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Nuclear Ataxia-Telangiectasia Mutated (ATM) Mediates the Cellular Response to DNA Double Strand Breaks in Human Neuron-like Cells*[S]

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The protein kinase ATM (ataxia-telangiectasia mutated) activates the cellular response to double strand breaks (DSBs), a highly cytotoxic DNA lesion. ATM is activated by DSBs and in turn phosphorylates key players in numerous damage response pathways. ATM is missing or inactivated in the autosomal recessive disorder ataxia-telangiectasia (A-T), which is characterized by neuronal degeneration, immunodeficiency, genomic instability, radiation sensitivity, and cancer predisposition. The predominant symptom of A-T is a progressive loss of movement coordination due to ongoing degeneration of the cerebellar cortex and peripheral neuropathy. A major deficiency in understanding A-T is the lack of information on the role of ATM in neurons. It is unclear whether the ATM-mediated DSB response operates in these cells similarly to proliferating cells. Furthermore, ATM was reported to be cytoplasmic in neurons and suggested to function in these cells in capacities other than the DNA damage response. Recently we obtained genetic molecular evidence that the neuronal degeneration in A-T does result from defective DNA damage response. We therefore undertook to investigate this response in a model system of human neuron-like cells (NLCs) obtained by neuronal differentiation in culture. ATM was largely nuclear in NLCs, and their ATM-mediated responses to DSBs were similar to those of proliferating cells. Knocking down ATM did not interfere with neuronal differentiation but abolished ATM-mediated damage responses in NLCs. We concluded that nuclear ATM mediates the DSB response in NLCs similarly to in proliferating cells. Attempts to understand the neurodegeneration in A-T should be directed to investigating the DSB response in the nervous system.

A-T[3] is a human autosomal recessive disorder characterized by progressive neuromotor dysfunction, immunodeficiency, genomic instability, radiation sensitivity, and predisposition to lymphoreticular malignancies. Cerebellar ataxia typically appears in infancy and is an early sign of a progressive neurological syndrome that includes choreoathetosis, dystonia, oculomotor apraxia, limitation on facial expressiveness, dysarthria, and difficulty in swallowing that often causes aspirations leading to sinopulmonary infections. Most A-T patients become wheelchair-bound by the end of the first decade of life. The ataxia reflects progressive degeneration of the cerebellar cortex with striking loss of the Purkinje and granule cells; impairment of the extrapyramidal movement system and peripheral neuropathy are also established features of A-T (1–3).

A-T results from null mutations in the *ATM* gene, which encodes the protein kinase ATM. ATM is the primary mobilizer of the cellular response to double strand breaks (DSBs) in the DNA, an elaborate network of signaling pathways that swiftly modulates many physiological processes (4, 5). DSBs are first sensed by *sensor/mediator* proteins that activate the *transducers*, which in turn convey the DNA damage alarm to many downstream *effectors* that control specific pathways. ATM, the chief transducer of the DSB alarm, is a member of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family (6). Following DSB induction ATM is rapidly autophosphorylated and activated (7) and phosphorylates numerous substrates, each of which in turn modulates a specific pathway (8, 9).

A-T is a multisystem disease but primarily a neurodegenerative disorder. While the defect in the DNA damage response could explain the defective cellular response to DSBs in A-T cells and several features of the disease, it has been conceptually difficult to relate it to the prominent symptom of A-T, the progressive neuronal degeneration. Understanding the role of ATM in the nervous system is crucial to understanding the disease but has remained largely elusive. ATM functions have been investigated mainly in cultured proliferating cells, in which an important role of the DNA damage response is to activate the cell cycle checkpoints (8, 10). Conceivably, these checkpoints are not activated in post-mitotic cells. Furthermore, several investigators suggested that ATM was cytoplasmic in human and murine neuronal tissues and hence was functioning in pathways unrelated to the DNA damage response (11–13).

Recent studies from our laboratory led us to the conclusion that the neuronal degeneration in A-T does in fact result from a defective response to DSBs (14). We therefore set out to study the subcellular localization of ATM and the ATM-mediated DSB response in human neurons. These experiments required the use of cultured cells that simulate human neuronal differentiation. Neuroblastoma cell lines provide extensively characterized models of neuronal differentiation in culture: they maintain their potential to undergo terminal neuronal differentiation upon treatment with a variety of

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[3] The abbreviations used are: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; A-TLD, A-T-like disease; DSB, double strand break; PIKK, phosphatidylinositol 3-kinase-like protein kinase; NLC, neuron-like cell; RA, retinoic acid; BDNF, brain-derived neurotrophic factor; NCS, neocarzinostatin; shRNA, small hairpin RNA; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; RARα, retinoic acid receptor α; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcription; GFP, green fluorescent protein.

agents. The resultant neuron-like cells (NLCs) are homogeneous populations of cells with the typical morphology and biochemical markers of primary cultures of neurons (15–18). We found that, similar to proliferating cells, NLCs are capable of activating a pronounced DSB response that is mediated by nuclear ATM.

EXPERIMENTAL PROCEDURES

Cell Cultures—We used two extensively characterized neuronal differentiation systems based on the human NB cell lines LA-N-5 and SH-SY5Y, which differentiate into NLCs upon treatment with retinoic acid (RA) or a combination of RA and brain-derived neurotrophic factor (BDNF), respectively (15–22). LA-N-5 and SH-SY5Y cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Human embryonic kidney cells, HEK293T, U2OS, and HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Differentiation of LA-N-5 and SH-SY5Y cells was carried out as described previously (15, 16). Briefly, LA-N-5 cells were treated with 10 μM RA (Sigma) for 7 days in complete medium and SH-SY5Y cells were treated with 10 μM retinoic acid in complete medium for 5 days and for another 2 days with 40 ng/ml BDNF (Cytolab, Rehovot, Israel) in serum-free medium.

DNA-damaging Agents and ATM Inhibitors—Neocarzinostatin (NCS) was obtained from Kayaku Chemicals Co. (Tokyo, Japan), etoposide was from Sigma. X-irradiation was carried out using an MG165 irradiator (Philips, Hamburg, Germany). The ATM inhibitor KU-55933 was a gift from Graeme Smith and Steve Jackson (KuDOS Pharmaceuticals Ltd. and Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, UK, respectively).

