Nucleolar disassembly occurs during mitosis and nucleolar stress, releasing several MDM2-interactive proteins residing in the nucleolus that share the common activity of p53 stabilization. Here, we demonstrate that mobilization of nucleostemin, a nucleolar protein enriched in cancer and stem cells, has the opposite role of stabilizing MDM2 and suppressing p53 functions. Our results show that nucleostemin increases the protein stability and nucleoplasmic retention of MDM2, and competes with L23 for MDM2 binding. These activities were significantly elevated when nucleostemin is released into the nucleoplasm by mutations that abolish its nucleolar localization or by chemotherapeutic agents that disassemble the nucleoli.

Nucleostemin was isolated as a gene enriched in neural stem cells (NSCs) but not in their differentiated progeny (Tsai and McKay, 2002). It encodes a nucleolar GTP-binding protein abundantly expressed in cancer and stem cells, and is required for maintaining the proliferation of embryonic NSCs and human cancer cells in vitro, as well as for early embryogenesis (Beekman et al., 2006; Zhu et al., 2006). The mechanism underlying the nucleostemin activity is not completely understood, but is indicated by its ability to bind and regulate p53 (Tsai and McKay, 2002) and telomeric repeat binding factor 1 (Zhu et al., 2006). The molecular basis of a nucleolar-related p53 regulation began to emerge when several nucleolar proteins were shown to exhibit the ability to bind MDM2 and stabilize p53. ARF (alternative reading frame), PML (promyelocytic leukemia protein), B23, L5, L11 and L23 all enhance p53 stability by inhibiting or sequestering MDM2 in the nucleolus (Bernardi et al., 2004; Dai et al., 2004; Jin et al., 2004; Kurki et al., 2004; Tao and Levine, 1999; Zhang et al., 2003).

A number of studies investigate the relationship between nucleostemin and p53, and show that knocking down the expression of nucleostemin increases the level of p53 (Ma and Pederson, 2007) and that the early embryonic lethal phenotype of nucleostemin-null mice cannot be rescued by p53 deletion (Beekman et al., 2006). Questions remain regarding how nucleolar nucleostemin and nucleoplasmic p53 come into contact with each other and what the molecular connection between these two proteins in tumor cells is. The association of nucleostemin and p53 in living cells can be envisaged in several ways. First, nucleostemin shuttles between the nucleolus and nucleoplasm in a GTP-driven cycle, thus allowing nucleostemin to interact with proteins residing in the nucleoplasm (Tsai and McKay, 2005). P53 has also been found in the active site of transcription within the nucleolus (Rubbi and Milner, 2000). In addition, nucleostemin can be relocated to the nucleoplasm upon nucleolar disassembly during mitosis or induced by drugs that block the RNA polymerase activity or de novo GTP synthesis. Finally, the interaction between nucleostemin and p53 might be mediated by other unidentified proteins.

While investigating the role of nucleostemin in p53 regulation, we discovered that the association between nucleostemin and p53 is mediated by MDM2, and we explored the mechanistic and biological relevance of the nucleostemin-MDM2 interaction. Upon completion of this work, another study was published that reported the same interaction between nucleostemin and MDM2 (Dai et al., 2008), but showed that both overexpression and knockdown of nucleostemin led to the same phenotypes of p53 activation, MDM2 upregulation and G1-S cell-cycle arrest, and that these findings depended on the L5 and/or L11 interaction with MDM2. In this study, we showed that the nucleostemin-MDM2 interaction occurs mainly when nucleolar nucleostemin is mobilized into the nucleoplasm in living cells. Nucleoplasmic relocation of
nucleostemin increases its MDM2 binding and the nucleoplasmic retention of MDM2. Contrary to the effect of other MDM2-interactive nucleolar proteins, nucleostemin is able to (1) stabilize MDM2 by preventing its ubiquitylation, (2) compete with L23 for MDM2 binding, and (3) lower the transcriptional activity of p53. Further analyses reveal a role of nucleostemin in promoting the G2-M transit and cell survival in U2OS cells.

Results
MDM2 binds nucleostemin independently of p53, and mediates association of nucleostemin and p53
To define the interaction between nucleostemin, MDM2 and p53, HEK293 cells were triple-transfected with HA-tagged nucleostemin, Flag-tagged MDM2, and/or Myc-tagged p53 expression plasmids, and immunoprecipitated with anti-tag antibodies. Although all three proteins showed up in the same protein complexes in the triple-transfected cells (Fig. 1A1), the binding between nucleostemin and p53 in the double-transfected cells was significantly reduced (Fig. 1A2). By contrast, the nucleostemin-MDM2 and MDM2-p53 interactions were unaffected by the coexpression of p53 or nucleostemin, respectively (supplementary material Fig. S1A; Fig. 1A1). We confirmed the in vivo binding of nucleostemin and MDM2 by showing that the endogenous nucleostemin and MDM2 coexisted in the same protein complexes in U2OS cells (Fig. 1B). These results demonstrate that MDM2 mediates part of the binding between nucleostemin and p53.

