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Over Expression of Nucleophosmin and Nucleolin Contributes to the Suboptimal Activation of a G2/M Checkpoint in Ataxia Telangiectasia Fibroblasts

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Abstract

Ataxia Telangiectasia (AT) cells exhibit suboptimal activation of radiation-induced cell cycle checkpoints despite having a wild type p53 genotype. Reducing or eliminating this delay could restore p53 function and reinstate normal cellular response to genotoxic stress. Here we show that the levels of Nucleophosmin (NPM), NPM phosphorylated at Serine 125, p53, p53 phosphorylated at Serine 15 and Serine 392 and the levels of Nucleolin (NCL) are high in AT fibroblasts compared to normal cells. Transfection of a functional ATM into AT fibroblasts reduced p53, phospho-p53, phospho-NPM and NCL levels to wild type fibroblasts levels. Our data indicate that ATM regulates phospho-NPM and NCL indirectly through the Protein Phosphatase 1 (PP1). Both, NPM and NCL interact with p53 and hinder its phosphorylation at Serine 15 in response to bleomycin. Moreover, NPM and NCL are phosphorylated by several of the same kinases targeting p53 and could potentially compete with p53 for phosphorylation in AT cells. In addition, our data indicate that down regulation of NCL and to a lesser extent NPM increase the number of AT cells arrested in G2/M in response to bleomycin. Together this data indicate that the lack of PP1 activation in AT cells result in increased NPM and NCL protein levels which prevents p53 phosphorylation in response to bleomycin and contributes to a defective G2/M checkpoint.

Keywords

Nucleophosmin; Nucleolin; p53; Ataxia Telangiectasia; ATM

Introduction

The Ataxia Telangiectasia (AT) syndrome is a rare cancer prone disease characterized by radio-sensitivity, progressive neurological degeneration, immune dysfunction and developmental disorder that manifest early in childhood. Mutations of the ATM kinase are responsible for the cellular and physiological abnormalities associated with this disease. Cells derived from AT patients have lost the normal capacity to arrest their cycle in response to ionizing radiation (IR) in spite of having a wild type p53 genotype (1). Because the p53 response is delayed and not totally absent in AT cells, it is likely that other kinases are eventually activating p53 in response to IR. This is supported by the fact that at higher doses of IR (10–20 Gy) the p53 response to IR in AT cells is not much different than the normal cells (2). Other stress-activated kinases such as ATR, DNA-PK and hSMG-1 are functional

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Conflicts of Interest

No potential conflicts of interest to disclose.

in AT cells and could eventually attempt to activate p53 (3). One possible reason for the delayed p53 response is that p53 is already phosphorylated in unstimulated AT cells (4). This may be due to increased basal levels of oxidative stress and chronic activation of signal transduction pathways that normally respond to cellular stress (4). Constitutive phosphorylation of stress-responsive proteins also points out to inactivated or defective phosphatases that normally regulate basal phosphorylation levels in order to maintain readiness to respond quickly to cellular insults. In that regard, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are both activated by IR in an ATM – dependent manner (5). A recent report (6) indicates that ATM regulates PP1 activity by phosphorylating its inhibitor (I-2) on Serine 43 which leads to I-2 dissociation from PP1. Activation of PP1 by ATM leads to a G2/M checkpoint through inhibition of Aurora-B kinase and down regulation of histone H3 phosphorylation at Serine 10. The combined effect of chronic stimulation of protein kinases and the inability to activate protein phosphatase is likely to impair the capacity of AT cells to respond to cellular stresses.

Activation of p53 by protein kinases other than ATM could also be prevented by interaction with abundant proteins that could either mask p53 phosphorylation sites or compete it out for phosphorylation. In that respect, we have shown that interaction of p53 with the calcium binding protein S100B prevents p53 activation in response to DNA damaging agents (7). We have also shown that levels of the nucleolar protein nucleophosmin (NPM) also known as B23, NO38 and numatrin (8), set a threshold for p53 phosphorylation in response to UV radiation (9). NPM mediates this effect by competing with p53 for phosphorylation by ATR. The capacity to be used as a sensor or threshold of stress is a new function for NPM. NPM was initially identified as an important player in ribosome biogenesis (10). Since then a number of cellular activities have been associated with NPM. NPM is thought to function as an oncogene when fused to a receptor tyrosine kinase (ALK) in anaplastic large cell lymphoma (11). NPM protein levels are 20 times higher in Novikoff hepatoma and 5 times higher in hypertrophic rat liver compared to normal rat liver (12). Another indication of NPM role in cell proliferation is its association with the silver-stained nucleolar organizer regions (AgNORs) that are used as markers for cancer progression in several tumors (13). Moreover, NPM has been shown to up-regulate the Proliferating Cell Nuclear Antigen (PCNA) and increase resistance to UV-induced cell-killing (8). NPM also binds to several proteins that are important for the regulation of cell proliferation. For example, NPM binds to pRb and synergistically stimulates DNA polymerase α (14). In addition, NPM binds to interferon regulatory factor-1 (IRF-1) and inhibits its tumor suppression function probably by preventing expression of the cyclin dependent kinase (CDK) inhibitor p21 (15). We have shown (9) that NPM interacts with p53 N-terminous and inhibits its transcriptional activity. All these observations indicate that NPM is associated with active cell proliferation.

Nucleolin (NCL) is another abundant p53 binding protein (16) that could prevent the early activation of p53. Like NPM, NCL was initially described as a nucleolar protein participating in ribosome biogenesis (reviewed in (17)). NCL is now recognized as an active player in several cellular processes (18). NCL is over-expressed in malignant tumors and its synthesis is associated with increased rates of cell division (13). In unstressed cells (19), reduction of NCL levels reduced cell proliferation. In addition to being a stress-responsive protein (20), NCL interacts with NPM (21), and responds to cellular stress by translocating from the nucleolus to the nucleoplasm. NCL translocation occurs following exposure to heat shock and IR and the latter is dependent on p53 (16). In a possible negative feedback loop, NCL interacts with p53 C-terminal end (16) and mRNA and prevents its induction and translation in response to IR (22). On the other hand, others have reported that NCL has rather a stabilizing effect on p53 in unstressed cells (23). Given that AT cells are continuously exposed to high levels of oxidative stress (4), we wanted to evaluate the role of NPM and NCL on p53 activation in these cells. Our data indicate that the inability of AT

cells to activate PP1 (6) results in increased NPM and NCL protein levels which prevents p53 phosphorylation and contributes to a defective G2/M checkpoint in response to bleomycin. Regulating the levels of NPM and/or NCL expression in AT cells could become a new mechanism to help restore p53 functions in AT patients.