Antibodies—Antibodies were purchased from the following manufacturers: α-vimentin monoclonal antibody, α-neurofilament 200 (NF 200) polyclonal antibody, α-MAP-2 monoclonal antibody, α-tau polyclonal antibody, α-GAP-43 monoclonal antibody, and α-tubulin monoclonal antibody (from Sigma); α-pS139-H2AX (from Upstate Biotechnology, Inc., Waltham, MA); α-Tuj1 monoclonal antibody (from Covance Research Products, Berkeley, CA); α-p53 monoclonal antibody DO-1, α-ATR polyclonal antibody, α-hsc70 monoclonal antibody, α-TrkA polyclonal antibody, and α-p27 polyclonal antibody (from Santa Cruz Biotechnology Inc., Santa Cruz, CA); α-pS15-p53 polyclonal antibody, α-pT68 Chk2 polyclonal antibody, α-pSQ/pTQ polyclonal antibody, α-pS1981 ATM monoclonal antibody (used for immunostaining) (from Cell Signaling Technology, Beverly, MA); α-aprataxin (from Bethyl Laboratories, Inc., Montgomery, TX); α-pS1981 ATM polyclonal antibody (for Western blotting analysis) (from Rockland, Gilbertsville, PA); α-pS957/SMC1 polyclonal antibody (from Novus Biologicals, Inc., Littleton, CO); α-DNA-PK monoclonal antibody (from NeoMarkers, Labvision, CA); α-Nbs1-polyclonal antibody and α-N-Myc monoclonal antibody (from Oncogene, San Diego, CA); α-ki-67 polyclonal antibody (from Zymed Laboratories Inc., San Francisco, CA). α-Rb monoclonal antibody (from BD Biosciences). α-53BP1 antibody was a gift from Thanos Halazonetis, α-pS2056/DNA-PK was a gift from David Chen, α-ATM 5C2 was a gift from Eva Lee, and α-SMC1 polyclonal antibody was a gift from Kyoko Yokomori. α-ATM monoclonal antibody MAT3 was produced in our laboratory in collaboration with N. Smorodinsky; α-ATM Y170 rabbit monoclonal antibody was obtained from Epitomics, Inc. (Burlington, CA); secondary antibodies α-mouse IgG and α-rabbit IgG were purchased from Molecular Probes (Leiden, Netherlands), and horseradish peroxidase-conjugated α-rabbit IgG and α-mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Vectors and Constructs—Small hairpin RNA (shRNA) containing a sequence complementary to a 19- or 21-nt sequence beginning at position 912 or 8538 of ATM mRNA (GenBank™ accession number U33841) was cloned into the retroviral vector pRetroSuper (23) (a gift from R. Agami). ATM was stably knocked down using two siRNA sequences corresponding to positions 912 (shRNA#1) and 8538 (shRNA#3) of the ATM transcript. Cells were infected with retroviral particles according to standard protocols and selected with 0.5 μg/ml (LA-N-5) or 1 μg/ml (SH-SY5Y) puromycin.

The following constructs were used: GFP, 5′-gatccccGGAGCGCACCATCTTCTTCttcaagagaGAAGAAGATGGTGCGCTCCtttttggaaa-3′; LacZ, 5′-gatccccAAGGCCAGACGCGAATTATttcaagagaataattcgcgtctggccttttttttggaaa-3′; ATM#1 (position 912), 5′-gatccccGACTTTGGCTGTCAACTTTCGttcaagagaCGAAAGTTGACAGCCAAAGTCtttttggaaa-3′; ATM#3 (position 8538), 5′-gatccccTGAAGATGGTGCTCATAAAttcaagagaTTTATGAGCACCATCTTCAttttttggaaa-3′.

RT-PCR—Five μg of total RNA were used for cDNA synthesis primed by oligo(dT) and carried out using Superscript II RNase H reverse transcriptase (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was used as control.

The primers used are as follows: calcitonin, 5′-GTCGCCGCCGCTTCCACA 3′ (forward) and 5′-ACTAGATTACCGCACCGCTTAGA-3′ (reverse); hGAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse); retinoic acid receptor α (RARα), 5′-GTGCATCATTAAGACTGTGGAGTTC-3′ (forward) and 5′-CTGCAGCATGTCCACCCGGTC-3′ (reverse).

Immunofluorescence Microscopy—Proliferating LA-N-5 and SH-SY5Y cells were seeded on 0.1% gelatin or collagen precoated glass coverslips, respectively, and underwent neuronal differentiation as described above. NLCs were treated with DSB-causing agents and 30 min after treatment were fixed in 4% buffered paraformaldehyde for 20 min followed by a 10 min incubation in phosphate-buffered saline containing 0.5% Triton X-100. Coverslips were blocked for 30 min with 1% bovine serum albumin and 10% normal donkey serum in phosphate-buffered saline and incubated overnight in 4 °C with primary antibodies diluted in primary antibody dilution buffer (Biomeda Corp., Foster City, CA), washed, incubated with secondary antibody for 30 min, and stained with DAPI. Immunostaining of ATM was carried as described previously (24). Immunofluorescence analysis was carried out using a Leica TCS SP2 confocal laser scanning microscope.

Immunoblotting Analysis—Cell were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA supplemented with a mixture of protease and phosphatase inhibitors. Gel electrophoresis, blotting, reaction with antibodies, and development of blots using enhanced chemiluminescence were carried out as described previously (14).

RESULTS

Characterization of Neuronal Differentiation of LA-N-5 and SH-SY5Y—Following treatment with RA (LA-N-5 cells) or sequential exposure to RA and BDNF (SH-SY5Y cells), these cell lines underwent morphological differentiation into NLCs resembling primary neurons, including cell dissociation, dendrite extension, and formation of extensive networks (Fig. 1*A*). These morphological changes were accompanied by the expression of several neuron-specific markers: neurofilament 200 (NF200), neuron-specific class III β-tubulin Tuj1, neuronal polarity markers such as tau, microtubule-associated protein (MAP2) and growth-associated protein 43 (GAP43), the neurotrophic receptor TrkA, the retinoic acid receptor, and the neuropeptide calcitonine (Fig.