Binding of MDM2 and nucleostemin requires the central domain of MDM2 and the coiled-coil and acidic domains of nucleostemin
To map the nucleostemin-binding domains of MDM2, non-overlapping deletions were made on MDM2 that correspond to its p53-binding (N, residues 1-108), intermediate-1 (I1, residues 109-222), acidic-zinc finger (AZ, residues 223-322), intermediate-2 (I2, residues 323-434), and RING-finger domains (R, residues 435-491) (Fig. 1C, top). Coimmunoprecipitation assays of Myc-tagged MDM2 mutants and HA-tagged nucleostemin showed that deleting the I1-domain (dI1) or the AZ-domain (dAZ) of MDM2 reduced its ability to bind nucleostemin (Fig. 1D1). To define the MDM2-interactive domain of nucleostemin, nucleostemin mutants deleted of the basic (B, residues 1-46), basic-coiled-coil (BC, residues 1-115), GTP-binding (G, residues 116-283), intermediate (I, residues 284-464), or acidic (A, residues 465-549) domain, as well as a single-residue mutant (G256V) lacking the GTP-binding and nucleolus-targeting capabilities, were generated (Fig. 1C, bottom). Coimmunoprecipitation assays of Myc-tagged MDM2 and HA-tagged nucleostemin mutants by anti-HA (Fig. 1D2) or anti-Myc antibody (supplementary material Fig. S1B) both demonstrated that deleting either the BC domain or the A domain of nucleostemin reduced its ability to bind MDM2, whereas deletion of the B domain (dB) alone did not. These findings indicate that the nucleostemin-MDM2 binding requires the central region (residues 109-322) of MDM2 and the C domain (residues 47-115) and the A domain of nucleostemin.

Nucleostemin binds MDM2 in the nucleoplasm and increases the nucleoplasmic retention of MDM2
Next, we used the BiFC (bimolecular fluorescence complementation) assay to show the actual formation of nucleostemin and MDM2 complexes in living cells. BiFC involves coexpression of two potentially interacting proteins fused individually to the N-terminal (VN173, Yn) or the C-terminal domain (VC155, Yc) of the Venus variant of yellow fluorescent protein (YFP), and measures the

Fig. 1. MDM2 mediates the association between nucleostemin and p53 via the central domain of MDM2 and the coiled-coil and acidic domains of nucleostemin. (A1) Triple-coimmunoprecipitation assays of HA-tagged nucleostemin (NS), Flag-tagged MDM2 and Myc-tagged p53 show that nucleostemin, MDM2 and p53 coexpress in the same protein complex. (A2) Binding between nucleostemin and p53 is significantly reduced without MDM2 coexpression. (B) In vivo binding of endogenous nucleostemin and MDM2 is confirmed by coimmunoprecipitation assays, which immunoprecipitated MDM2 or nucleostemin complexes from U2OS cells. (C) MDM2 (top) and nucleostemin (bottom) deletion mutants. Gray lines indicate the deleted regions. (D) Coimmunoprecipitation assays showed that nucleostemin fails to bind MDM2 mutants with deleted I1 or AZ domains (D1), and that deleting the A domain or the BC domain of nucleostemin abolishes its ability to bind MDM2 (D2). Abbreviations: A, acidic domain; AZ, acidic/zinc finger; B, basic domain; C, coiled-coil domain; G, GTP-binding domain; I, intermediate; N, p53-binding domain; R, RING finger; Sup, supernatant; WB, western blot.
reconstitution of a functional YFP complex when the interactive protein pairs bring the Yn and Yc fragments into close proximity (Fig. 2A1). In our experiments, HeLa cells were cotransfected with plasmids encoding the Yn- and Yc-fused proteins and a nucleolar localization signal (NoLS)-tagged cyan fluorescent protein (noCFP). The BiFC efficiencies were measured by counting the percentages of YFP+ cells in the CFP+ population by fluorescence-activated cell sorting (FACS) analyses. Whereas a 48.7% BiFC efficiency was observed between wild-type MDM2 and nucleostemin, the nucleostemin mutant lacking the BC and A domains (NS-GI) displayed only a 27.2% BiFC efficiency with the wild-type MDM2. The BiFC efficiencies between the wild-type nucleostemin and the MDM2 mutants lacking the AZ-domain (dAZ) or the I1- and AZ-domains (dIAZ) were reduced to 15% and 9.4%, respectively (Fig. 2A2). Western blots showed that the expression levels of wild-type and mutant Flag-tagged MDM2-Yn (or Myc-tagged NS-Yc) were the same (supplementary material Fig. S2A), excluding the possibility that the observed findings were caused by different expression levels of the fusion proteins.