Material and Methods

Cell culture and treatments

The normal human fibroblasts (GM 05659) and the SV-40 transformed (GM 05849) and non SV-40 transformed (GM 02052) AT fibroblasts cells were obtained from the Human Genetic Mutant Cell repository (Camden, NJ). The cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Where indicated, the cells were exposed to 5mUnits/ml of bleomycin (BioMol, Plymouth Meeting, PA) for different periods of time. Small interfering (40 nM) RNAs (siRNA) for NPM, NCL, Scrambled and (200 nM) PP1 were transfected with TransMessenger (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Transfections were performed twice on two consecutive days before treating and harvesting the cells. The NPM siRNA was composed of 21 nucleotides (nt) double stranded RNA synthesized by Xeragon Inc. (Huntsville, AL). The sequence (5'>CCACAGAAAAAA GUAAAAC<3') corresponds to 19 nt from NPM open reading frame (nt 547 to 565) and two dT-overhang. The NCL siRNA duplexes were synthesized by Qiagen-Xeragon (Germantown, MD). The siRNA sequence-targeting NCL corresponded to nucleotides 626-646 of the coding region relative to the first nucleotide of the start codon (sense; 5'-GCUAUGGAGACUACACC AG-3'; antisense; 5'CUGGUGUAGUCUCCAUAGC-3'). The PP1 pan specific siRNA was composed of six target specific RNAs of 20–25 nt and was synthesized by Santa Cruz (Santa Cruz, CA). The scrambled RNA was composed of four random sequences of 19 nt with 2 UU overhang on each side (non-Specific Control Duplexes-XIII pool of 4, Dharmacon, Inc., Boulder, CO). The NPM antisense expression vector harboring a hygromycin selectable gene (24) was stably transfected in AT fibroblast cells. Selection was accomplished with 400 µg/ml hygromycin.

For cell cycle analysis, the cells were plated at 5×10^4 cells/100-mm-diameter Petri dish and transfected with siRNA as described above. Following transfection, the cells were exposed to bleomycin (4 µg/ml) for 3h, the media was replenished and the cells were analyzed by FACS analysis 24 h later.

Protein purification and in vitro kinase assays

The human NPM and NCL recombinant proteins were engineered by PCR in the BamH1/Xho1 sites of a pGEX-4T2 vector (Amersham, Piscataway, NJ) and purified on glutathione Sepharose beads as recommended by the manufacturer (Amersham). The NCL amino acid residues were numbered according to the gene bank protein sequence NM_005381.

Expression vectors for FLAG-ATM and its kinase dead (KD) mutant were obtained from Dr. Mike B Kastan (St. Jude Children's Research Hospital, Memphis, TN). The Flag-hSMG-1 expression vector was obtained from Alan P Fields (University of Texas Medical Branch, Galveston, TX). Each of the kinase expressing vectors was stably transfected in RKO cells. The cells were exposed to bleomycin to activate the kinase and harvested 4h later. Cellular extracts were immunoprecipitated with a FLAG antibody and used as a source of kinase activity to phosphorylate recombinant proteins *in vitro*. The *in vitro* kinase reactions were performed essentially as described before (9). Briefly, 500 ng of immunoprecipitated kinase was incubated with 300 ng of recombinant NPM or NCL in the presence of 2 µCi of (γ - ^{32} P)ATP in 30 µl of kinase reaction buffer (10 mM Tris-HCl (pH

7.4), 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol) for 30 min at 37°C. The reactions were stopped with the addition of protein loading buffer (25) and run on a 12% SDS-PAGE. The gels were dried and exposed to X-ray sensitive films.

Western Blots

Cellular extracts (10–20 µg) were loaded on a 12% SDS-PAGE and transfer on Immobilon P-PVDF membrane (Millipore, Burlington, Mass). Proteins were reacted with the following antibodies: Rabbit p53 phospho Ser 15, Cell Signaling (Beverly, Mass), rabbit p53 phospho Ser392 Cell Signaling, mouse p53 Ab-6, Calbiochem (San Diego, CA) and mouse NPM Chemicon (Temecula, CA) antibodies at 1:1000 dilution. Mouse NCL Santa Cruz (Santa Cruz, CA) and mouse Actin (Cell Signaling) were used at 1:5000 dilutions and the mouse PP1 antibody (Santa Cruz) was used at 1:500 dilution. The mouse monoclonal phospho specific NPM Ser125 antibody was developed by Rockland Immunochemicals, Inc, (Gilbertsville, PA) with a phospho peptide (H₂N-CVEEDAE(pS)EDEE-OH) and selected against the same unphosphorylated peptide. The antibody was used at 1:100 dilution. The blots were then reacted with a corresponding secondary antibody conjugated to horseradish peroxidase and revealed with a chemiluminescent substrate (ECL: Amersham, Piscataway, NJ). Fold induction was calculated by densitometry and normalized to actin.

Results

Basal levels of stress-responsive proteins are high in AT cells

Over expression of proteins interacting with p53 could prevent its activation (9). Here, we evaluated the effect of NPM and NCL, two proteins over expressed in cancer cells and known to interact with p53, on p53 activation in AT cells. We initially measured the basal levels of NPM, NCL, p53 and p53 phosphorylated at Serine 15 and at Serine 392 as well as NPM phosphorylated at Serine 125. NPM Serine 125 was previously identified as an *in vitro* phosphorylation site for ATM/ATR (9). Because the Protein Phosphatase 1 (PP1) is activated by ATM (5), its basal levels were also analyzed. Data shown in Figure 1 indicate that the basal levels of all the proteins measured are higher in non-SV40 (lanes 2, 5 and 8) as well as SV-40 transformed (lanes 3, 6 and 9) AT fibroblasts compared to wild type cells (lanes 1, 4 and 7). These data thus indicate that increased expression level of these proteins is an intrinsic characteristic of AT cells fibroblasts that is reemphasized in transformed cells.

A functional ATM is required to prevent constitutive activation of stress-responsive proteins

The constitutive high basal levels of p53 phosphorylation (Figure 1) could contribute to the suboptimal activation of radiation-induced cell cycle checkpoints in AT cells (2). As mentioned earlier, chronic activation of stress activated kinases (4) and defective activation of protein phosphatases (5) are the likely culprits for these high basal levels of phosphorylated proteins. PP1 and Protein Phosphatase 2A (PP2A) are both activated by IR in an ATM-dependent manner (5). To determine whether an ATM regulated phosphatase was responsible for the constitutive expression of p53, NPM and NCL, we first stably transfected ATM in an ATM defective SV-40 transformed cell line (GM 05849) and compared the basal levels of these proteins. The SV-40 transformed cell line was chosen because the basal levels of the proteins of interest are also elevated (Figure 1) in this cell line and because it is easier to transfect and manipulate than untransformed cells. This cell line (GM 05849) was analyzed in great details previously (26) and found to be suitable for p53 studies. While it could be argued that the SV40Tag can bind to p53 and inactivates its function (27) it was also demonstrated that in this cell line (GM 05849) a pool of p53 (~50%) is not bound to SV40 and can be activated by DNA damaging agent (26). Actually, several reports have demonstrated that the p53 response to DNA damage (G1 arrest,

phosphorylation at Serine 15 and Serine 37, activation of DNA binding activity and transcriptional activity) is intact in SV40 transformed human fibroblasts (28). We thus performed the majority of the experiments described here with SV-40 transformed AT fibroblasts (GM 05849). The data shown in Figure 2A (lanes 1 and 2) indicate that introducing a functional ATM in this cell line brings down the basal levels of NPM phosphorylated at Serine 125, p53 and NCL markedly while affecting to a lesser extent the levels of phosphorylated p53. The levels of total NPM were not affected. To investigate whether PP1 was involved, we down regulated the levels of PP1 in an ATM proficient cell line (Fig.2B) by siRNA. Our data indicate that down regulating PP1 clearly brought back up the levels of NPM phosphorylated at Serine 125, p53 phosphorylated at Serine 392 and NCL and had only marginal effects on total p53 and p53 phosphorylated at Serine 15. Once again, the levels of total NPM were not affected. These data suggest that the inability to activate PP1 in AT cells (6) could be responsible for the chronic expression of NCL and NPM phosphorylated at Serine 125.

