

MYCN Sensitizes Human Neuroblastoma to Apoptosis by HIPK2 Activation through a DNA Damage Response

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Abstract

MYCN amplification occurs in approximately 20% of human neuroblastomas and is associated with early tumor progression and poor outcome, despite intensive multimodal treatment. However, MYCN overexpression also sensitizes neuroblastoma cells to apoptosis. Thus, uncovering the molecular mechanisms linking MYCN to apoptosis might contribute to designing more efficient therapies for MYCN-amplified tumors. Here we show that MYCN-dependent sensitization to apoptosis requires activation of p53 and its phosphorylation at serine 46. The p53^{S46} kinase HIPK2 accumulates on MYCN expression, and its depletion by RNA interference impairs p53^{S46} phosphorylation and apoptosis. Remarkably, MYCN induces a DNA damage response that accounts for the inhibition of HIPK2 degradation through an ATM- and NBS1-dependent pathway. Prompted by the rare occurrence of p53 mutations and by the broad expression of HIPK2 in our human neuroblastoma series, we evaluated the effects of the p53-reactivating compound Nutlin-3 on this pathway. At variance from other tumor histotypes, in MYCN-amplified neuroblastoma, Nutlin-3 further induced HIPK2 accumulation, p53^{S46} phosphorylation, and apoptosis, and in combination with clastogenic agents purged virtually the entire cell population. Altogether, our data uncover a novel mechanism linking MYCN to apoptosis that can be triggered by the p53-reactivating compound Nutlin-3, supporting its use in the most difficult-to-treat subset of neuroblastoma. *Mol Cancer Res*; 9(1); 67–77. ©2010 AACR.

Introduction

Neuroblastoma is the most common extracranial solid tumors of childhood (1) and is characterized by a broad spectrum of clinical behaviors ranging from spontaneous regression to very aggressive disease (2). Clinical and pathologic features (i.e., age at diagnosis, stage, tumor grade, histology, and ploidy) contribute to the identification of high-risk patients (3). At the molecular level, *MYCN*

amplification (MNA) represents one of the best independent parameters predicting adverse outcome and very poor survival, despite intensive multimodal treatment (4, 5). Therefore, new treatment perspectives urge for MNA neuroblastoma patients.

MYCN is a helix-loop-helix/leucine zipper transcription factor belonging to the MYC family that can affect the expression of a number of genes involved in cell cycle progression, metabolism, invasion, and angiogenesis (6, 7). MYCN expression is deregulated in several human neoplasia and significantly contributes to cancer development. Its pivotal role in neuroblastic carcinogenesis is supported by a mouse model where human MYCN expression targeted to the neural crests leads to development of tumors closely resembling human neuroblastoma (8). Furthermore, MNA neuroblastoma cells are addicted to MYCN and its depletion profoundly affects their survival, proliferation, and differentiation *in vitro* and *in vivo* (9–11).

MYCN overexpression is also directly linked to apoptosis in cell cultures and animal models (12–14). A largely validated cell system for conditional MYCN overexpression, the SHEP Tet21/N, has extensively been used to show that MYCN sensitizes MYCN single-copy (MNSC) neuroblastoma cells to apoptosis (15, 16). On the basis of the observation that impaired caspase 8 expression preferentially occurs in human MNA neuroblastomas (17), it has been proposed that loss of proapoptotic molecules and

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org>).

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doi: 10.1158/1541-7786.MCR-10-0227

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impairment of the apoptotic machinery are required for MNA-dependent tumor progression (18). More recently, however, Fulda and coworkers reported that loss of caspase 8 does not correlate with MNA (19) and many evidences support the idea that cell death can be efficiently triggered in MNA neuroblastomas. Indeed, primary MNA neuroblastomas are not insensitive to radio- and chemotherapy, at diagnosis. Consistent with this, MNA does not correlate with drug resistance *in vitro* (20) and MNA cell lines retain a higher sensitivity to ionizing radiations (IR)-induced apoptosis compared with MNSC cells (21).