Because the BiFC binding is irreversible, we applied the FLIP (fluorescence loss in photobleaching) approach to determine the dynamic interaction between nucleostemin and MDM2 in living cells. The FLIP paradigm was set up to measure the rate of fluorescence loss in the nucleoplasm while bleaching one nucleolus with repetitive bleaching pulses. The validity of using the C-terminally GFP-fused MDM2 to track the distribution of endogenous MDM2 protein was verified by results showing that the C-terminally GFP-fused MDM2, similar to the wild-type protein, was able to reduce p53 protein (supplementary material Fig. S2C) (P=0.95, Repeated Measures ANOVA). FLIP analyses demonstrated that coexpression of wild-type nucleostemin (mean decay half-time, \( T_{1/2}=51.5 \) seconds), dB (\( T_{1/2}=47.4 \) seconds), G256V (\( T_{1/2}=56.0 \) seconds), or dB(256) mutant (\( T_{1/2}=69.5 \) seconds) all increased the nucleoplasmic retention time of MDM2 compared to the control-transfected cells (\( T_{1/2}=37.0 \) seconds) (Fig. 2B) (P<0.0001 for all). Among them, the dB(256) mutant had the most ability to retain
MDM2 in the nucleoplasm ($P<0.0001$ compared with wild-type nucleostemin and dB; $P<0.01$ compared with G256V).

To demonstrate that this MDM2-retaining effect by overexpressing wild-type and mutant nucleostemin proteins can also be seen with the native nucleostemin protein, we used doxorubicin (ADR, 2 μM for 4 hours) and mycophenolic acid (MPA, 40 μM for 4 hours) to mobilize the endogenous nucleostemin from the nucleolus to the nucleoplasm. ADR and MPA trigger nucleolar stress by inactivating the transcriptional activity and blocking de novo GTP synthesis, respectively. Our FLIP results showed that when cells were exposed to ADR or MPA, their nucleoplasmatic retention time of MDM2 was significantly prolonged (blue traces; $T_{1/2}=59.4$ seconds and 68.0 seconds for ADR- and MPA-treated cells, respectively) compared with that of mock-treated samples (black traces; $T_{1/2}=37.2$ seconds) ($P<0.0001$ for both drugs) (Fig. 2C, left panel for ADR treatment and right panel for MPA treatment). To determine how much of this drug-induced increase of MDM2 nucleoplasmic retention is mediated by nucleostemin translocation, we compared the drug effects between control (siScr, blue traces) and nucleostemin-knockdown (siNS, red traces) cells. Our results showed that knocking down the endogenous nucleostemin reverses a major portion of this drug-induced MDM2 retention ($T_{1/2}=44.7$ seconds and 47.7 seconds for ADR and MPA-treated cells, respectively) ($P<0.0001$ for both). By contrast, nucleostemin knockdown in mock-treated cells did not affect the nucleoplasmic retention time of MDM2 significantly (supplementary material Fig. S2D) ($P=0.97$). These results demonstrate that the interaction between nucleostemin and MDM2 occurs when the endogenous nucleostemin is released from the nucleolus to the nucleoplasm, and that nucleostemin binding increases the nucleoplasmic residence of MDM2.

Nucleostemin increases MDM2 protein by decreasing its degradation and ubiquitylation

To address the functional importance of nucleostemin-MDM2 binding, we first asked how nucleostemin affects the protein level of MDM2. H1299 cells were cotransfected with the same amount of MDM2 and increasing amounts of nucleostemin expression plasmids. MDM2 protein levels were compared between different samples after normalization to a coexpressed GFP control. Western