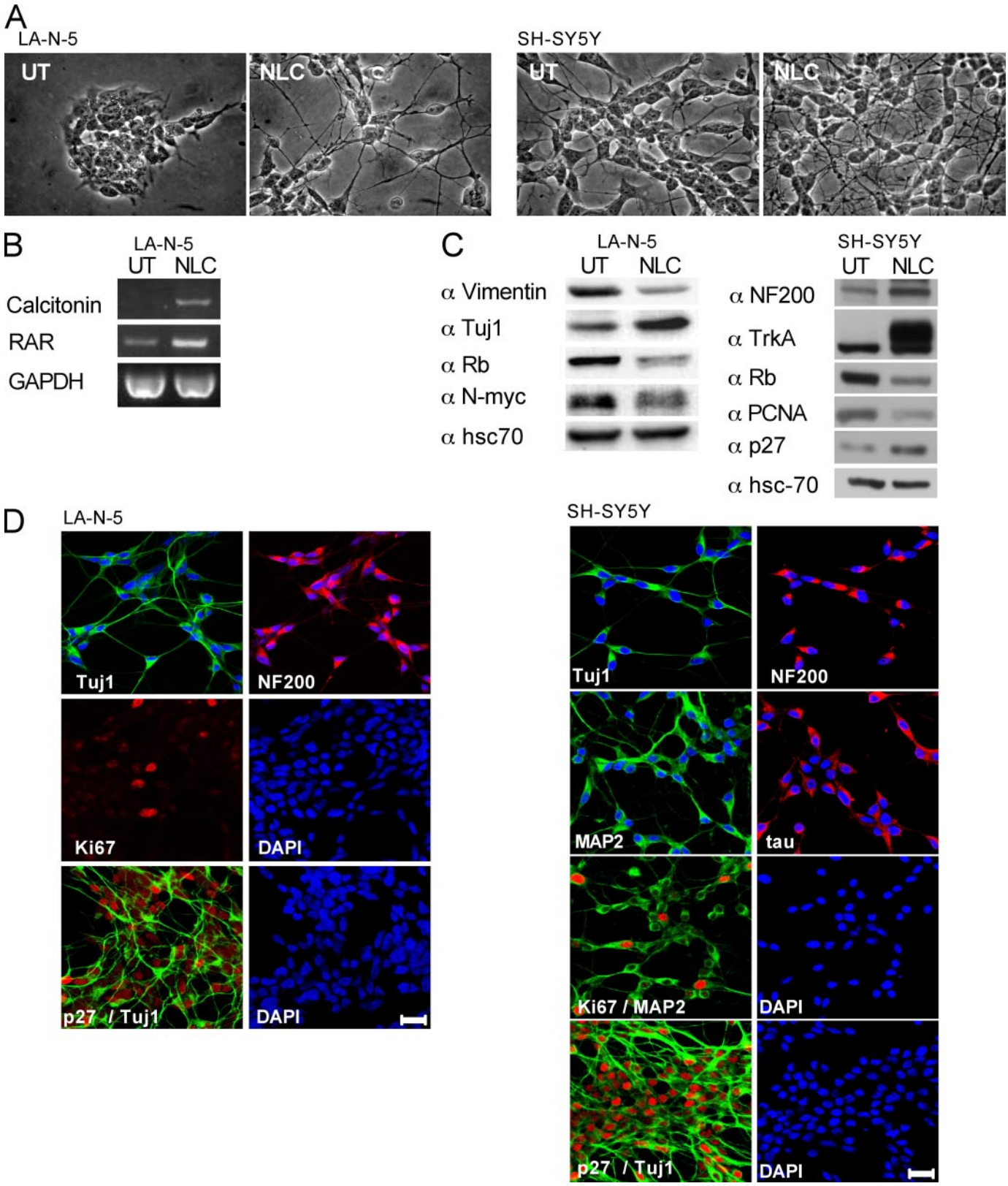

FIGURE 1. **LA-N-5 and SH-SY5Y differentiate into NLCs.** LA-N-5 (*left panel*) and SH-SY5Y (*right panel*) cells underwent neuronal differentiation as described under "Experimental Procedures." Morphological and molecular markers of neuronal differentiation were monitored. *A*, phase contrast micrographs show untreated LA-N-5 cells as adherent clusters cells and untreated SH-SY5Y cells as mainly neuroblastic (N-type). After 7 days of treatment the cells morphologically resemble neurons forming extensive networks. *B*, RT-PCR shows increased levels of the transcripts of the neuropeptide calcitonin and the RARα in differentiated LA-N-5 cells. *C*, Western blotting analysis reveals decreased levels of the proteins vimentin, pRb, N-Myc, and proliferating cell nuclear antigen and an increase in the neuronal markers Tuj1, NF200, TrkA, and the Cdk-inhibitor $p27^{kip1}$ in NLCs. *D*, immunostaining of NLCs shows expression of the neuronal cell markers NF200, Tuj1, MAP2, and tau. While most of the NLCs express the Cdk-inhibitor $p27^{kip1}$, only a small fraction of them express the proliferation marker ki-67. It should be noted that expression of NF200, tau, and also GAP43 were detected also in the long axonal-like process of the NLCs (see supplemental Fig. 1). *UT*, untreated with differentiation inducers. DAPI, nuclear staining. *Bar* = 15 μm.

1, *B–D*, and supplemental Fig. 1). Notably, there was a decrease in the level of vimentin, an intermediate filament expressed in mesenchymal cells but not in neurons (Fig. 1*C*). Importantly, the majority of differentiated NLCs were no longer cycling, evidenced by the small fraction of cells exhibiting the proliferation marker ki-67 (Fig. 1*D*); the reduced levels of proliferating cell nuclear antigen, the retinoblastoma protein pRb and the oncoprotein N-Myc, and the induction of the Cdk-inhibitor $p27^{kip1}$ (Fig. 1, *C* and *D*). These changes in morphology and molecular markers are typical of differentiated NLCs as described previously (15, 16, 19, 25).

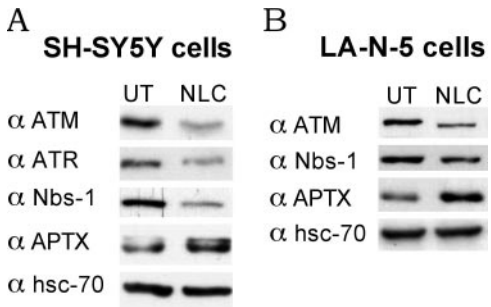

FIGURE 2. **Changes in the levels of DNA damage response proteins in NLCs.** Western blotting analysis indicates that during neuronal differentiation there is a reduction in the cellular levels of the PIKK kinases ATM and ATR and of Nbs1, a member of the MRN complex, a sensor of DSBs required for ATM activation, while APTX, which is involved in single strand break processing, is elevated. The heat shock protein hsc-70 served as loading control.

Changes in the Levels of DNA Damage Response Proteins upon Differentiation into NLCs—It was previously reported that ATM expression is highest in the embryonic nervous system and markedly reduced in the adult nervous system (11, 26, 27). We asked whether the differentiation into NLC affected the cellular levels of several key players in the DNA damage response: the ATM protein; the Nbs1 protein, a member of the MRN (Mre11/Rad50/Nbs1) complex, a DSB sensor that is involved in the initial processing of the breaks and is required for ATM activation (14, 28–30); the protein kinase ATR, which belongs to the PIKK family and responds to replication stress and UV damage but also acts redundantly with ATM in the late stages of the DSB response (31); and the aprataxin protein (APTX1), which is involved in the repair of single strand breaks and is defective in patients with ataxia oculomotor apraxia 1 (AOA1) (32–35). Notably, neuronal differentiation in culture was followed by various degrees of reduction in the cellular levels of all the DSB response proteins tested, with the exception of the aprataxin protein, which increased (Fig. 2).