High basal levels of NPM and NCL prevent p53 phosphorylation in AT cells

We have previously shown that increased levels of NPM could prevent p53 activation in human colorectal cancer cells (9). To determine whether NPM and NCL could affect p53 response to DNA damage in AT cells, we first down regulated NPM and treated the cells with the x-ray mimetic agent bleomycin. Our data (Figure 3A) indicate that down regulation of NPM resulted in a two-fold increase in p53 phosphorylation at Serine 15 in response to bleomycin (lane 2). The effect is in agreement with and in the same range as a previous report on NPM effects on p53 activation in response to hypoxia (29). The specificity of the NPM effect is demonstrated by the inability of the scrambled RNA sequence (lanes 3–4) to affect the levels of p53 phosphorylated at Serine 15 in response to bleomycin. We also transiently transfected the AT fibroblasts with a NPM antisense vector and an empty vector (pCDNA3.1) as control. The data shown in Figure 3D indicate that NPM was successfully (~ 70%) down regulated in the AT fibroblasts. Transient transfection of NPM antisense vector resulted in a 2.5 fold increased phosphorylation of p53 at Serine 15 in response to bleomycin (Figure 3B). The increase was noticeable as soon as 30 min after bleomycin treatment and, as with siRNA (Figure 3A, lane 2), was still measurable 3 h after treatment. A much weaker (1.4–1.5 fold) effect was observed in cells transfected with the control vector pCDNA3.1. The kinetics of p53 phosphorylation in the presence of reduced NPM levels is similar to the one reported in normal cells (30). These data thus indicate that down regulation of NPM results in increased p53 phosphorylation in response to DNA damage.

NCL has also been shown to prevent p53 phosphorylation and translation in response to IR in breast cancer cells (22). To determine whether NCL alone or in combination with NPM could affect the p53 response to DNA damage in AT cells, we down regulated each protein either separately or in combination and exposed the cells to bleomycin. Our data (Figure 3C) indicate that in agreement with the data shown in Figure 3A, down regulation of NPM (Fig. 3C, lanes 3–4) resulted in increased phosphorylation of p53 at Serine 15 in response to bleomycin. The fold induction of p53 phosphorylated at Ser15 obtained under these conditions is very reproducible (compare Figure 3A to Figure 3C). Down regulating NCL (Fig. 3C, lanes 5–6) had a milder effect, in a range similar to the scrambled siRNA (lanes 1,2) but this may be due to the fact that only 50% of NCL were down regulated by the siRNA (compare lane 1 and 5). Nonetheless, the 0.5 fold difference (lanes 5–6) is comparable to a 0.6 fold (60%) effect of NCL on p53 translation in response to IR (22). Most importantly, down regulating both proteins while keeping the total amount of siRNA constant (40 μ M) almost triple the levels of p53 phosphorylation in response to bleomycin (lane 8) when compared to untreated cells (lane 7). This effect is greater than NPM alone where twice as much NPM siRNA was used (lanes 3, 4). The greater difference with the double down

regulations is due to a decrease in basal levels of p53 phosphorylated at Ser 15 (lane 7). Neither NCL nor NPM individual down regulation affected basal p53 phosphorylation (lanes 3 and 5). Nonetheless, the concurrent down regulation of both proteins had an intermediary effect (1.7 fold, lane 8) when compared to control RNA (lane 1). These data thus suggest that over-expression of NCL and NPM work in concert to impair p53 response to DNA damaging agent in AT cells.

NPM and NCL are *in vitro* substrates for PI-3 kinases

In addition to suppressing p53 translation (22), NCL and possibly NPM could prevent p53 phosphorylation in AT cells by competing out p53 for phosphorylation by the remaining active kinases. To verify this possibility, we aimed at determining whether NPM and NCL could be phosphorylated *in vitro* by PI-3 kinases known to target p53 (3). We first stably transfected expression vectors for FLAG-ATM and its kinase dead (KD) mutant in colorectal carcinoma RKO cells. The cells were exposed to bleomycin to activate the kinases and harvested 4h later. Cellular extracts were immunoprecipitated with a FLAG antibody and used as a source of kinase activity to phosphorylate recombinant NPM. The data shown in Figure 4A indicate that NPM is a good *in vitro* substrate for ATM and that this phosphorylation is specific since the ATM kinase dead mutant (ATM-KD) did not phosphorylate it.

We have already determined that Serine 125 of NPM is an ATR *in vitro* phosphorylation site (9). Here, we proceeded to determine if Serine 125 is also phosphorylated by ATM and another ATM related kinase, hSMG-1^{ATX}. The data presented in Table 1 indicate that Serine 125 is also phosphorylated by ATM since this site was not phosphorylated when Serine 125 was replaced by Alanine. Interestingly, Serine 125 is a major NPM phosphorylation site *in vivo* as determined by ³²P labeling (31), a source of IR. In addition to Serine 125, ATM may also phosphorylate other sites on NPM. As reported for ATR (9), our data indicate that the NPM fragment 175–222 is also phosphorylated (Table 1). Moreover, the fragment containing the residues 212–293, which was not phosphorylated by ATR (9), is also phosphorylated to some extent by ATM. Serine 125 is not part of the conventional Ser/Thr-Gln-Glu (s/t-q-e) ATM consensus sequence but is surrounded by acidic residues (Figure 4B) that may facilitate phosphorylation *in vitro* (32). Not all ATM substrates adhere to the consensus sites. For example BRCA1 is phosphorylated at Ser-Gln clusters as well as on Serine residues that are not part of the conventional consensus sites (33). ATM itself can autophosphorylate at a Ser-Glu (Serine 1893) in addition to the conventional Ser-Gln sites (34). Nonetheless, the NPM Serine 125 phosphorylation is specific since the NPM fragments 1–180 and 175–222 are not phosphorylated by Cdc2 (35). Other smaller molecular weight bands were also detected with the full length and 1–180 fragments but these probably represented break down products of the Serine 125 site since they were not detected when Serine 125 was replaced by Alanine (data not shown).

The hSMG-1^{ATX} protein kinase is another member of the ATM family of protein kinases and displays several functional overlaps with ATM including activation by IR and phosphorylation of p53 at Serine 15 (36). To determine whether NPM is also a substrate for hSMG-1^{ATX} we stably transfected RKO cells with a Flag-hSMG-1^{ATX} expression vector and performed a kinase assay as described above. Our data (Table 1) indicate that indeed NPM is also a good *in vitro* substrate for hSMG-1^{ATX}. As with ATM, replacement of Serine 125 with Alanine (lane 13) completely abolished phosphorylation of the 1-180 fragment. The fragment containing residues 175 to 222 is also phosphorylated but in contrast to ATM, the fragment containing residues 212 to 293 is not. NPM thus appears to be another potential target for hSMG-1^{ATX}. It has been suggested that strategies that could augment hSMG-1^{ATX} activity could rescue at least in part the phenotypic defects caused by loss of ATM in AT

cells (37). Down regulation of NPM could thus achieve this aim by increasing the chances that hSMG-1^{ATX}, or other kinases, targets p53 rather than NPM.