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Nucleostemin increases the protein level of MDM2 by preventing its degradation and ubiquitylation. (A) Coexpression of nucleostemin (HA-tagged) increased the protein levels of exogenously expressed MDM2 in a dose-dependent manner. (B) Knocking down the expression of nucleostemin with nucleostemin-targeting shRNA constructs (shNS-1 and shNS-2) decreased the amount of MDM2 protein. (C) MDM2 protein stability, with or without nucleostemin overexpression (NS vs Ctrl), was measured in H1299 cells. After cycloheximide treatment, cell lysates were collected from 0 to 4 hours at 0.5-1 hour intervals. The amount of MDM2 protein at every time point was measured in three independent experiments, adjusted based on their α-tubulin amounts, and expressed as a percentage of the MDM2 at the 0 time-point. (D) Nucleostemin depletion by shNS-2 cotransfection increased the degradation of MDM2. Protein degradation assays were performed over a 50 minute window. (E) HEK293 cells were transfected with (His)6-tagged ubiquitin, MDM2 and/or nucleostemin (wild-type or mutant) plasmids as specified. Ubiquitylated MDM2 products were pulled down by Ni²⁺ Sepharose (His PD) and detected by anti-MDM2 (SMP14) antibody. Overexpression of wild-type nucleostemin slightly decreased the polyubiquitylation of MDM2 compared with the control sample. Protein degradation assays were performed over a 50 minute window. (F) Conversely, depleting the endogenous nucleostemin by the nucleostemin-targeting siRNA (siNS) increased the ubiquitylation of MDM2 compared with cells treated with a scrambled siRNA (siScr).
blots showed that MDM2 protein was increased by coexpression of wild-type nucleostemin in a dose-dependent manner (Fig. 3A). The ability to increase MDM2 protein was abolished by deleting the MDM2-binding domains of nucleostemin (supplementary material Fig. S3A) but preserved in the nucleoplasmic mutants (supplementary material Fig. S3B). To confirm that the MDM2 protein level is also regulated by the endogenous nucleostemin, two micro-RNA-adapted short hairpin RNA constructs (shRNAmir) were created that exhibited a 54% (shNS-1) and 84% (shNS-2) knockdown efficiency of nucleostemin protein (Fig. 3B, bottom panel). Compared with the sample treated with a scrambled shRNAmir construct (shScr), cells transfected with the shNS-1 or shNS-2 construct had reduced amounts of exogenous MDM2 protein at levels comparable to their nucleostemin knockdown efficiencies (Fig. 3B, top panel). Nucleostemin depletion also showed the same effect on endogenous MDM2 (Fig. 5A). Since the expressions of exogenous MDM2 and GFP were both driven by the same EF1α promoter, we reasoned that the nucleostemin effect on MDM2 protein must occur post-transcriptionally, and tested this idea by measuring the protein stability of MDM2 in nucleostemin-perturbed cells. For overexpression experiments, H1299 cells were transfected with MDM2 and with or without nucleostemin expression plasmid. Thirty-six hours after transfection, cells were treated with cycloheximide (CHX, 100 μg/ml), and lysates were collected at 0.5-1 hour intervals. Western analyses showed that MDM2 in the nucleostemin-overexpressing cells was degraded much more slowly than that in the control cells (Fig. 3C) (P<0.0001 by Repeated Measures ANOVA). To confirm these findings by the knockdown approach, the protein stability of MDM2 was measured in the shNS-2 and shScr-transfected cells, and was found to be decreased upon nucleostemin depletion (Fig. 3D) (P=0.04).

MDM2 protein is degraded by the ubiquitin-proteasome-mediated mechanism. To determine how nucleostemin influences the ubiquitylation of MDM2, in vivo ubiquitylation assays were conducted in which HEK293 cells were transfected with (His)₆-tagged ubiquitin, MDM2 and nucleostemin (wild-type or mutant) expression plasmids. Ubiquitylated proteins were captured from protein extracts by Ni²⁺-chelating Sepharose. Anti-MDM2 western blots showed that overexpression of wild-type nucleostemin, but not the non-MDM2-binding dA mutant, slightly and consistently reduced the amount of ubiquitylated MDM2 in the pull-down fraction (Fig. 3E). Notably, this activity of nucleostemin was significantly enhanced in the nucleoplasmic mutants of nucleostemin NSdB, G256V and dB(256). Confirming these findings, knocking down the endogenous nucleostemin by a

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**Fig. 4.** Nucleoplasmic nucleostemin competes with L23 for MDM2 binding. (A1) Triple coimmunoprecipitation of nucleostemin (HA), MDM2 (Myc) and L23 (Flag) by the indicated antibodies (IP) showed that L23 coexpression reduces the interaction between nucleostemin and MDM2. Double coimmunoprecipitation shows that L23 interacts with MDM2 (A2) but not with nucleostemin (A3). (B) The ability of nucleostemin to bind MDM2 in the presence of L23 overexpression is significantly increased by the combined mutations of dB and G256V [dB(256)]. (C) The dB(256) mutant is capable of competing with L23 for MDM2 binding in the triple coimmunoprecipitation experiments. MDM2 protein complexes (top panel) immunoprecipitated from lysates (bottom panel) containing the same amount of L23 and increasing amounts of dB(256). (D) Both nucleostemin (HA) and L23 (Flag) reside primarily in the nucleus under normal growth conditions (Ctrl). When exposed to ADR (2 μM), actinomycin D (ActD, 0.05 μg/ml) and MPA (40 μM) for 4 hours, L23 is redistributed to the nucleolus, as is nucleostemin, but to a lesser extent. Distribution of nucleostemin and L23 is shown in different ADR-treated cells because of the autofluorescent property of ADR. Scale bar: 10 μm. (E) Compared with mock-treated cells, ADR and MPA significantly increased the binding between nucleostemin and MDM2. ActD had a lesser effect. (F) Proteins were extracted from cells coexpressing HA-tagged nucleostemin, Myc-tagged MDM2 and Flag-tagged L5, L11 or L23, and immunoprecipitated by anti-Myc antibody. Western blots showing that L23 competes with nucleostemin for MDM2 binding better than L5 and L11.
nucleostemin-targeting siRNA duplex (siNS) (Tsai and McKay, 2002) increased the ubiquitilation of MDM2 (Fig. 3F). These data demonstrate that nucleostemin stabilizes MDM2 by reducing its ubiquitilation, and such activity is more evident with the nucleoplasmic mutants of nucleostemin than with the wild-type protein.

