDNA encoding HDM2 as template. The PCR products were cloned into the bacterial expression vector pGEX-2T (Amersham). In a similar manner, the entire coding region of HDM2 was also fused downstream of the GST cDNA in pGEX-2T. B23-GFP (Dundr et al., 2000) expression vector was a kind gift from Dr. Mark Olson, and B231.1-pCHA and B231.2-pCHA were kindly provided by Dr. Kyosuke Nagata (Okuwaki et al., 2001). NPM-pcDNA3 was generated by EcoRI excision of NPM1.1 from B23-GFP and ligation to pcDNA3. Myc-tagged K cyclin expression vector was originally received from Dr. Sibylle Mittnacht (Ellis et al., 1999). SUMO-1 expression vector was from Dr. Jorma Palvimo. CRP1 cDNA was obtained from American Type Culture Collection (clone no. 6900545), and cloned into pGEX-4T to generate GST-CRP1.

Cells and transfections

SaOS-2 and U2OS osteosarcoma cells, WS-1 human fibroblasts, and *p53*$^{-/-}$*mdm2*$^{-/-}$ mouse embryo fibroblasts (Montes de Oca Luna et al., 1995) were cultured in a humidified atmosphere containing 5% CO_2 at 37°C and were transfected by either lipofection (Lipofectamine 2000, Invitrogen) or by electroporation (Gene Pulser II, Bio-Rad). Cells were treated with 35 J/m^2 UVC (Stratalinker 2400, Stratagene) and incubated for the given lengths of time.

siRNA

The siRNA duplex sequences used for depletion of NPM were as described by Colombo et al. (2002), and were purchased from Dharmacon Research, Inc. Scrambled NPM siRNA duplexes were used as a control for the siRNA reactions. HDM2 RNAi duplexes targeting HDM2 mRNA sequence 5′UGGU UGCAUUGUCCAUGGC3′ and SMARTpool HDM2 siRNA mix were purchased from Dharmacon Research, Inc. RNA duplexes (final concentration 70 nM) were transfected into the cells by lipofection according to manufacturers protocol (Oligofectamine, Invitrogen). Cells transfected with HDM2 and NPM siRNA duplexes were incubated for one and three days, respectively. A decrease in the respective protein levels was verified by immunofluorescence staining and Western blotting.

Immunofluorescence

Cells were fixed with 3.5% paraformaldehyde followed by permeabilization with 0.5% NP-40. For detection of HDM2, an antibody mix containing the following primary antibodies was used: IF-2 (Oncogene Sciences), SMP-14 (Santa Cruz Biotechnology), and 2A10. Other primary antibodies were as follows: NPM, C-19 (Santa Cruz Biotechnology) and mouse anti-nucleophosmin (Zymed); Myc-tag, 9E10 (Biosite); p53, FL393 (Santa Cruz Biotech). In coimmunostainings, swine anti-rabbit FITC, rabbit anti-goat FITC or rabbit anti-mouse TRITC (DAKO), or goat anti-mouse conjugated Alexa 594 or goat anti-rabbit conjugated Alexa 488 (Molecular Probes) were used as fluorochromes, and DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and RNA with Syto 12 green fluorescent nucleic acid stain (Molecular Probes). Absence of crossreactivity of the antibodies and conjugates was verified in separate experiments. The fluorochromes were visualized with Zeiss Axioplan 2 Imaging MOT (Jena, Germany) equipped with appropriate filters (Chroma) with 63× magnification, and images were captured with Zeiss Axiocam CCD-videocamera followed by image processing and multilayer analysis with AxioVision program version 3.0. Quantitation of the staining intensities was performed by KS Run 3.0 analysis program (KS 400, Zeiss) from 100 nuclei per time point.

Immunoprecipitation and immunoblotting

Cells were lysed into NP-40 lysis buffer containing 25 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethyl-

sulfonyl fluoride, 100 KIU/ml aprotinin, and 10 μg/ml leupeptin. Cellular NP-40 lysate was separated to insoluble and soluble fractions by centrifugation. The insoluble pellet fraction was boiled in LSB containing dithiothreitol (100 mM). To obtain total cellular lysates, cells were resuspended in urea-Tris buffer (9 M urea, 75 mM Tris-HCl [pH 7.5] and 0.15 M 2-mercaptoethanol) and sonicated briefly. Total protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein concentrations of NP-40 lysates were determined by Bio-Rad D_c protein assay kit (Bio-Rad, Hercules, CA) and after normalization of the protein concentrations, lysates were immunoprecipitated with specific antibodies (NPM, anti-nucleophosmin; HDM2, mix of IF-2, SMP-14, and 2A10; and p53, an antibody mix of DO-1, 421, and 1801). The immunocomplexes were collected on GammaBind-G Sepharose (Pharmacia Biotech). Cellular lysates or immunocomplexes were separated by 9% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Trans-Blot, Transfer Medium, Bio-Rad). Immunoblotting was carried out by using mouse anti-nucleophosmin (Zymed), the above HDM2 antibody mix, FL-393 (Santa Cruz Biotechnology) for p53, mouse anti-SUMO-1 (GMP-1, Zymed), mouse anti-ubiquitin (P4D1, Santa Cruz Biotechnology), GAPDH (Europa Bioproducts, Cambridge, UK), and monoclonal anti-GST (clone BC8E8), followed by secondary antibodies containing horseradish peroxidase (HRP), after which the proteins were detected with enhanced chemiluminescence (ECL, Amersham Life Sciences).

In vitro translation and competition assays

HDM2, p53, and NPM were translated in vitro (TnT coupled Reticulosyte lysate system, Promega) in the presence of 20 μCi of ^{35}S-methionine (specific activity 1000 Ci/mmol, Amersham) when indicated, mixed, and incubated for 90 min at 30°C. In competition assays, in vitro translated p53 and HDM2 were mixed with a lysate from *p53*$^{-/-}$*mdm2*$^{-/-}$ murine embryo fibroblasts and the amounts of reticulosyte lysate added to the reactions were balanced to equal volumes. Subsequently, the mixes were precipitated with HDM2 antibodies followed by analyses of the protein complexes by Western blotting.

GST pulldown assays

The encoded HDM2 GST-fusion proteins and full-length HDM2 and irrelevant GST-protein control (GST-CRP1) were expressed in BL-21 cells following induction with IPTG. The fusion proteins were captured on glutathione-Sepharose 4B beads (Amersham) for the GST pulldown experiments, and in vitro translated ^{35}S-NPM was added. Binding was performed in TNE-buffer (140 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 1 mM PMSF) overnight under rotation at +4°C, and the beads were washed ten times with TNE-buffer and finally with PBS. Beads were boiled in 1× LSB containing dithiothreitol (100 mM) for 5 min, and the supernatant was loaded to gels followed by autoradiography. The amount of GST-HDM2 fusion products and GST-controls used in the reactions were controlled by Western blotting of parallel samples with antibody against GST.

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