Nucleolin is composed of three functionally different domains: the N-terminal acidic region that controls rDNA transcription, the central RNA binding domain (RBD) consisting of 4 RNA binding motifs that determine the RNA binding specificity, and the C-terminal arginine-glycine rich domain (RGG boxes) that regulates RNA or protein interactions (38). The data shown in Table 2 indicate that none of the RBD or the RGG boxes located in the C-terminal domains were phosphorylated *in vitro* by ATM or ATR. On the other hand, the N-terminal acidic region is readily phosphorylated by ATM, ATR and h-SMG-1^{ATX} (Figure 5A, Table 2). The fragment 1–151 (Fig.5A, lanes 2) is the preferred substrate for all three kinases. To narrow down the phosphorylation site we engineered N-terminal deletion mutants fused to GST and repeated the *in vitro* kinase assay. Our data (Table 2) indicate that no phosphorylation was observed in the first 100 residues of Nucleolin and that most phosphorylation occurred between residues 101 and 151. ATM also phosphorylated fragments 152–183 and 184–219 but to a much lesser extent. Fragments 101–219 contain 4 Serines all surrounded by acidic residues and 6 Threonines. Interestingly, even though fragment 184–219 contains the highest number of acidic residues and two Serines residues, this fragment was the least preferred substrate for all three kinases (Figure 5B). The most preferred substrate, fragment 101–151, contains only one Serine residue, Serine 145, that when mutated to an Alanine abolishes phosphorylation by all three kinases (Figure 5B, lanes 3–4). This indicates that Serine 145 is targeted by all three kinases and that none of the Threonine residues are *in vitro* substrate for these kinases. Mutations of Serine 153 (lanes 6) and Serine 184 (lanes 8) also abolished the residual phosphorylation on these fragments indicating that other minor sites could also be targeted by these kinases. These data thus indicate that both NPM and NCL are good *in vitro* substrates for protein kinases targeting p53.

High expression levels of NCL and NPM contributes to the defective G2/M checkpoint in AT fibroblasts

To determine whether the high expression levels of NPM and NCL could contribute to the suboptimal cell cycle checkpoints in AT cells, we down regulated each protein by siRNA and analyzed cell cycle distribution of Non-SV40 and SV40 transformed AT fibroblasts in response to bleomycin. The data shown in Figure 6 indicate that transfection with NCL or NPM siRNA in non SV-40 transformed cells did not affect cell cycle distribution at the 0h time point nor did it increase toxicity (sub G1). However, down regulating NCL (si-NCL) almost triple the number of cells arrested in G2/M 24h after exposure to bleomycin as compared to cells transfected with control siRNA (si-Scrambled). Down regulating NPM had a milder effect where 3% more cells were arrested in G2/M (14 % vs 11 %) as compared to control siRNA 24h after exposure to bleomycin. Down regulating both proteins, while keeping the amount of siRNA constant more than double the number of cells arrested in G2/M as compared to control. The combined effect of down regulating both proteins simultaneously is probably additive since by adding half the cells arrested in G2/M with each siRNA (7% + 15.5%) we obtain the same percentage of cell as using half of each siRNA simultaneously (23%). Similar data were also obtained in SV-40 transformed cells (data not shown). These data and the report on PP1 regulation of the G2/M checkpoint (6) thus suggest that PP1 could also regulate the G2/M checkpoint by controlling the levels of NPM and NCL in an ATM dependent manner (Figure 2).

Discussion

It has long been suspected that endogenous mechanisms must be in place to prevent premature activation of the tumor suppressor p53 (39). Such restraint is necessary to allow

normal growth and development but at the same time it should not compromise the capacity to respond rapidly to stress associated with tumor progression. Masking of p53 activation sites by abundant p53 binding proteins may provide such flexibility. In that respect, we have previously shown that over-expression of NPM prevents the activation of p53 in response to low doses of UV radiation (9). Here we show (Figure 3) that down regulation of NPM also increased p53 phosphorylation at Serine 15 in response to bleomycin in AT cells. NPM could mediate this effect by at least two mechanisms; the first one would be by competing with p53 for phosphorylation by other kinases. In addition to ATR and hSMG-1^{ATX} (9) and Figure 4), NPM could also compete for phosphorylation by Casein Kinase 2 (CK2) since NPM and p53 are both phosphorylated by this kinase (31). Alternatively, NPM binding to p53 N-terminal end (9), could mask p53 phosphorylation sites at residues targeted by stress-activated kinases. These two possibilities are not mutually exclusive and are supported by the data presented in Figures 3, 4 and Table 1. Nonetheless, since NPM Serine 125 but not total NPM is affected by ATM and PP1 (Figure 2) the first mechanism seems more likely.

Interestingly, NPM Serine 125 was the major NPM phosphorylation site identified *in vivo* (31). As the levels of p53 Serine 15, the levels of NPM Serine 125 are also elevated in unstressed AT cells (Figure 1). This is probably due to the high basal levels of oxidative stress and the chronic activation of signal transduction pathways in AT cells (4). Kinases such as ATR and hSMG1^{ATX} (Table 1), and perhaps CK2, are most likely responsible for the NPM Serine 125 constitutive phosphorylation in AT cells. In addition to the constitutive activation of protein kinases, the inability to activate protein phosphatase can lead to increased phosphorylation as well. Our data indicate that down regulation of PP1 in ATM corrected cells (Figure 2B) returned the levels of NPM phosphorylated at Serine 125 and NCL to the high basal levels observed in ATM deficient cells. A recent report clearly demonstrated that a functional ATM is required to regulate PP1 activity (6). It thus seems likely that the inability to activate PP1 contributes to the increased NPM phosphorylated at Serine 125 and NCL levels in AT cells. On the other hand, while an ATM-regulated phosphatase is apparently necessary to maintain low expression levels of p53 (Figure 2, lanes 2), PP1 does not seem to be the main phosphatase involved in the modulation of total p53 and p53 phosphorylated at Ser15 (Figure 2B, lanes 2). The protein phosphatase PP2A is a possible alternative since this phosphatase is also activated by ATM and can dephosphorylate p53 following ionizing radiation (5,40). Nonetheless, it is also possible that the partial down regulation of PP1 obtained by siRNA procedure (Figure 2B) prevented the measure of a full effect on p53, NPM and NCL. Moreover, both NPM and NCL are highly abundant proteins that are regulated at several levels, regulation of posttranslational modification by PP1 while an important contributor may not be sufficient to affect the total levels of these proteins expression.

Nucleolin (NCL) is another abundant protein that can affect p53 activation and expression levels (22). Our data indicate that NCL is over-expressed in AT cells and, as NPM, its down regulation allows p53 phosphorylation in response to bleomycin treatments (Figure 3C). This is in good agreement with previously reported NCL effects on p53 translation in breast cancer cells (22). One mechanism by which NCL could prevent p53 phosphorylation would be by competing with p53 for phosphorylation by the remaining active kinases in AT cells. In that regard we have identified NCL Serine 145 as a preferred *in vitro* target for ATR and hSMG1^{ATX} (Figure 5). All NCL *in vitro* phosphorylation sites identified in our study (Serine 145, Serine 153, Serine 184 and Serine 206) are also recognized to some extent as potential DNA damage kinase (ATM, DNA-PK) sites by Scansite (Massachusetts Institute of Technology). Most importantly, these sites are also identified as CK2 sites with high stringency. This indicates that as it is the case for NPM, NCL has the potential to compete with p53 for phosphorylation by these kinases. Nonetheless, even though NCL does not contain any perfectly match ATM/ATR consensus sites (32) three of the identified

phosphorylation sites (Serine 145, Serine 153 and Serine 206) are recognized at low stringency as ATM sites. Non consensus ATM/ATR sites have been identified in *bonafide* ATM substrates such as BRCA1 (33) and ATM itself (34) and proximity to the ATM/ATR kinases rather than sequence context is believed to play a pivotal role in the selection of the substrates *in vivo* (32). Therefore we cannot rule out the possibility that the abundance of NCL and NPM or their proximity to ATM could favor some direct phosphorylation.