Mutations in the TP53 gene are extremely rare in neuroblastomas and the p53 pathway is functionally—rather than genetically—inactivated in neuroblastic carcinogenesis (22), indicating the p53 restoration strategy as an interesting option for neuroblastoma treatment. Very recently, MYCN was shown to transcriptionally regulate p53 expression (23), leading to the hypothesis that p53 upregulation might be involved in MYCN-dependent sensitization to apoptosis. However, p53 induction was reproducibly shown to occur at similar levels in MYCN-overexpressing and -nonoverexpressing neuroblastoma cells, on treatment with clastogenic drugs (21, 24) suggesting that, although necessary, p53 expression might not be sufficient for the induction of apoptosis in neuroblastoma cells. Additional mechanisms might contribute to the MYCN-dependent sensitization to apoptosis, some of which might be directly linked to p53 activation via post-translational modifications. Indeed, we show here that MYCN-dependent sensitization to apoptosis induced by clastogenic agents requires p53 and its phosphorylation at serine 46. This p53 proapoptotic posttranslational modification is due to the accumulation of the Homeodomain Interacting Protein Kinase 2 (HIPK2) that is necessary for MYCN-dependent apoptosis induced by different anticancer drugs. Remarkably, we show that MYCN activates HIPK2 through an oncogene-dependent DNA damage response (DDR) pathway that relies on ATM activity. Eventually, we report that the HIPK2-p53 pathway is largely maintained in primary MNA neuroblastomas and can be activated by the nongenotoxic p53-reactivating compound Nutlin-3 (Nut-3), with a strong apoptotic effect either alone or in combination with clastogenic agents, suggesting that the exploitation of this pathway might be a therapeutic relevant strategy in MNA neuroblastomas.

Materials and Methods

Cell lines, culture conditions, and cell death assays

Human neuroblastoma cells (obtained from either Dr. M. Ponzoni, Gaslini Institute, Genoa; Dr. Carol J. Thiele, CMBS, NCI, Bethesda, MD; Dr. D. Fruci, Istituto Bambino Gesù, Rome; BBCF, Istituto Nazionale Ricerca sul Cancro, Genoa, between 1996 and 2009) were grown in standard conditions and validated by genetic search of MNA or other genetic aberrations. SHEP Tet21/N cells, received from Dr. Schwabb, DKFZ, Heidelberg, Germany were cultured and validated for MYCN inducibility as reported

(25). LAN-1 cells were a kind gift of Dr. Nicole Gross, Department of Pediatrics, University Hospital, Lausanne, Switzerland. Subconfluent cells were treated with bleomycin (Calbiochem; 5 μ g/mL for 24 hours, unless differently specified), adriamycin (ADR), cisplatin (CDDP), Nut-3 (Sigma Chemical Co.), or γ -irradiated (at 4 Gy/min using a ^{60}Co source at room temperature) and collected at the indicated time points for further analysis. Cell death was measured by trypan blue exclusion counting at least 200 cells, in duplicate samples. For the analysis of nuclear morphology, cells were fixed in 4% formaldehyde/PBS for 10 minutes, permeabilized in 0.25% Triton/PBS for 10 minutes, counterstained with 1 mg/mL Hoechst 33258, and mounted in PBS/50% glycerol. At least 200 cells were counted in each dish, and experiments were conducted in duplicate.

RNA preparation and quantitative reverse transcription-PCR

Total RNA extraction was carried out with TRIzol reagent (Invitrogen). For quantitative reverse transcription-PCR (Q-RT-PCR), total RNA (1 μ g) was reverse transcribed using Gene Amp kit (Applied Biosystems) and subjected to PCR amplification using SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are given in the supplementary materials. Samples underwent 40 amplification cycles (95°C for 10 seconds; 60°C for 1 minute) monitored by an ABI Prism 7900 sequence detector (Applied Biosystems). All amplification reactions were conducted at least in duplicate and averages of threshold cycles were used to interpolate standard curves and calculate transcript amount using the SDS version 2.3 software (Applied Biosystems).