ATM Knockdown Does Not Affect Neuronal Differentiation—To examine the ATM dependence of DSB response in NLCs it was essential to knock down their endogenous ATM and obtain isogenic NLCs that

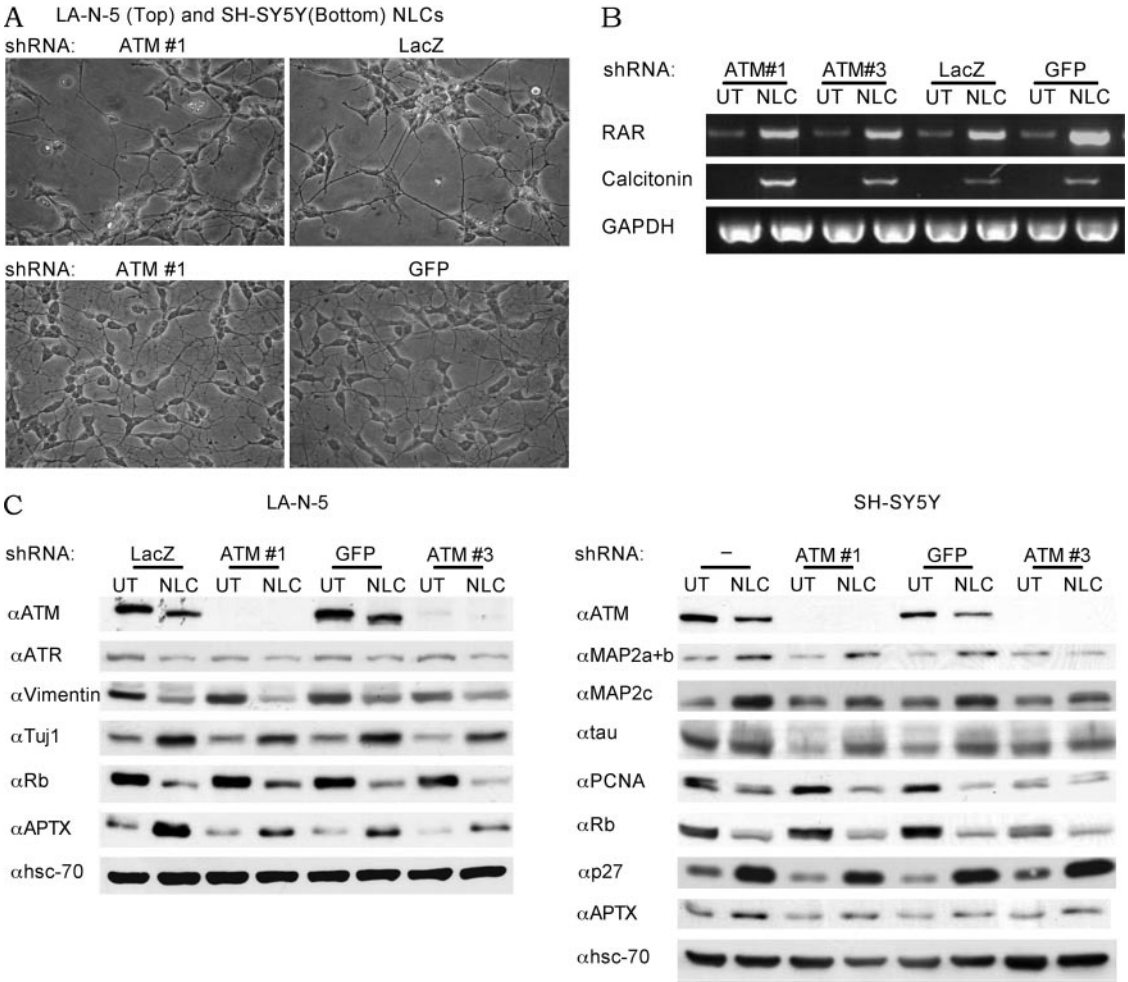

FIGURE 3. **Uninterrupted neuronal differentiation of neuroblastomas knocked down for ATM.** ATM was stably knocked down in LA-N-5 and SH-SY5Y cells by expressing in them two shRNAs against ATM mRNA (ATM#1 and ATM#3). Two irrelevant shRNAs (against LacZ and GFP) were used as controls. *A*, phase contrast micrographs show typical neuronal morphology of NLCs expressing various shRNAs. *B*, RT-PCR analysis shows the typical increase in transcripts of the RARα and the neuropeptide calcitonin in these NLCs. *C*, Western blotting analysis demonstrates the different molecular markers of neuronal differentiation in NLCs expressing various shRNAs. In general, the parameters of neuronal differentiation of cells expressing the various shRNAs are comparable with those of non-manipulated cells (Fig. 1).