The suboptimal activation of radiation-induced cell cycle checkpoints in AT cells is responsible for a variety of cellular aberrations associated with the AT syndrome. Here, we described a new mechanism to allow p53 phosphorylation in cells deprived of a functional ATM kinase. This system takes advantage of the wild type p53 genotype in these cells and the remaining active stress inducible protein kinases. Our data suggest that both proteins, NPM and NCL share similar mechanisms to impede p53 functions since no synergistic effect was observed (Figure 6). The model proposed in Fig.7 indicates that in normal cells, ATM can phosphorylate PP1 Inhibitory factor 2 (I-2) and activate PP1. Once activated, PP1 represses NPM Ser125 phosphorylation and NCL levels. The resulting low levels of NPM and NCL allow p53 to respond to stress by activating cellular checkpoints. PP2A can also dephosphorylate p53 in an ATM dependent manner. In AT cells, the absence of a functional ATM prevents the activation of PP1 and results in high NPM Ser125, NCL and p53 Ser15 phosphorylation levels probably through the remaining active kinases such as ATR and SMG1^{ATX}. High levels of NCL and NPM could restrain p53 phosphorylation in response to DNA damage by a number of mechanisms including masking p53 phosphorylation sites (9), competing out with p53 for phosphorylation (Figures 4,5) or in the case of NCL preventing p53 translation (22). The down regulation of NPM and NCL levels in AT cells could reduce p53 steric hindrance and decreases the pool of competing substrates for the remaining active kinases which would allow p53 phosphorylation and activation of a G2 checkpoint. As mentioned earlier, the ATM-regulated PP1 (6) leads to a G2/M checkpoint through inhibition of Aurora-B kinase and down regulation of histone H3 phosphorylation at Serine 10. Our data indicate that PP1 could also affect the G2/M checkpoint through regulation of NCL and NPM protein levels. These mechanisms are not mutually exclusive and could be exploited to restore at least in part p53 function in AT cells

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References

1. Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*. 1992; 71:587–97. [PubMed: 1423616]
2. Canman CE, Wolff AC, Chen CY, Fornace AJ Jr, Kastan MB. The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia. *Cancer Res*. 1994; 54:5054–8. [PubMed: 7923116]
3. Wright JA, Keegan KS, Herendeen DR, Bentley NJ, Carr AM, Hoekstra MF, Concannon P. Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. *Proc Natl Acad Sci U S A*. 1998; 95:7445–50. [PubMed: 9636169]
4. Gatei M, Shkedy D, Khanna KK, Uziel T, Shiloh Y, Pandita TK, Lavin MF, Rotman G. Ataxia-telangiectasia: chronic activation of damage-responsive functions is reduced by alpha-lipoic acid. *Oncogene*. 2001; 20:289–94. [PubMed: 11313957]
5. Guo CY, Brautigan DL, Larner JM. Ionizing radiation activates nuclear protein phosphatase-1 by ATM-dependent dephosphorylation. *J Biol Chem*. 2002; 277:41756–61. [PubMed: 12202491]

6. Tang X, Hui ZG, Cui XL, Garg R, Kastan MB, Xu B. A novel ATM-dependent pathway regulates protein phosphatase 1 in response to DNA damage. *Mol Cell Biol.* 2008; 28:2559–66. [PubMed: 18250156]
7. Lin J, Blake M, Tang C, Zimmer D, Rustandi RR, Weber DJ, Carrier F. Inhibition of p53 transcriptional activity by the S100B calcium-binding protein. *J Biol Chem.* 2001; 276:35037–41. [PubMed: 11454863]
8. Wu MH, Chang JH, Yung BY. Resistance to UV-induced cell-killing in nucleophosmin/B23 over-expressed NIH 3T3 fibroblasts: enhancement of DNA repair and up-regulation of PCNA in association with nucleophosmin/B23 over-expression. *Carcinogenesis.* 2002; 23:93–100. [PubMed: 11756229]
9. Maiguel DA, Jones L, Chakravarty D, Yang C, Carrier F. Nucleophosmin sets a threshold for p53 response to UV radiation. *Mol Cell Biol.* 2004; 24:3703–11. [PubMed: 15082766]
10. Chang JH, Olson MO. A single gene codes for two forms of rat nucleolar protein B23 mRNA. *J Biol Chem.* 1989; 264:11732–7. [PubMed: 2745414]
11. Drexler HG, Gignac SM, von Wasielewski R, Werner M, Dirks WG. Pathobiology of NPM-ALK and variant fusion genes in anaplastic large cell lymphoma and other lymphomas. *Leukemia.* 2000; 14:1533–59. [PubMed: 10994999]
12. Chan WY, Liu QR, Borjigin J, Busch H, Rennert OM, Tease LA, Chan PK. Characterization of the cDNA encoding human nucleophosmin and studies of its role in normal and abnormal growth. *Biochemistry.* 1989; 28:1033–9. [PubMed: 2713355]
13. Derenzini M, Sirri V, Trere D, Ochs RL. The quantity of nucleolar proteins nucleolin and protein B23 is related to cell doubling time in human cancer cells. *Lab Invest.* 1995; 73:497–02. [PubMed: 7474921]
14. Takemura M, Sato K, Nishio M, Akiyama T, Umekawa H, Yoshida S. Nucleolar protein B23.1 binds to retinoblastoma protein and synergistically stimulates DNA polymerase alpha activity. *J Biochem.* 1999; 125:904–9. [PubMed: 10220582]
15. Kondo T, Minamino N, Nagamura-Inoue T, Matsumoto M, Taniguchi T, Tanaka N. Identification and characterization of nucleophosmin/B23/numatrin which binds the anti-oncogenic transcription factor IRF-1 and manifests oncogenic activity. *Oncogene.* 1997; 15:1275–81. [PubMed: 9315094]
16. Daniely Y, Dimitrova DD, Borowiec JA. Stress-dependent nucleolin mobilization mediated by p53-nucleolin complex formation. *Mol Cell Biol.* 2002; 22:6014–6022. [PubMed: 12138209]
17. Srivastava M, Pollard HB. Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *Faseb J.* 1999; 13:1911–22. [PubMed: 10544174]
18. Ying GG, Proost P, van Damme J, Bruschi M, Introna M, Golay J. Nucleolin, a novel partner for the Myb transcription factor family that regulates their activity. *J Biol Chem.* 2000; 275:4152–8. [PubMed: 10660576]
19. Mi Y, Thomas SD, Xu X, Casson LK, Miller DM, Bates PJ. Apoptosis in leukemia cells is accompanied by alterations in the levels and localization of nucleolin. *J Biol Chem.* 2002; 27:27.
20. Yang C, Maiguel DA, Carrier F. Identification of nucleolin and nucleophosmin as genotoxic stress-responsive RNA-binding proteins. *Nucleic Acids Res.* 2002; 30:2251–60. [PubMed: 12000845]
21. Li YP, Busch RK, Valdez BC, Busch H. C23 interacts with B23, a putative nucleolar-localization-signal-binding protein. *Eur J Biochem.* 1996; 237:153–8. [PubMed: 8620867]
22. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell.* 2005; 123:49–63. [PubMed: 16213212]
23. Saxena A, Rorie CJ, Dimitrova D, Daniely Y, Borowiec JA. Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization. *Oncogene.* 2006; 25:7274–88. [PubMed: 16751805]
24. Yang C, Carrier F. The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. *J Biol Chem.* 2001; 276:47277–84. [PubMed: 11574538]
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–5. [PubMed: 5432063]
26. Turenne GA, Paul P, Laflair L, Price BD. Activation of p53 transcriptional activity requires ATM's kinase domain and multiple N-terminal serine residues of p53. *Oncogene.* 2001; 20:5100–10. [PubMed: 11526498]