Protein extraction and Western blot

Total protein extracts were obtained in RIPA buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.5% Sodiodeoxycolate, 0.1% SDS, 1% NP40, 0.001 mol/L EDTA and a mix of protease inhibitors]. Protein extracts (30 μ g/sample) were separated by SDS-PAGE and blotted onto nitrocellulose membrane (PerkinElmer). Membranes were blocked with 5% nonfat dry milk and incubated with primary antibodies (Abs) at the appropriate dilutions. Abs were as follows: polyclonal Ab anti-p85 (cleaved) fragment of the PARP protein (cPARP, Promega Corporation); rabbit anti-phosphorylated p53^{S46} polyclonal and rabbit anti-phosphorylated p53^{S15} polyclonal Abs (Cell Signaling Technology); mouse anti-p53 (DO-I) and mouse anti- α -tubulin monoclonals and goat anti- β -actin, rabbit anti-MYCN and rabbit anti-p21^{WAF1} polyclonal Abs (Santa Cruz Biotechnology); rabbit anti-Cul3 polyclonal Ab (Zymed Laboratories); rabbit HIPK2 polyclonal Ab (kindly provided by Prof. M.L. Schmitz). Immunoreactive bands were visualized by enhanced chemoluminescence (Perkin Elmer).

Transfections, RNA interference, and luciferase reporter assay

The plasmids for wild type and mutant p53 expression (pCAGp53, pCAGp53S15A, pCAGp53s46A) were pre-

viously described (26). The pSUPER-LacZ, pSUPER-p53, and pSUPER-HIPK2 plasmids for sh-RNA interference were previously described (27). SHEP Tet21/N cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. KCNR and LAN5 cells were transfected by electroporation with Nucleofector Solution V and the A-020 and T-020 programs, respectively, in a Nucleofector II (Amaxa Biosystems). ATM and NBS1 knockdown was obtained with (25 nmol/L) stealth RNA interfering oligos (ATM, custom Smart Pool, Invitrogen, HSS181472, HSS181473 and HSS181474; NBS1, see ref. 28) transfected by Dharmafect 2 Reagent (Dharmacon) according to the manufacturer's protocol. Control no-target stealth duplex oligos were as follows: sense, 5'-UAAUAAUCCUAACCGUUGUAA-CAGC-3'; antisense, 5'-GCUGUUACAACGGUUAG-GAUUAUUU-3'.

Primary tumor samples and immunohistochemical analysis

Twenty-four frozen neuroblastoma tumors selected for HIPK2 transcript expression analysis and 13 MNA paraffin-embedded neuroblastoma tumors subjected to immunohistochemistry for HIPK2 protein expression were obtained from newly diagnosed patients with neuroblastoma admitted to the Department of Oncology at Alder Hey Children's NHS Trust. Final clinicopathologic diagnosis fulfilled the International Criteria for Neuroblastoma Diagnosis. MYCN copy number was evaluated routinely and MNA was defined as more than 10 copies per haploid genome. Institutional written informed consent was obtained from the patient's parents or legal guardians. After blocking with unrelated antiserum, formalin-fixed and paraffin-embedded tissue sections were challenged with rabbit anti-HIPK2 polyclonal Ab (29) at concentration ranging between 1:200 and 1:500, in a moist chamber at 4°C followed by a secondary anti-rabbit IgG antisera according to the manufactory instruction of the LSAB detection Kit (DakoCytomation). The enzymatic activity was visualized by using 3,3'-diamino-benzidine (Dako).

Results

MYCN sensitizes neuroblastoma cells to apoptosis induced by clastogenic agents through a p53-dependent pathway

MNA neuroblastomas are sensitive to chemo- or radiotherapy at diagnosis and MNA neuroblastoma cell lines were shown to be more sensitive to IR-induced apoptosis than MNSC cells (20, 21). In keeping with these observations, a short pulse of the radiomimetic drug bleomycin induced apoptosis more efficiently in MNA neuroblastoma cells (i.e., LAN-5, IMR32, GILIN, and KCNR) compared with MNSC cells (i.e., GIMEN, SK-N-SH, and SHEP) as indicated by dead cell count and PARP cleavage (cPARP; Fig. 1A). Unfortunately, the contribution of MYCN to clastogenic agent-induced apoptosis cannot be tested by MYCN depletion in MNA neuroblastoma cells, due to their