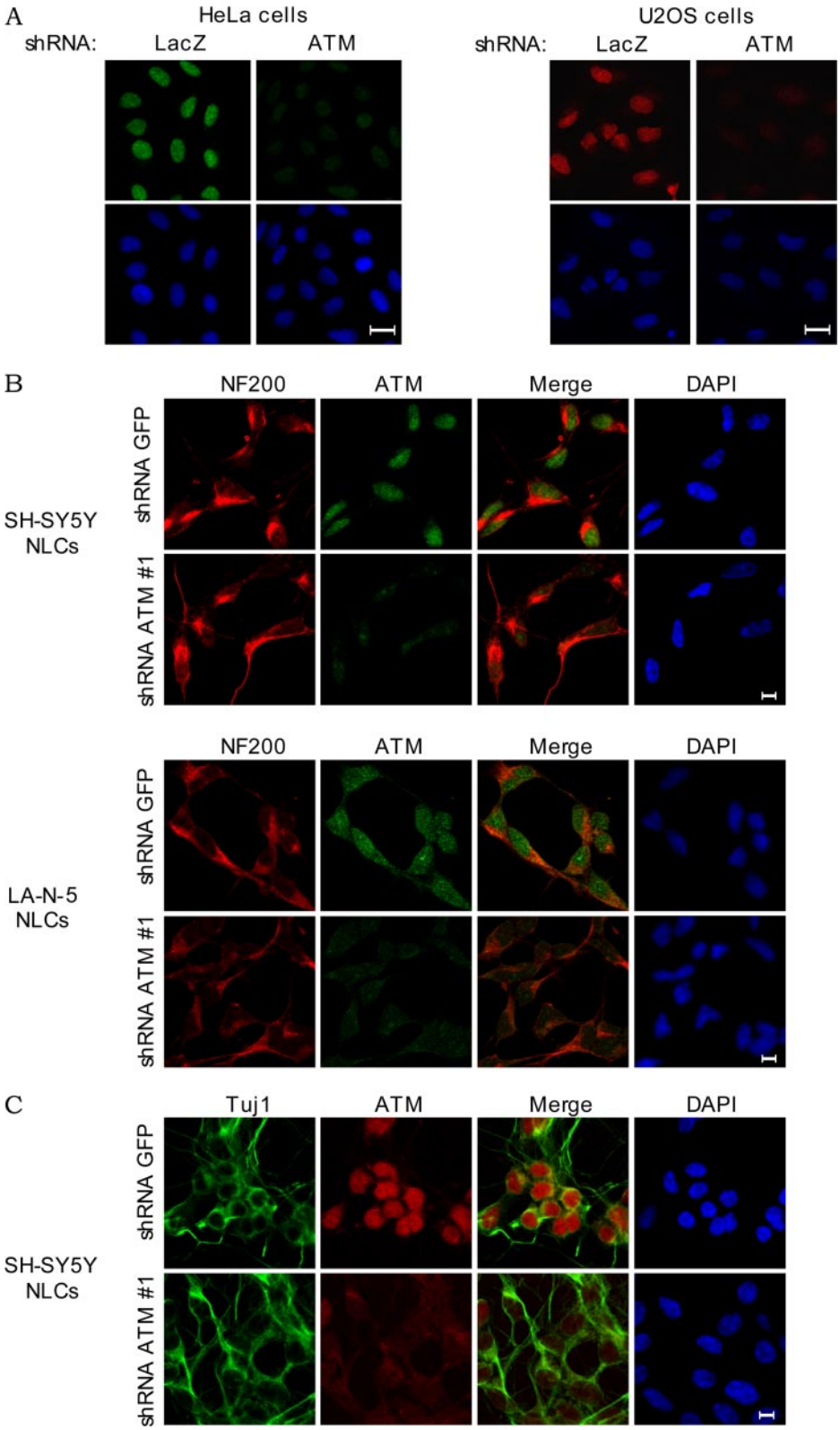

FIGURE 4. **Nuclear ATM in NLCs.** *A*, demonstration of the ATM specificity of the mouse monoclonal antibody 5C2 (*green*) and rabbit monoclonal antibody Y170 (*red*) used in this study. ATM was knocked down in HeLa and U2OS cells in which ATM is largely nuclear. Note the considerable reduction in nuclear staining in ATM-deficient cells in both antibodies. *Bar* = 10 μm. *B*, NLCs derived from ATM-deficient and proficient SH-SY5Y cells and LA-N-5 were immunostained with the 5C2 antibody (*green*). NF200 (*red*) served as a neuronal marker. *C*, NLCs derived from ATM-deficient and proficient SH-SY5Y cells were immunostained with the Y170 antibody (*red*). NF200 (*green*) served as a neuronal marker. The vast majority of ATM is nuclear in NLCs. DAPI, nuclear staining. *Bar* = 5 μm.

differ only in their ATM content. We stably knocked down ATM in LA-N-5 and SH-SY5Y cells by expressing in them two shRNAs against ATM mRNA (see "Experimental Procedures") using the retroviral vector pRETRO-SUPER (23). Irrelevant shRNAs against bacterial LacZ or GFP served as controls. We first monitored the differentiation process of ATM-deficient neuroblastomas.

Following the differentiation procedure, ATM-deficient NLCs were morphologically similar to ATM-proficient NLCs and exhibited the same neuronal markers (Fig. 3). It should be noted that although retroviral silencing may occur upon cellular differentiation (36, 37), and this could potentially affect the stability of shRNA expression, ATM knockdown remained stable in our NLCs throughout their life in culture. We