27. Hess RD, Brandner G. DNA-damage-inducible p53 activity in SV40-transformed cells. *Oncogene*. 1997; 15:2501–4. [PubMed: 9395246]
28. Kohli M, Jorgensen TJ. The influence of SV40 immortalization of human fibroblasts on p53-dependent radiation responses. *Biochem Biophys Res Commun*. 1999; 257:168–76. [PubMed: 10092528]
29. Li J, Zhang X, Sejas DP, Bagby GC, Pang Q. Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. *J Biol Chem*. 2004; 279:41275–9. [PubMed: 15310764]
30. Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*. 1998; 281:1677–9. [PubMed: 9733515]
31. Chan PK, Aldrich M, Cook RG, Busch H. Amino acid sequence of protein B23 phosphorylation site. *J Biol Chem*. 1986; 261:1868–72. [PubMed: 3944116]
32. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev*. 2001; 15:2177–96. [PubMed: 11544175]
33. Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science*. 1999; 286:1162–6. [PubMed: 10550055]
34. Kozlov SV, Graham ME, Peng C, Chen P, Robinson PJ, Lavin MF. Involvement of novel autophosphorylation sites in ATM activation. *Embo J*. 2006; 25:3504–14. [PubMed: 16858402]
35. Cha H, Hancock C, Dangi S, Maiguel D, Carrier F, Shapiro P. Phosphorylation regulates nucleophosmin targeting to the centrosome during mitosis as detected by cross-reactive phosphorylation-specific MKK1/MKK2 antibodies. *Biochem J*. 2004; 378:857–65. [PubMed: 14670079]
36. Brumbaugh KM, Otterness DM, Geisen C, Oliveira V, Brognard J, Li X, Lejeune F, Tibbetts RS, Maquat LE, Abraham RT. The mRNA surveillance protein hSMG-1 functions in genotoxic stress response pathways in mammalian cells. *Mol Cell*. 2004; 14:585–98. [PubMed: 15175154]
37. Abraham RT. The ATM-related kinase, hSMG-1, bridges genome and RNA surveillance pathways. *DNA Repair (Amst)*. 2004; 3:919–25. [PubMed: 15279777]
38. Bouvet P, Diaz JJ, Kindbeiter K, Madjar JJ, Amalric F. Nucleolin interacts with several ribosomal proteins through its RGG domain. *J Biol Chem*. 1998; 273:19025–9. [PubMed: 9668083]
39. Vousden KH. Activation of the p53 tumor suppressor protein. *Biochim Biophys Acta*. 2002; 1602:47–59. [PubMed: 11960694]
40. Shouse GP, Cai X, Liu X. Serine 15 phosphorylation of p53 directs its interaction with B56gamma and the tumor suppressor activity of B56gamma-specific protein phosphatase 2A. *Mol Cell Biol*. 2008; 28:448–56. [PubMed: 17967874]

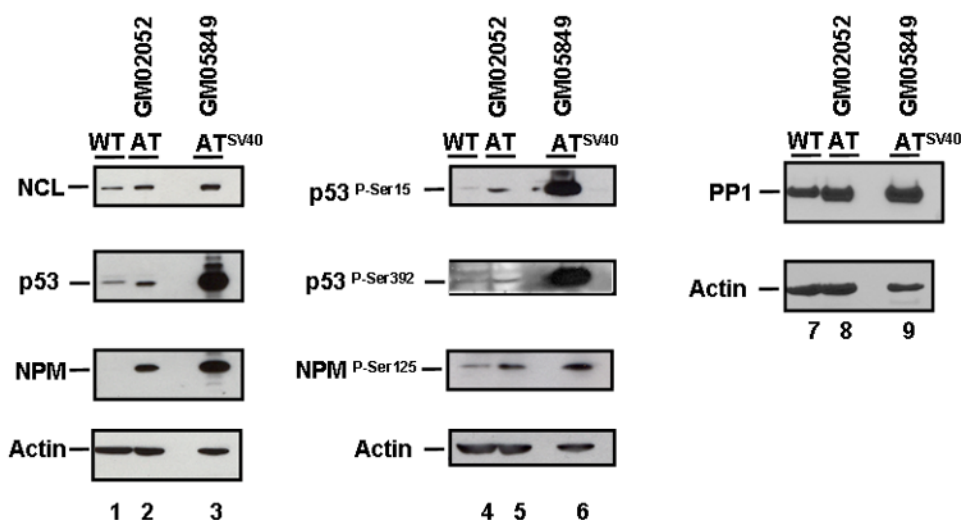


Figure 1. Basal levels of stress proteins are high in AT cells. Western blots

Normal human fibroblasts (GM 05659) and SV-40 transformed (GM 05849) and non SV-40 transformed (GM 02052) AT fibroblasts cells were grown as described under Material and Methods. Cellular extracts (10 μ g) were prepared and analyzed with the indicated antibodies as described in the text.

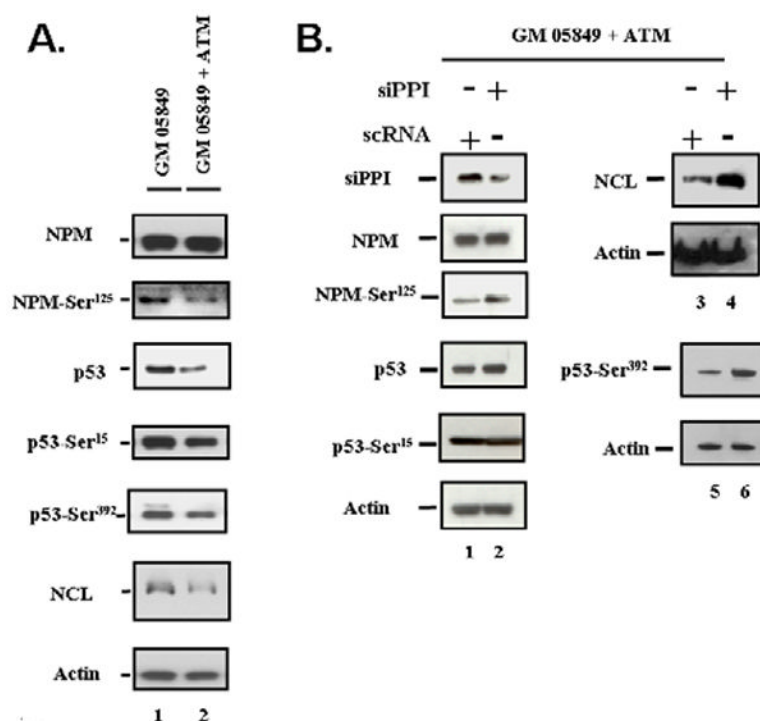


Figure 2. A functional ATM is required to prevent over-expression of stress-proteins

Western blots. (A) SV-40 transformed (GM 05849) AT fibroblasts stably transfected with a Flag vector (lane 1) or a Flag-ATM vector (lane 2) were used. Cellular extracts (10 μ g) from exponentially growing cells were prepared and analyzed with the indicated antibody as described in the text. (B) Same as (A) except that scrambled siRNA (scRNA, lanes 1, 3 and 5) or PP1 siRNA (lanes 2, 4 and 6) were transiently transfected in ATM corrected AT fibroblasts.