oncogene addiction (9–11). However, the well-characterized MYCN-inducible SHEP Tet21/N cell model has been extensively used to show that MYCN sensitizes neuroblastoma cells to apoptosis (15, 16, 25). Using these cells, we found that both IR and bleomycin induced cell death in MYCN-overexpressing (since now on MYCN+) cells but not in uninduced (since now on MYCN–) cells (Fig. 1B and C and; Supplementary Fig. S1). The very similar results obtained with IR and the radiomimetic bleomycin (Supplementary Fig. S1) allowed us to use bleomycin as a surrogate for IR in the following experiments.

Apoptosis induced by radio- or chemotherapy is, at least in part, mediated by the p53 oncosuppressor. Among the MNA neuroblastoma cell lines, the mutant p53 carrying SK-N-BE2c cells failed to undergo bleomycin-induced cell death (Fig. 1A) suggesting a role for p53 in MYCN-dependent apoptosis. Indeed, p53 depletion by p53 RNA interference (p53i), although incomplete, reduced of approximately 30% the number of apoptotic nuclei induced by bleomycin in MYCN+ cells, having no consequences in MYCN– cells (Fig. 1D). Consistently, reintroduction of a wild type p53 (wtp53) in the p53-null and apoptosis-resistant MNA LAN-1 neuroblastoma cells resulted in massive apoptosis (not shown), whereas transfection of sublethal amounts of wtp53 still compatible with cell survival under basal conditions restored the apoptotic response to bleomycin (see in the following text and Fig. 2C).

Sensitization to apoptosis by MYCN is linked to p53^{S46} phosphorylation

Bleomycin induced p53 accumulation in both MYCN+ and MYCN– cells (Fig. 2A), indicating that p53 is required but not sufficient for an effective apoptotic response. However, p53 activity can be modulated by posttranslational modifications. In particular, phosphorylation at serine 15 is involved in p53 stabilization and activation on DNA damage, thus allowing it to participate in downstream cellular responses, including cell cycle arrest, senescence, and apoptosis (reviewed in refs. 30, 31). In contrast, phosphorylation at serine 46, eventually occurring at later times on DNA damage, commit p53 to proapoptotic promoters, thus favoring apoptosis rather than cell cycle arrest (32, 33). Interestingly bleomycin specifically induced p53^{S46} phosphorylation in MYCN+ cells, whereas p53^{S15} phosphorylation occurred at similar levels in the presence and in the absence of MYCN (Fig. 2A), suggesting that p53^{S46} phosphorylation was indeed specifically associated to the proapoptotic phenotype induced by MYCN. Consistent with the presence of phosphorylated p53^{S46} we observed a stronger increase in the expression of the proapoptotic p53 targets AIP-1, Noxa, Bax, and DR5 and a more modest induction of the p21^{WAF1} transcript in the bleomycin-treated MYCN+ apoptotic cells as compared with MYCN– cells (Fig. 2B). To further assess the relevance of p53^{S46} phosphorylation in the context of the MYCN-dependent apoptotic phenotype, we transfected the p53-null MNA LAN-1 cells with wtp53 and its nonphosphor-