Nucleoplasmic nucleostemin competes with ribosomal protein L23 for MDM2 binding

The nucleostemin-interactive domain of MDM2 overlaps with its binding sites for L5, L11 and L23. Here, we used L23 as an example to determine the MDM2-binding relationship between nucleostemin and this group of proteins. HEK293 cells were triple-transfected with HA-tagged nucleostemin, Myc-tagged MDM2 and Flag-tagged L23 plasmids. Protein complexes were immunoprecipitated by anti-tag antibodies. Compared with the samples expressing only nucleostemin and MDM2 (supplementary material Fig. S1A1), the interaction between nucleostemin and MDM2 was significantly reduced when L23 was coexpressed (Fig. 4A1). Binding between MDM2 and L23 did not require coexpression of wild-type nucleostemin (Fig. 4A2), and no direct interaction was detected between nucleostemin and L23 (Fig. 4A3), indicating that in normal growing cells, more MDM2 proteins are bound by L23 than by nucleostemin. As most nucleostemin proteins are localized in the nucleolus in the interphase cells and only bind MDM2 when it is translocated into the nucleoplasm, we next examined the abilities of the three nucleoplasmic mutants of nucleostemin [dB, G256V, and dB(256)] to compete with L23 for MDM2 binding. Triple coimmunoprecipitation experiments revealed that dB(256) had the strongest activity to bind MDM2 in the presence of L23 (Fig. 4B), consistent with its stronger ability to change the nucleoplasmic retention and ubiquitylation of MDM2 than that of wild-type nucleostemin, dB and G256V. To determine whether dB(256) can compete with L23 for MDM2 binding, MDM2 protein complexes were immunoprecipitated by anti-MDM2 antibody from cells expressing the same amount of L23 but different levels of dB(256). The coimmunoprecipitation results showed that increased binding of dB(256) to MDM2 reduced the amount of L23 bound by MDM2 (Fig. 4C).

To confirm that the increased MDM2 binding by dB(256) can also be seen with the native nucleostemin protein, we used ADR (2 µM, 4 hours), actinomycin D (ActD, 0.05 µg/ml, 4 hours) and MPA (40 µM, 4 hours) to mobilize the endogenous nucleostemin from the nucleolus to the nucleoplasm based on the previously described rationale, and measured the coimmunoprecipitation efficiency between nucleostemin and MDM2. Confocal analyses showed that these drugs trigger nucleoplasmic translocation of both nucleostemin and L23. Notably, the nucleoplasmic relocation of nucleostemin was more sensitive to these treatments than that of L23 (Fig. 4D). To test the drug effects on nucleostemin-MDM2 binding, coimmunoprecipitation assays were performed in cells triple-transfected with MDM2, nucleostemin and L23 plasmids, treated with ADR, ActD or MPA, and immunoprecipitated by anti-Myc antibody for MDM2. Western blots showed that the coimmunoprecipitation efficiency between nucleostemin and MDM2 was increased by these drugs even in the presence of L23, and that this effect was more significant in the ADR and MPA-treated samples than in the ActD-treated sample (Fig. 4E). To determine whether L5 and L11 show the same activity as L23 in competing with nucleostemin for MDM2 binding, triple coimmunoprecipitation experiments were performed, which showed that L23 competes with nucleostemin for MDM2 binding better than L5 and L11 (Fig. 4F). This result indicates that the relationships between nucleostemin, MDM2 and these three ribosomal proteins are not exactly the same.

Nucleostemin depletion reduces MDM2 protein and increases p53 transcriptional activity without changing p53 protein level

To address how nucleostemin might affect the protein level and transcriptional activity of p53, U2OS and H1299 stable cell lines with doxycycline (Dox)-inducible nucleostemin knockdown capabilities were established (supplementary material Fig. S4A), both of which displayed comparable knockdown efficiencies of nucleostemin proteins after Dox treatment (Fig. 5A; supplementary material S4B). Cell lysates were collected from control (shScr) and nucleostemin-knockdown (shNS) U2OS cells, receiving no treatment or Dox (20 µg/ml) treatment for 4, 7 or 10 days. Compared with the non-treated cells, the Dox-treated U2OS-shNS cells showed a time-dependent reduction of nucleostemin protein along with a decrease in MDM2 protein, whereas the U2OS-shScr cells did not (Fig. 5A). Although the p53 protein level was unchanged, its transcriptional activity, as assessed by two of its transcriptional targets (p21 and Bax), was upregulated. The increase in Bax expression was paralleled by elevated protein levels of cleaved caspase-3, the convergent point of both the intrinsic and extrinsic cell death pathways. These results demonstrate that nucleostemin depletion decreases MDM2 protein and enhances the transcriptional activity without changing the protein level of p53.