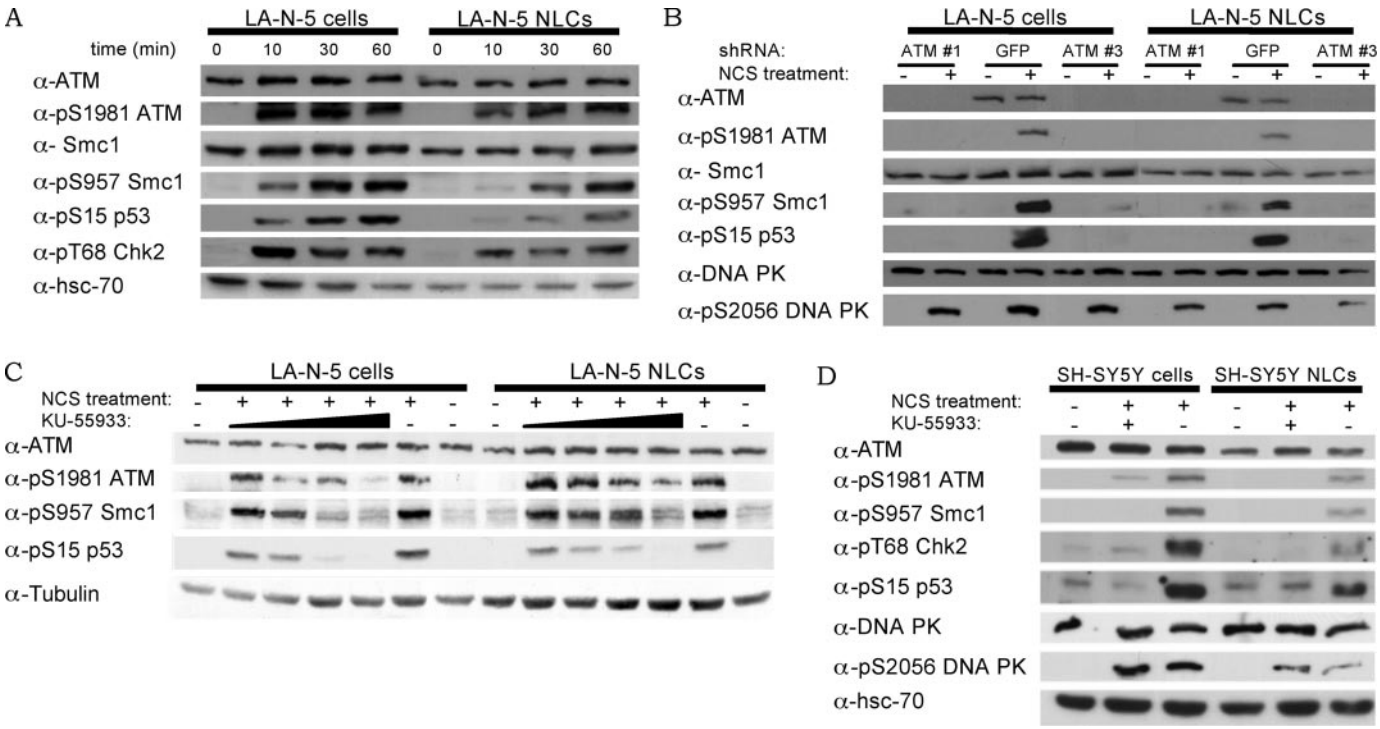

FIGURE 5. **ATM-dependent DNA damage responses in NLCs.** Western blotting analysis was used to monitor the phosphorylation of the following ATM targets: ATM autophosphorylation on Ser[1981] (7), phosphorylation of the cohesin subunit Smc1 on Ser[957] (54, 55), phosphorylation of p53 on Ser[15] (85, 86), phosphorylation of the Chk2 protein kinase on Thr[68] (87). *A*, comparison of DNA damage-induced phosphorylation of ATM targets in LA-N-5 cells and LA-N-5-derived NLCs. The cells were treated with 100 ng/ml the radiomimetic drug NCS for the indicated time points, and cellular lysates were subjected to immunoblotting analysis. *B*, similar analysis with ATM-deficient and proficient LA-N-5 cells and derived NLCs. The cells were treated with 100 ng/ml NCS for 30 min. We also monitored a DNA damage response that is not ATM-dependent: the autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) on Ser[2056] (88). DNA-PK is a member of the PIKK family, which is involved in DSB repair (89). As expected, this phosphorylation was not affected by ATM absence. All other responses are ATM-dependent in both cell types. *C*, cell cultures were preincubated for 1 h with increasing amounts (0.5–10 μM) of the specific ATM inhibitor KU-55933 (40) and subsequently treated with 200 ng/ml NCS for 30 min. Note the similar effect of the inhibitor on the phosphorylation of ATM targets in LA-N-5 cells and NLC derivatives. *D*, SH-SY5Y cells and NLC derivatives were pretreated with 10 μM KU-55933 for 1 h and then with 100 ng/ml NCS for 30 min. Note the lack of effect of the inhibitor on autophosphorylation of DNA-PKcs on Ser[2056].

concluded that in this system neuronal differentiation does not require normal ATM levels. The availability of ATM-deficient NLCs now allowed us to explore the ATM-mediated DNA damage response in this type of cells.

Nuclear ATM in NLCs—It is well accepted that ATM in cultured proliferating cells is predominantly a nuclear protein with a minor cytoplasmic fraction (38). However, several reports claimed that ATM in neuronal tissues was mainly cytoplasmic (11–13). We used immunostaining to examine the subcellular localization of ATM in our NLCs. Although most of the currently available anti-ATM antibodies were tested by us and found not to be ATM-specific in immunostaining tests, the monoclonal antibody 5C2 and the monoclonal antibody Y170 that were used in this study were more reliable. Knocking down ATM in HeLa and U2OS cells considerably reduced their nuclear signals (Fig. 4*A*), and we attributed the remaining signal to residual ATM or low cross-reactivity with another nuclear protein. Importantly, in ATM-proficient NLCs these antibodies exhibited strong nuclear staining, which turned into faint cytoplasmic or pan-cellular staining in ATM-deficient NLCs (Fig. 4*B*, C). We regard the staining pattern in ATM-deficient NLCs as background caused by nonspecific reactions of the antibody in the absence of its primary antigen. Similar results were obtained with the other ATM shRNA. We concluded that while a minor cytoplasmic fraction of ATM in NLCs could not be ruled out, the vast majority of its content in these cells was nuclear.

Activation of the ATM-mediated DSB Response in NLCs—The above results set the scene for close examination of the ATM-mediated DNA damage response in NLCs. This elaborate signaling network can be monitored by following the phosphorylation of its various components, including the autophosphorylation of ATM that marks its activation (7), and phosphorylations of ATM targets. The main tools in this analysis are specific anti-phospho antibodies. We treated LA-N-5 cells and their NLC derivatives with the radiomimetic drug NCS, a potent inducer of DSBs in the DNA (39). Western blotting analysis using anti-phospho antibodies showed ATM activation and phosphorylation of several downstream substrates in both the parental LA-N-5 cells and the derived NLCs, with some attenuation in NLCs compared with the parental cells (Fig. 5*A*). Importantly, these responses were ATM-dependent: in both the parent cells and the NLCs, knocking down ATM abolished these phosphorylations (Fig. 5*B*). Similar results were obtained with SH-SY5Y cells and derived NLCs (data not shown). Administration of a specific ATM inhibitor, KU-55933 (40) into the cultures abrogated these responses in a dose-dependent manner (Fig. 5, *C* and *D*).

We further characterized the DSB response in NLCs by immunostaining, which allows subcellular localization of the response and demonstration of one of its important facets, the formation of nuclear foci at the damage sites by recruited damage response proteins. The staining readouts of the DSB response in NLCs were similar to those obtained in commonly used proliferating cell lines and were typically nuclear (Fig. 6*A*) and ATM-dependent (Fig. 6*B* and supplemental Figs. 2 and 3). Either ATM knockdown (Fig. 6*B*) or the ATM inhibitor (supplemental Fig. 3) abrogated these responses or abolished them completely. We concluded that our NLCs possess a DSB response with similar characteristics to that of proliferating cells, which is similarly mediated by activated nuclear ATM.