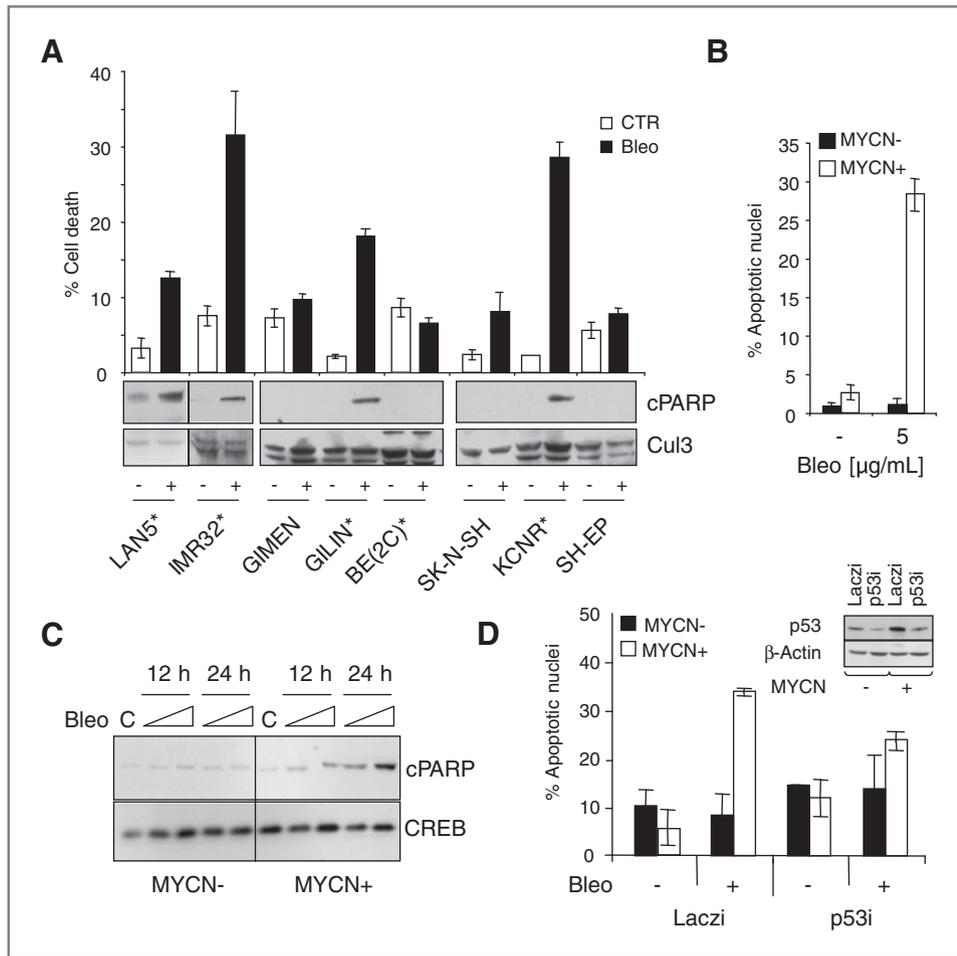


Figure 1. Sensitization of neuroblastoma cells to bleomycin-induced apoptosis by MYCN requires p53. A, cell death occurring 24 hours after bleomycin administration (5 μg/mL, 1-hour pulse) in a panel of neuroblastoma cell lines was measured by trypan blue exclusion test (top) and confirmed to occur via apoptosis by immunoblot detection of the cleaved fragment of PARP (cPARP, bottom). *, MNA cell lines; Cullin-3 (Cul-3) expression was used as a loading control for all extracts, with the exception of LAN-5, normalized with β-actin (not indicated in the figure). B and C, MYCN-dependent cell death induced by continuous treatment with bleomycin for 48 hours in MYCN+ and MYCN- SHEP Tet21/N cells, quantified by counting Hoechst 33258 stained apoptotic nuclei of adherent cells (B), and by immunoblot detection of cPARP (C). D, p53 knockdown was achieved by transient transfection with sh-RNAi in MYCN+ and MYCN- cells (inset). Count of apoptotic nuclei showed that p53 specific RNAi (p53i), but not LacZ control RNAi (LacZi), impairs bleomycin-induced apoptosis occurring in MYCN+ cells.

ylatable mutants p53^{S15A} and a p53^{S46A}. As expected for an early step of p53 activation (34, 35), loss of S15 phosphorylation, as obtained by p53^{S15A} mutant overexpression, impaired p53 activity, despite a constitutive phosphorylation at p53^{S46} (Fig. 2C). Of relevance, similarly impaired apoptotic rates were induced by p53^{S46A} mutant overexpression in spite of p53^{S15} phosphorylation (Fig. 2C), confirming that phosphorylation at p53^{S46} is required for the MYCN-dependent apoptotic phenotype induced by bleomycin in neuroblastoma cells. Taken together, these data indicate that p53 can be recruited into a drug response in neuroblastoma cells and that MYCN might drive p53 toward the apoptotic pathway, possibly through the induction of kinase/s responsible for p53^{S46} phosphorylation.