Nucleostemin promotes cell survival and G2-M transit during nucleolar stress

To determine the biological functions of nucleostemin, we measured the cell proliferation rates of nucleostemin-depleted U2OS (p53-wild-type) and H1299 (p53-null) cells. The population-doubling levels (PDLs) were calculated daily over a 6-day period using the formula: \[ \Delta \text{PDL} = \log(n_f/n_0)/\log2, \] where \( n_0 \) is the initial number of cells and \( n_f \) is the final number of cells. The time (in days) for one population doubling was calculated as \( 1/\Delta \text{PDL} \). Our results showed that nucleostemin-depleted U2OS cells had a longer doubling time compared with shScr cells and non-treated shNS cells (Fig. 5B, black bars). The doubling time of non-treated U2OS-shNS cells was slightly longer than that of U2OS-shScr cells, indicating a possible leakage expression of the shNS-2 construct before Dox treatment. In contrast to the inhibitory effect of nucleostemin knockdown on the proliferation of U2OS cells, nucleostemin depletion did not slow down the proliferation rate of H1299 cells (Fig. 5B, grey bars), suggesting that the ability of nucleostemin to promote cell proliferation might be partially mediated by a p53-dependent mechanism in human cancer cells.

A reduced PDL can be caused by an increase in cell death, cell-cycle arrest or elongation of cell-cycle length. These possibilities were addressed by propidium-iodide-labeled cell-cycle analyses of control (shScr) and nucleostemin-knockdown (shNS) U2OS cells (Fig. 5C). Before Dox treatment, the S-phase cell percentage of the U2OS-shNS culture was lower and its sub-G1-cell percentage was higher than that of the U2OS-shScr culture, consistent with low expression of shNS-2 before Dox induction. After Dox treatment for 7 or 10 days, the nucleostemin-knockdown cells displayed lower G1-G0 cell percentages (\( P<0.01 \)) and higher G2-M cell percentages (\( P<0.001 \)) compared with the time-matched shScr cells, indicating cell-cycle arrest at the G2-M stage. Most significantly, nucleostemin depletion increased the percentage of sub-G1 (apoptotic) cells (\( P<0.001 \). To
determine whether the G2-M arrest occurs before, during or after mitosis, prophase cells with condensed chromatin and anti-phospho-Histone H3 labeling were measured in the 7 and 10 day Dox-treated shScr and shNS cultures (Fig. 5D). We found that the nucleostemin-knockdown culture contained more G2-M-phase cells but fewer prophase cells than did the control culture, suggesting that the G2-M arrest occurs before the mitotic entry. These results indicate that nucleostemin depletion blocks mitotic entry and triggers apoptosis, which was not seen in the nucleostemin-knockdown H1299 cells (supplementary material Fig. S4C).

One notable change in the nucleoli during cell-cycle progression is that they dissemble during prophase and reform at the late stage of mitosis, suggesting that in normal dividing cells, nucleostemin mainly interacts with MDM2 during mitosis when nucleostemin is released from the nucleolus. To test this idea, binding of endogenous MDM2 and nucleostemin was examined by coimmunoprecipitation experiments in S-phase- or M-phase-synchronized U2OS cells. Coimmunoprecipitation results showed that the interaction between nucleostemin and MDM2 was increased in the mitotic cells compared with the S-phase cells or the non-synchronized cells (Fig. 5E), supporting the idea that the nucleostemin-mediated MDM2 binding and stabilization mainly occur during mitosis in non-stressed cells. Nucleoplasmic relocation of nucleostemin also occurs during nucleolar stress induced by ADR and ActD or during MPA-triggered GTP depletion. To demonstrate that cells with more nucleostemin will be better protected from drug-induced cell death or cell-cycle arrest than cells with less nucleostemin, nucleostemin-overexpression U2OS stable cells (NS#12) (Zhu et al., 2006) and vector-transfected U2OS stable cells (grey bars) were exposed to ActD (0.05 μg/ml) or MPA (40 μM) for 18 hours and their cell-cycle profiles were analyzed. Here, we did not use ADR because the autofluorescence of ADR overlapped with that of propidium iodide. In mock-treated cells, ActD treatment triggered cell-cycle arrest within the S-phase, and both ActD and MPA increase the apoptotic cell percentages (sub-G1). In non-treated cultures, nucleostemin overexpression arrested cells at the G1-S stage. Notably, overexpression of nucleostemin reduced ActD- or MPA-induced cell death and reversed ActD-dependent S-phase delay. These findings show that nucleostemin-overexpressing cells are better protected from cell death induced by drugs that trigger nucleolar stress or GTP depletion.