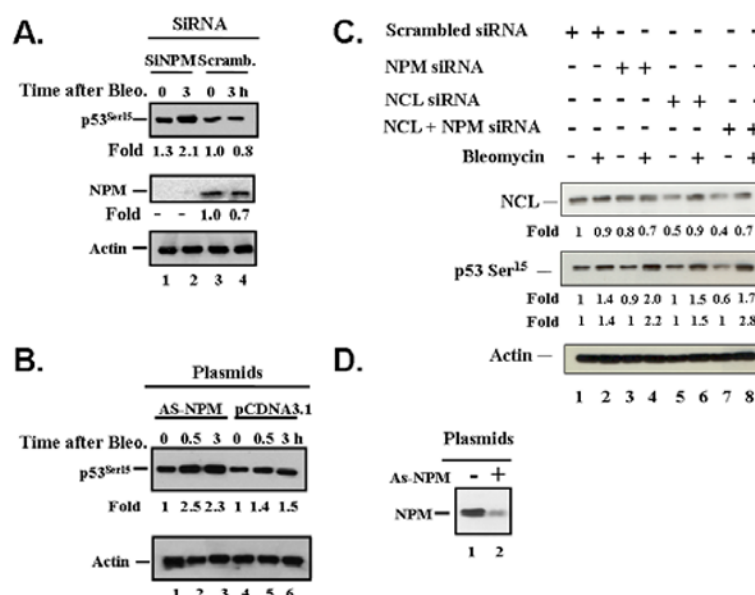


Figure 3. Down regulation of NPM and NCL allows p53 phosphorylation in response to bleomycin

(A) Western blots. AT fibroblasts (GM 05849) were treated with 40 nM siNPM or Scrambled RNA (Scramb.) and exposed to 5 mUnits/ ml bleomycin for the indicated period of time. Western blots were performed with the indicated antibodies. Actin was used as a control for loading. Fold induction was determined by densitometry and normalized to actin. (B, D) The cells were transiently transfected with 7.5 μ g antisense NPM (As-NPM) vector or empty vector (pCDNA3.1) and exposed to 5 mUnits/ ml bleomycin for the indicated period of time. Actin was used as a control for loading. Fold induction was determined by densitometry and was normalized to actin. (C) Western blots performed as in A with the exception that where indicated, NCL siRNA (40 nM) were used. NCL + NPM siRNA (40 nM total).

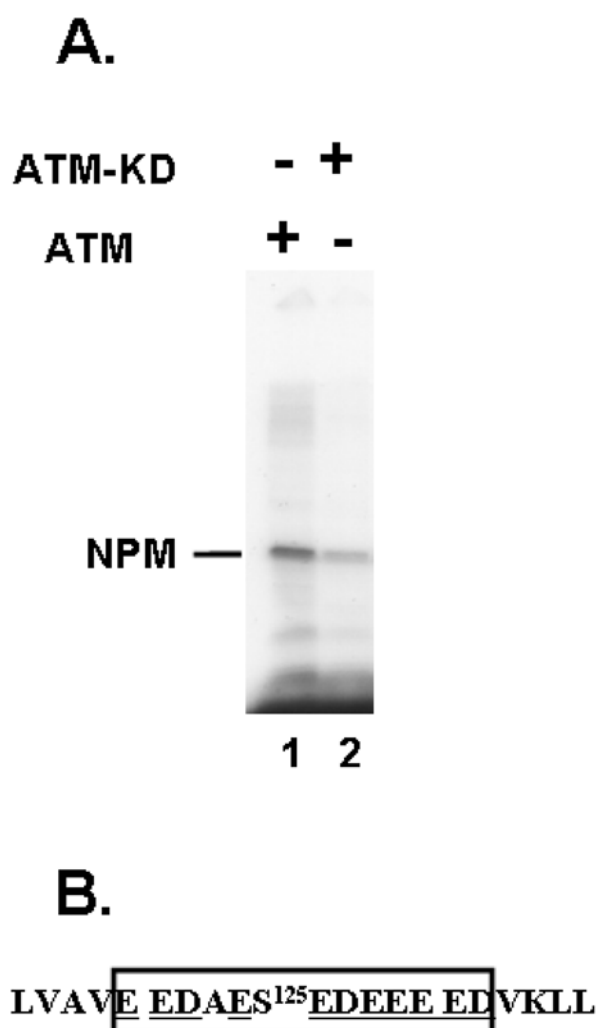


Figure 4. NPM is an in vitro substrate for ATM and SMG1^{ATX}

(A) In vitro kinase assay on recombinant NPM (300 ng) with Flag-ATM (lane 1) or its kinase dead (KD, lane 2) mutant immunoprecipitated from RKO cells. (B) Primary sequence of residues surrounding human NPM Serine 125. Acidic residues are underlined.

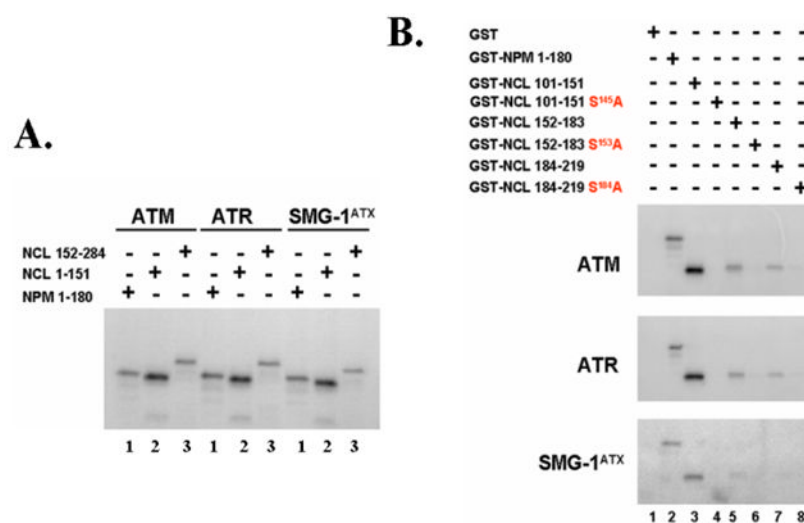


Figure 5. Nucleolin Serine 145 is an in vitro substrate for ATM, ATR and SMG-1^{ATX}
 (A) In vitro kinase assay performed as in Fig.4 with the indicated kinase and N-terminal GST-NCL recombinant proteins (lanes 2-3). GST-NPM fragment 1-180 (lanes 1) was used as a positive control. (B) Three point mutations were analyzed as in A): GST-NCL 101-151 S145A: Serine 145 was replaced by Alanine (lane 4), GST-NCL 152-183 S153A: Serine 153 was replaced with Alanine (lane 6), GST-NCL 184-219 S184A: Serine 184 was replaced with Alanine (lane 8).