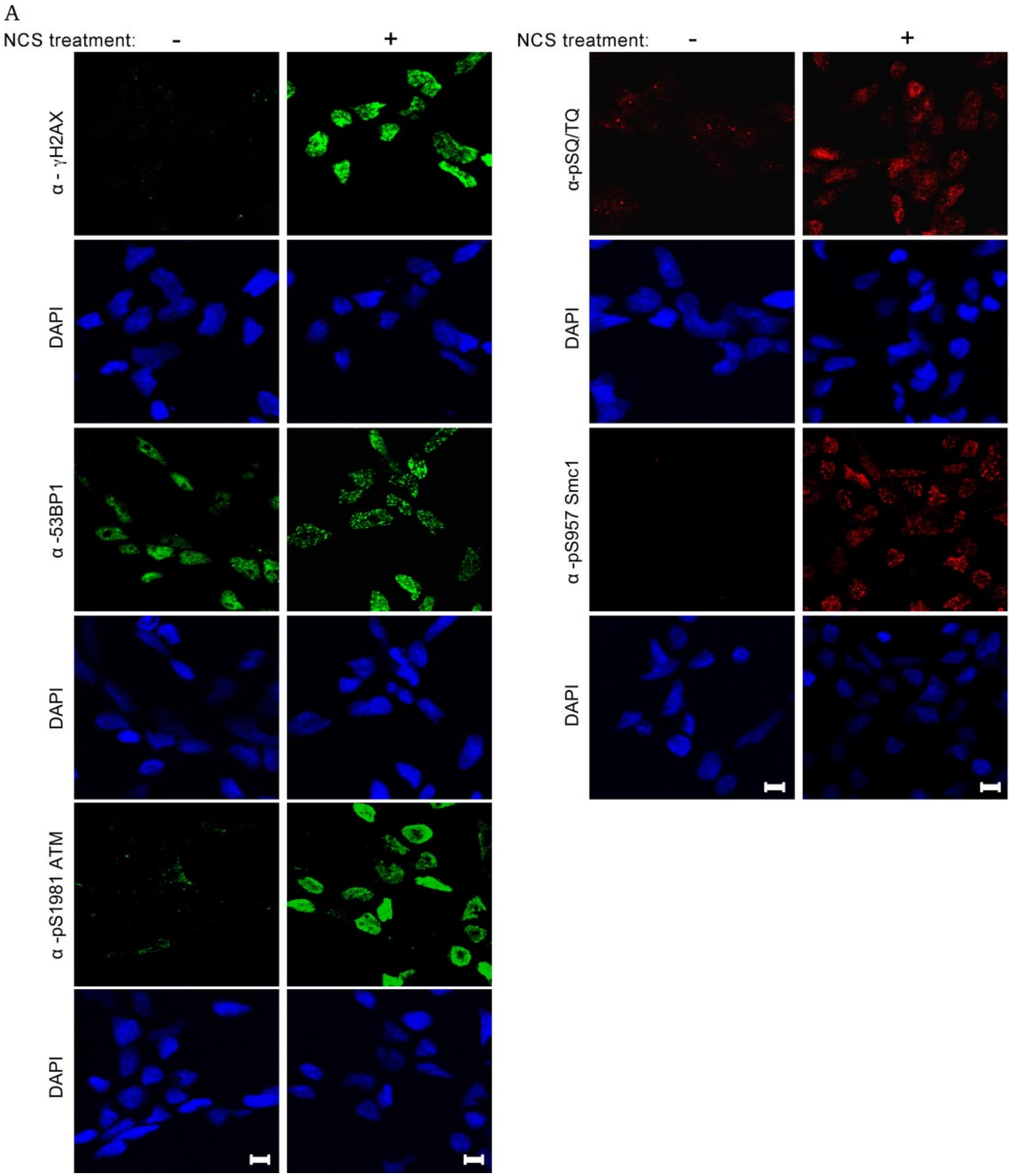

FIGURE 6. **Nuclear DNA damage responses in NLCs are ATM-dependent.** *A,* initial characterization of the DSB response in LA-N-5- derived NLCs using immunostaining. The cells were treated with 200 ng/ml NCS, and the following nuclear responses were monitored 30 min later: phosphorylation of histone H2AX at the DSB sites (90); recruitment of the sensor/mediator protein 53BP1 to DSB sites, evidenced by formation of 53BP1 foci (91); ATM autophosphorylation on Ser^{1981} (7); phosphorylation of several PIKK targets collectively detected by an antibody against phosphorylated SQ or TQ motifs (14); phosphorylation of Smc1 on Ser^{957} (54, 55). The nuclear localization and appearance of these responses are similar to those observed in proliferating cells. *Bar* = 5 μm. *B*, abolishment of the above responses in NLCs knocked down for ATM. Similar results obtained with the shRNA ATM#3 (data not shown). DAPI, nuclear staining. *Bar* = 10 μm.

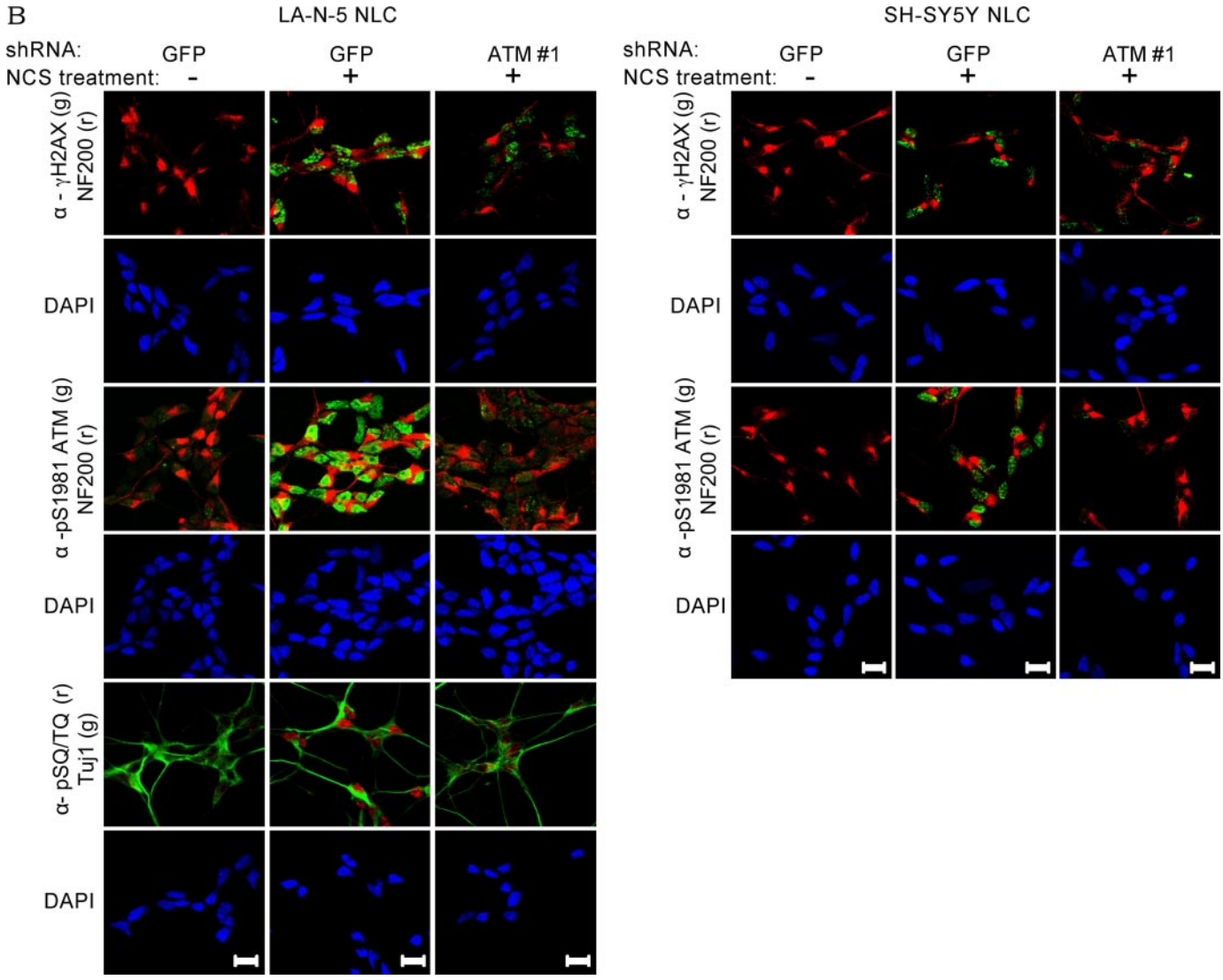

FIGURE 6—*continued*

DISCUSSION

A-T is caused by loss of ATM function. Its prominent symptom is the progressive neurological deterioration. Therefore, understanding the role of ATM in neuronal cells is central to understanding the disease. The present study was aimed at disclosing and characterizing the ATM-mediated DNA damage response in human NLCs. This attempt grew out of our recognition, that the neuronal degeneration in A-T results from defective DNA damage response and not from lack of other, putative ATM functions. This conclusion was based on investigation of another genomic instability syndrome, A-T-like disease (A-TLD), which is similar to A-T and shares with A-T the neuronal degeneration but exhibits later age of onset and slower progression (41). In A-TLD the defective protein is Mre11, a component of the MRN complex that serves as a sensor and initial processor of DSBs (28, 41, 42). Previous studies from our laboratory showed that the MRN complex was required for ATM activation in response to DSBs (14). We therefore concluded that in A-TLD, and probably in A-T, the neuronal degeneration is due to defective ATM-mediated response to DSBs (14).