**Discussion**

This work identifies MDM2 as a nucleostemin-binding protein responsible for most of the nucleostemin-p53 interaction, and locates their protein-binding sites in the central acidic-zinc finger domain of MDM2 and the coiled-coil and acidic domains of nucleostemin. Our findings on the interactive domains of these two proteins are largely consistent with the results described by the recent study on nucleostemin-MDM2 interaction (Dai et al., 2008), except for the involvement of the acidic domain of nucleostemin. We show further evidence for the association between nucleostemin and MDM2 in living cells and determine that their binding occurs in the nucleoplasm. As a result of this interaction, MDM2 protein degradation and ubiquitylation are reduced. A major role of MDM2 is to suppress p53 function by either increasing its protein degradation or directly inhibiting its transcriptional activity. Our data demonstrate that loss of nucleostemin enhances the transcriptional activity without changing the p53 protein level in vivo and triggers G2-M arrest and cell death in U2OS cells.
Mobilized nucleostemin regulates MDM2 protein stability and p53 activity

In interphase cells, MDM2 is localized in the nucleoplasm and nucleostemin resides in the nucleolus. Nucleolar sequestration of MDM2 has been proposed as a potential mechanism that controls its activity via association with several nucleolar proteins, including ARF, L5, L11 and L23. Our findings show that MDM2 binding of nucleostemin does not require the nucleolar distribution of nucleostemin. In fact, the nucleoplasmic mutants of nucleostemin show stronger activities in binding and retaining MDM2 in the nucleoplasm (Fig. 2B, Fig. 4B) and inhibiting MDM2 ubiquitylation (Fig. 3E) than wild-type nucleostemin does. In addition, overexpression of nucleostemin does not promote the nucleolar accumulation of MDM2 (data not shown). Based on this, we conclude that the nucleostemin-mediated regulation of MDM2 occurs when nucleostemin is mobilized from the nucleolus, which happens during mitosis or nucleolar stress. Under normal growth conditions, the majority of cells are in interphase, and therefore, their nucleostemin proteins are inactive in stabilizing MDM2. This might account for why overexpression of wild-type nucleostemin or knockdown of endogenous nucleostemin showed only a mild but reproducible effect on MDM2 ubiquitylation in non-synchronized cultures.

Nucleostemin depletion increases the transcriptional activity of p53 but not its protein level, indicating that this nucleostemin-mediated MDM2 stabilization might regulate p53 function by direct inhibition rather than a ubiquitylation-dependent mechanism. Because MDM2 serves as an E3 ubiquitin ligase for itself and for p53, the activity of nucleostemin to regulate the ubiquitylation of MDM2 might at the same time affect the ability of MDM2 to ubiquitylate p53, which might explain why a decrease in MDM2 upon nucleostemin knockdown does not lead to an increase in p53 protein. An alternative mechanism for nucleostemin-mediated p53 inhibition is by neutralizing the p53-stabilizing activity of L23. In normal cultures, more MDM2 proteins were bound by L23 than by nucleostemin. When cells are exposed to stimuli that release nucleostemin from the nucleolus to the nucleoplasm, the overall binding between nucleostemin and MDM2 increases, even though some L23 proteins also relocate to the nucleoplasm in the same process. Such findings may be caused by the differential sensitivities of nucleostemin and L23 to drug-induced nucleoplasmic translocation (Fig. 4D). Another explanation is that the MDM2 binding of nucleostemin is highly regulated by both its GTP-binding status and nucleolar distribution, whereas a measurable amount of MDM2-L23 interaction already occurs at the baseline level in the nucleoplasm and cytoplasm (Dai et al., 2004; Jin et al., 2004). Finally, MDM2 is known to interact and affect (or be affected by)
a number of other proteins that take part in p53 regulation. These proteins include L5, L11, PML and ARF, and not all of them are expressed in the same cell type or at the same level as nucleostemin. Although the whole picture of this nucleostemin-mediated MDM2 regulation requires further investigation, this work provides a molecular basis to begin to address these questions.

Biological roles of nucleostemin in safeguarding G2-M progression and preventing drug-induced cell death

Consistent with that reported by Dai et al., we observed a G1-S arrest effect associated with nucleostemin overexpression. Although these authors described an increase of MDM2 protein and G1-S arrest by nucleostemin knockdown, our results showed that nucleostemin depletion leads to MDM2 decrease and G2-M arrest. The first finding is supported by both gain- and loss-of-function experiments in this study. The latter finding was consistently observed and is also supported by our previous FACS analyses of nucleostemin+/– MEF cells (Zhu et al., 2006). Although the p53-observed and is also supported by our previous FACS analyses of nucleostemin+/– MEF cells (Zhu et al., 2006). Although the p53-observed and is also supported by our previous FACS analyses of nucleostemin+/– MEF cells (Zhu et al., 2006).

In conclusion, this study shows that nucleostemin is a unique MDM2-interactive nucleolar protein that stabilizes MDM2, inhibits p53 function and promotes cell proliferation and survival. It does so by binding and retaining the MDM2 protein in the nucleoplasm during mitosis and nucleolar stress.

Materials and Methods

**Epitope-tagged full-length, deletion and point-mutation cDNA constructs**

Deletions and point mutations were introduced by stitching PCR reactions as described previously (Tsai and McKay, 2002; Tsai and McKay, 2005). cDNAs were subcloned into pcDNA expression vectors containing Myc, hemagglutinin (HA), or Flag epitopes at the N- or C-terminus. The N- and C-terminally GFP-fused MDM2 constructs were created in the pC13 and pEGFP-N1 vectors, respectively.