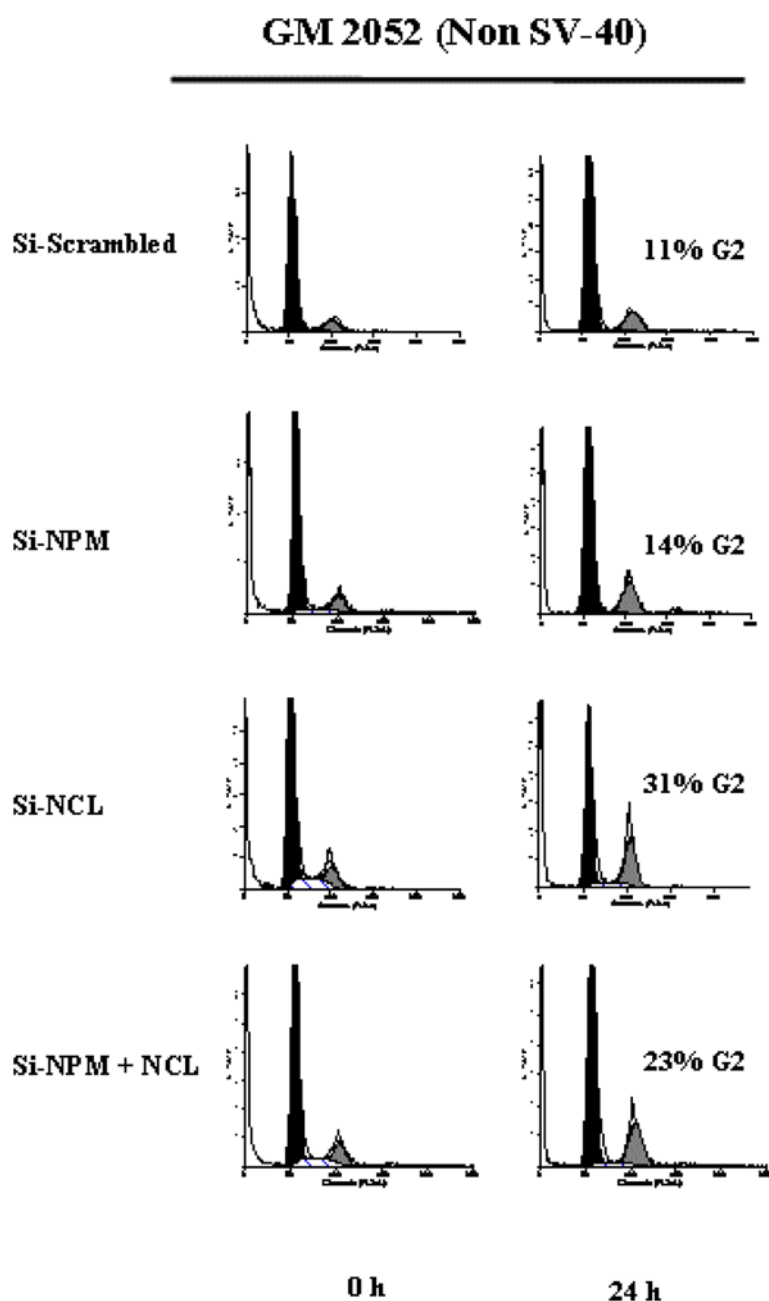


Figure 6. Overexpression of NCL and NPM contributes to the suboptimal G2/M checkpoint in AT cells

The non SV-40 transformed (GM 02052) AT fibroblasts were transfected with the indicated siRNA (40 nM) for two consecutive days and then treated with bleomycin (4 μ g/ml) for 3h. The cells were then either harvested (0h) or maintained in cultured for an additional 24 h and analyzed by FACS analysis.

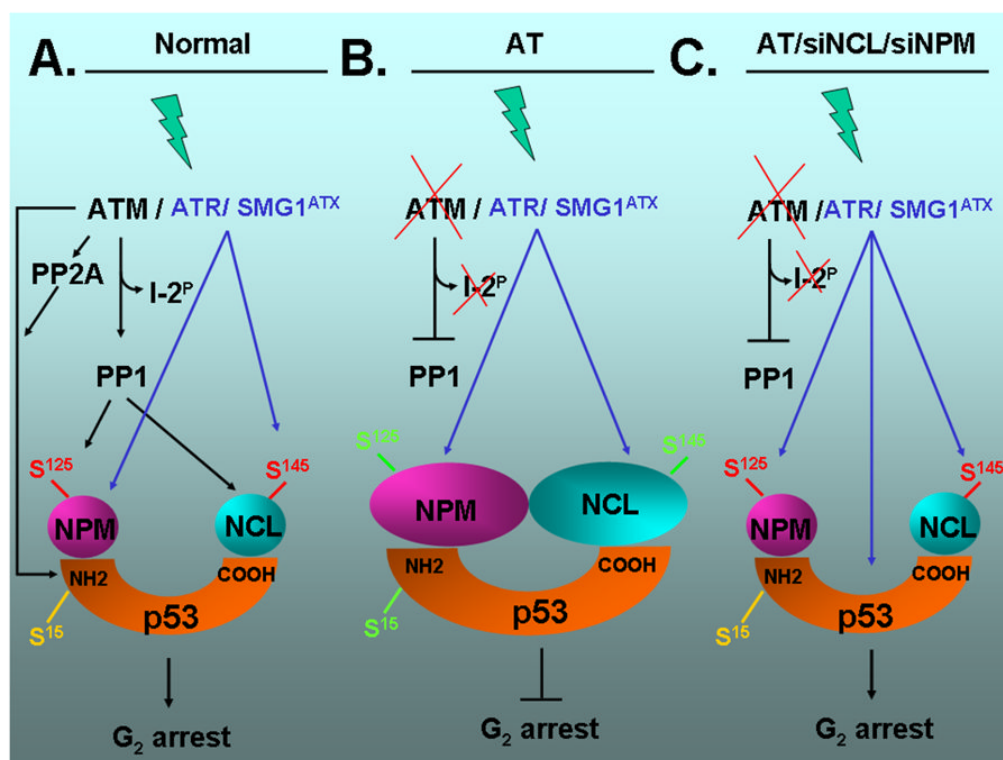


Figure 7. Schematic summary and proposed model

(A) In normal cells, ATM can phosphorylate PP1 Inhibitory factor 2 (I-2) and activate PP1. PP1 represses NPM Ser125 phosphorylation and NCL levels. Low levels of NPM and NCL allow p53 to respond to stress by activating cellular checkpoints. PP2A can also dephosphorylate p53 in an ATM dependent manner. (B) In AT cells, the absence of a functional ATM prevents the activation of PP1 and results in high NPM Ser125, NCL and p53 Ser15 phosphorylation levels probably through the remaining active kinases such as ATR and SMG1^{ATX}. The high expression levels of NPM and NCL could compromise p53 activation through binding to either p53 amino (NPM) or carboxy (NCL) terminal end or through competition for the remaining active kinases. (C) Down regulation of NPM and NCL levels in AT cells reduces p53 steric hindrance and decreases the pool of competing substrates for the remaining active kinases which allow p53 phosphorylation and activation of a G₂ checkpoint. PP1: protein phosphatase 1; PP2A; Protein Phosphatase 2A; NPM: Nucleophosmin; NCL: Nucleolin. Phosphorylated Serines are indicated; red indicates low level of phosphorylation, green indicates hyper-phosphorylation and yellow indicates p53 inducible levels.

Table 1

Ser 125 is the preferred ATM and SMG-1^{ATX} phosphorylation site on NPM

NPM Fragment	ATM	SMG-1A ^{TX}
Full Length	+++	+++
GST-1-180	+++	+++
GST-1-180 *A125	-	-
GST-175-222	++	+++
GST-212-293	+	-
GST-1-60	-	-
GST-55-120	-	-
GST	-	-

ATM preferred phosphorylation site is located between Nucleolin residue 101 and 151

Table 2

NCL Fragment	ATM
GST-1-50	-
GST-51-100	-
GST-101-151	+++
GST-152-183	++
GST-184-219	+
GST-284-707	-
GST	-