Here we used a model system in which neuronal differentiation is induced in cultured cells. While being a culture-based system, the NLCs obtained exhibit many characteristics typical of neurons and are considered to faithfully represent neuronal cells *in vivo* (15, 16, 19).

Our model system mimics another *in vivo* characteristic of neuronal differentiation, the reduction in cellular levels of several DNA damage response proteins (Fig. 2) (11, 26, 27, 43–46). This decline does not detract, however, from the critical importance of these proteins in neurons. Further reduction in their amount has been associated with various neurodegenerative pathologies (47, 48). Interestingly, the opposite trend was observed for APTX1 (aprataxin), the product of the AOA1 gene (32, 33), which is involved in single strand break repair (34, 35). It has been suggested that single strand breaks are more predominant DNA lesions in mature neurons than in dividing cells (45), hence the increase in APTX1 level may reflect the increased intensity of this repair pathway in NLCs.

When we knocked down ATM in the parental neuroblastoma cells and let them differentiate, the reduction in ATM amounts below detectable levels did not interfere with differentiation (Fig. 3). This result is not surprising since lack of ATM does not seem to exert a marked effect on the development of the human nervous system. A-T patients usually appear normal at birth and their neurological symptoms develop gradually over the first decade of life. Furthermore, post-mortem histological studies have suggested that initial differentiation and maturation of neurons were undisturbed in A-T patients and these cells degenerated later in life (1). This is in contrast to previous findings in mice, that Atm

is required for differentiation of hippocampus neural progenitors cells into neurons but not into astrocytes (26). Currently there is no evidence of this phenomenon in humans.

Determination of the subcellular localization of ATM in neurons is central to understanding its role in these cells. Immunohistochemical analyses in human and murine neuronal tissues showed cytoplasmic localization of ATM in Purkinje cells and other types of neurons (11, 13). This is in contrast to the primary nuclear localization of ATM in proliferating cultured cells (49–51). The studies of Oka and Takashima (1998) and Barlow *et al.* (2000) were based on analysis of neuronal tissues while the present work employed immunostaining of cultured cells. Immunofluorescence analysis is sensitive to experimental conditions and is strictly dependent on the specificity of the antibody for the target protein. Furthermore, the low level of ATM in mature neurons (11, 26, 27) probably requires a highly specific antibody with very high affinity for ATM for such analysis. We demonstrated the specificity of our two antibodies using ATM-deficient cells (Fig. 4), and we used Wilson and Bianchi's (1999) (24) protocol, which had been adapted for immunostaining of nuclear mammalian proteins and enabled the detection of previously undetectable or poorly detectable proteins. Our results demonstrated that the vast majority of cellular ATM in NLCs is nuclear (Fig. 4).

The subcellular localization of ATM in neurons suggests nuclear functions, most likely similar to those in proliferating cells. Indeed, using a variety of readouts we identified in our NLCs a vigorous, highly responsive ATM-mediated DNA damage response. ATM activation, evidenced by its autophosphorylation as well as the phosphorylation of several ATM targets (Figs. 5 and 6 and supplemental figures 2 and 3) were remarkably similar to what we and others observed repeatedly in commonly used cell lines. We concluded that ATM transduces the DNA damage alarm in NLCs similarly to in proliferating cells. Notably, another study has recently showed the existence of functional ATM-mediated damage response in proliferating myoblasts and their differentiated counterparts, myotubes (52). Additional functions of ATM still cannot be ruled out in any cell type, but we feel it is safe to say that activating the DSB response is the major role of ATM in proliferating as well as differentiated cells.

Among the ATM substrates whose phosphorylation was monitored in this study were the checkpoint kinase Chk2 (53) and the cohesion subunit Smc1, whose ATM-mediated phosphorylation has been associated with the intra-S cell cycle checkpoint (54, 55). Given the post-mitotic nature of neurons, the DNA damage response was not expected to activate the cell cycle checkpoints. However, evidence is emerging that in neurodegenerative diseases and following various insults including DNA damage, neurons attempt to re-enter the cell cycle and this attempt is followed by programmed cell death (56–62). Expression of cell cycle proteins was reported in human and murine ATM-deficient neurons (63). The exact roles of cell cycle signaling in the DNA damage response in neurons is unclear, and the corresponding proteins may be involved in these cells in other processes such as DNA repair (56). Significantly, ATM suppression was found to attenuate the damage-induced recycling attempts and consequently the apoptotic process, in neuronal cells following DNA damage (57). This observation can be linked to earlier findings on resistance of neuronal tissues in $Atm^{-/-}$ mice to DNA damage-induced apoptosis (64–66). Of note, we too observed resistance of ATM- deficient NLCs to DNA damage-induced apoptosis when compared with ATM-proficient NLCs.[4] In the developing nervous system this process may serve to remove cells from the system that suffer irreparable damage (64, 66–69). Under experimental conditions these processes are induced by acute genotoxic stress. In everyday life they are probably induced also by the constant exposure of neuronal cells to high oxidative stress (59, 70–74).

The importance of maintaining genomic stability in neurons cannot be overemphasized. Their finite number, long life, high metabolic rate, and continuous exposure to oxidative stress on the one hand, and extensive gene transcription on the other hand, call for stringent control of genomic integrity. It is not surprising that defects in various branches of the DNA damage response lead to severe neurological demise (75–78). In this regard, Atm-knock-out mice were an unexpected exception: while exhibiting most of the characteristics of human A-T, they barely show the cerebellar degeneration and associated neuromotor dysfunction (79–81). A severe cerebellar phenotype that mimics the human one was eventually obtained by conditionally knocking out the Nbs1 protein in the nervous system (82). This phenotype can be explained by the requirement of the MRN complex for full activation of both ATM and ATR (14, 29, 83, 84). Thus, the concomitant abrogation of these two axes of the DNA damage response in the nervous system might be necessary to evoke an effect in the murine cerebellum similar to that of ATM loss in humans. Importantly, these results further support the notion that in both instances the neuronal demise is caused by a defect in the DNA damage response.

Recognition that the neuronal degeneration in A-T is caused by the deficiency in the DNA damage response, and the results shown here highlight the importance of studying the ATM-mediated network in neurons to understanding A-T. This insight into the molecular basis of A-T is expected to guide the search for novel treatment modalities to alleviate the symptoms of the disease and slow its relentless progression.

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