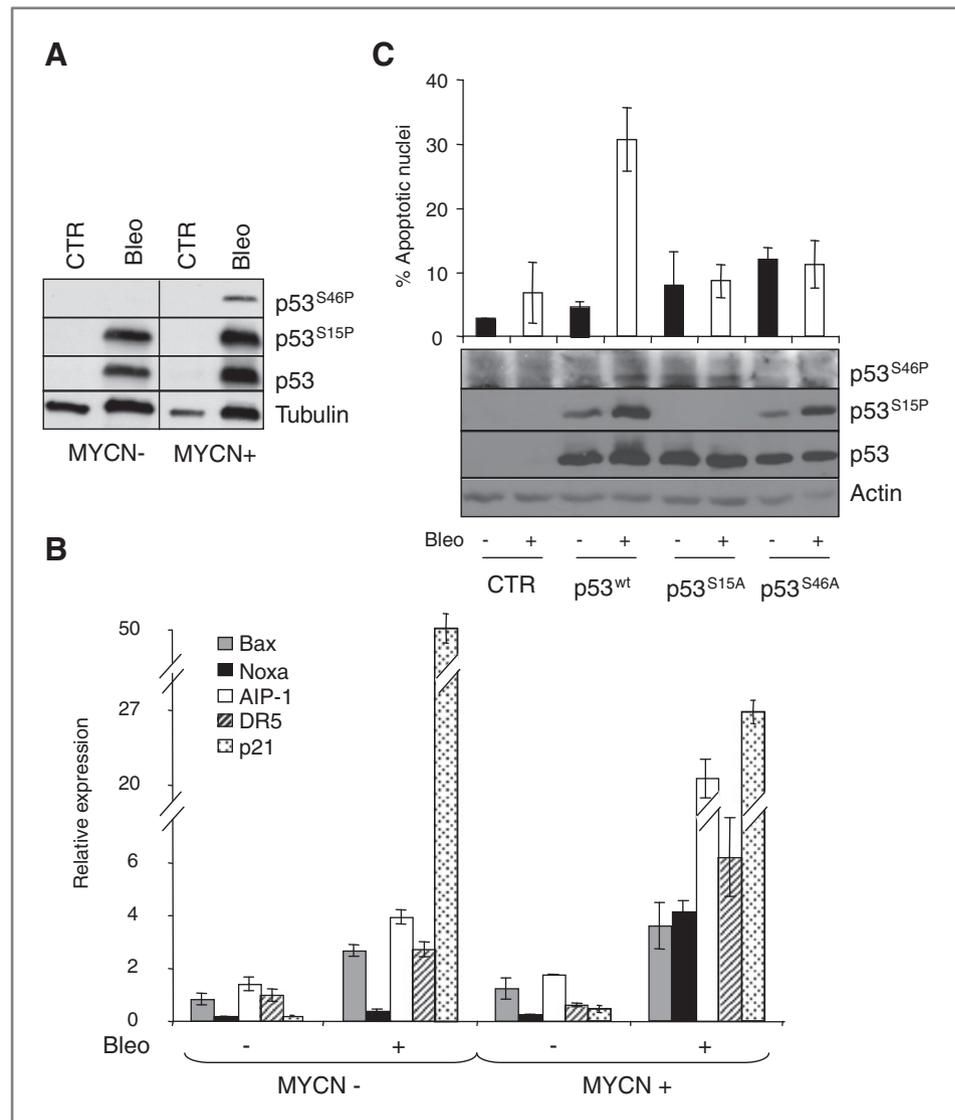
MYCN promotes accumulation of the p53^{S46} proapoptotic kinase HIPK2 by activating a DDR pathway

HIPK2 is one of the p53^{S46} kinases and a potent activator of p53 apoptotic functions (26, 36). Remarkably, HIPK2 depletion by specific sh-RNAi strongly reduced the occurrence of bleomycin-induced apoptosis, cPARP accumulation, and p53^{S46} phosphorylation in MYCN+ cells (Fig. 3A and B). In contrast, no significant modification of total p53

and p21^{WAF1} protein accumulation occurred under these conditions (Fig. 3B). HIPK2 depletion also impaired MYCN-dependent sensitization to apoptosis induced by different drugs, such as ADR and CDDP (Supplementary Fig. S2), indicating that HIPK2 is a key element through which MYCN sensitizes neuroblastoma cells to clastogenic agents.