**Cell culture, transfection and western blot**

Cells culture and plasmid transfection procedures were described previously (Meng et al., 2007). Primary antibodies used in western analyses include anti-HA (HA.11), anti-Myc (9E10), anti-Flag (Sigma), anti-MDM2 (SMP14), anti-p53 (DO-1), anti-p21 (Santa Cruz), anti-Bax (Santa Cruz), anti-cleaved caspase-3 (Cell Signaling), anti-B23 (Zymed), and anti-nucleostemin antibodies raised in chicken (Ab2438) or rabbit (Ab138).

**Short hairpin RNA, siRNA duplex and inducible nucleostemin-knockdown cells**

Transient knockdown experiments were performed by transfection of shRNAmir constructs or siRNA duplexes. shRNAmir constructs were generated in the pShag Magic vector (pSM2c) based on a mir-30 hairpin design that targets 21 bp sequences of nucleostemin, capped by mir-5′- and mir-3′-sequences and driven by a U6 promoter. Two or more shRNAmir constructs were tested for their nucleostemin knockdown efficiencies. The targeted sequences for nucleostemin are: 5′-GCT GTA CCG AGA ACT TA3′-3′ (shNS-1), 5′-GCT GAT AAT CCA ACA AAT-3′ (shNS-2). The shScram construct targets a scrambled sequence of 5′-BTC CTG GTG GGG AGA GTG AA-3′.

**Protein degradation and in vivo ubiquitination assays**

Protein degradation assays were performed in cycloheximide-treated H1299 cells as described (Zhu et al., 2006). For in vivo ubiquitination assays, His-tagged ubiquitin and MDM2 expression plasmids were coexpressed with or without nucleostemin or shNS-2 in HEK293 cells. Two days after transfection, cells were treated with MG132 (10 μM) for 6 hours before protein extraction in 6 M guanidinium buffer. Ubiquitlated proteins were pulled down by Ni2+-chelating Sepharose.

**Cell-cycle profile and synchronization**

Cell-cycle profiles were analyzed by counting the PI-labeled cells with a Coulter Epics XL flow cytometer and the XL System II software (Zhu et al., 2006). Each cell-cycle profile was compiled from 2×10⁶ gated events, and analyzed using the Multi Cycle AV software. Early S-phase synchronization was achieved by incubation with 2 mM thymidine for 20 hours, and mitotic arrest was achieved by incubation with 0.5 μM nocodazole for 20 hours.

**Coimmunoprecipitation**

Cells were harvested in NTEN buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.1 mM DTT, supplemented with 1 mM PMSF, 1 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin A and 1 μM E64). Lysates were incubated with primary antibody for 1 hour at 4°C, followed by incubation with protein G Sepharose beads (Pharmacia) for an additional 4 hours at 4°C. Immunoprecipitates were washed 5 times with RIPA buffer (1% PBS, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 1 mM PMSF, 1 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin A and 1 μM E64), fractionated by 10% SDS-PAGE, and detected by western blot.

**Fluorescence loss in photo-bleaching (FLIP)**

Bleaching experiments were performed on HeLa cells grown on Nalgene Lab Tek II chamber slides by using a Zeiss LSM510 confocal microscope equipped with a 63× plan-apochromat oil objective as described previously (Meng et al., 2007). The nucleostemin retention time was measured by the rate of fluorescence loss in the nucleoplasm while bleaching a 2 μm circular region within one nucleolus with repetitive bleaching pulses of 150 msec duration and 0.59 second intervals. The relative fluorescence index (RFI) in the nucleoplasm of bleached cells was normalized to the nucleoplasmic intensity of neighboring non-bleached cells after background subtraction by the following calculation: RFI=(I1/I0)×(C/C0), where I1 and I0 are the background-subtracted intensities of the nucleoplasm in the bleached cell at time-
point t and before photobleaching, respectively. C1 and C0 are the background-subtracted intensities of the nucleoplasm in the neighboring control cell at time-point t and before photobleaching, respectively.

Bimolecular fluorescence complementation (BiFC)
Protein pairs were individually fused to a Flag-tagged Venus YFP-terminal fragment (residues 1-173, Yn) and a Myc-tagged YFP C-terminal fragment (residues 156-239, Yc), and coexpressed with a nucleolar localization signal-tagged CFP (noCFP) in HeLa cells grown on Nalgene Lab Tek II chamber slides. After a 24 hour incubation at 37°C and a 15 hour incubation at 30°C, cells were collected for fluorescence-activated cell sorting (FACS) analyses. Live cell images were recorded on a Zeiss Axiovert 200 fluorescence microscope, equipped with a 63x oil objective (NA 1.4), a Zeiss AxioCam MRm CCD camera, and filter sets described as below: YFP (excitation, BP 500/20; emission, BP 535/30), CFP (excitation, BP 436/20, emission, BP 480/40).

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References
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