Because bleomycin specifically induced p53^{S46} phosphorylation in MYCN+ cells and HIPK2 was required for MYCN-dependent apoptosis, we tested whether MYCN might control HIPK2 expression. Increased HIPK2 protein, but not mRNA levels, was detected on MYCN induction in Tet21/N cells or MYCN overexpression in SK-N-MYC stable transfectant (Fig. 3C; Supplementary Fig. S3A) suggesting a nontranscriptional type of regulation. Interestingly, in MYCN- cells, inhibition of protein degradation by the proteasome inhibitor MG132 induced HIPK2 accumulation (Fig. 3D), suggesting that HIPK2 induction by MYCN might occur at the level of protein stability. HIPK2 expression is largely controlled via proteasomal degradation by several E3 ubiquitin ligases (37–40). Importantly, in response to DNA damage, the ATM and ATR kinases were shown to promote HIPK2 accumulation by inhibiting its ubiquitin ligases Siah1 and WSB1 (38, 40).

Figure 2. p53^{S46} phosphorylation is important for the induction of apoptosis in MYCN⁺ cells. A, MYCN⁺ and MYCN⁻ cells were treated with bleomycin (20 μ g/mL, 48 hours) and p53 expression, its serine-15 and serine-46 phosphoepitopes were detected by immunoblot. B, Q-RT-PCR analysis of known p53 target genes in bleomycin-treated (20 μ g/mL, 48 hours) MYCN⁻ and MYCN⁺ cells. C, sublethal amounts (40 ng) of plasmids containing WT, a S15A and a S46A mutant p53 were transfected in the apoptosis-resistant, p53-null MNA LAN-1 cells. Cells were treated with bleomycin and then analyzed for the appearance of apoptotic nuclei and for the expression of the indicated proteins and phosphoepitopes.



By inducing replication stress, several oncogenes were shown to initiate a DDR sustained by the activation of the ATM/ATR kinases (41); thus, we postulated that MYCN might promote HIPK2 accumulation as a consequence of DDR. Indeed, MYCN induction in SHEP Tet/21N cells was associated with typical signs of DDR such as an increase in the number of phospho-ATM^{S1981} and γ H2AX nuclear foci per cell, in the number of positive cells (Fig. 4A and B), in a time-dependent increase of H2AX and p53^{S15} phosphorylation and in an accumulation of total p53 (Fig. 4D; Supplementary Fig. S3C). Apparently, MYCN-induced DDR was independent of oxidative stress, because it occurred also in the presence of N-acetyl-cysteine (Supplementary Fig. S3B). Turning off MYCN expression from MYCN⁺ cells resulted in a time-dependent decrease of γ H2AX and p53, indicating a repression of the MYCN-dependent DDR (Supplementary Fig. S3C and D). Under

these conditions also HIPK2 quickly declined (Supplementary Fig. S3D), this being consistent with the hypothesis that DDR activation may in turn be responsible for HIPK2 accumulation. To directly address this issue, we depleted ATM via siRNAs in MYCN⁺ cells. ATM knockdown resulted in the inhibition of the MYCN-induced DDR as indicated by the reduction of both p53 expression and p53^{S15} phosphorylation (Fig. 4D). At the same time it also led to HIPK2 repression (Fig. 4D). In addition, ATM knockdown resulted in the inhibition of bleomycin induced p53^{S46} phosphorylation and in the impairment of p53^{S15} phosphorylation and p53 accumulation (Supplementary Fig. S3E). Remarkably, by depleting MYCN⁺ cells of NBS1, another important mediator of DDR, we obtained similar results (Supplementary Fig. S3F), confirming that MYCN induces HIPK2 protein accumulation via an ATM- and NBS1-dependent DDR pathway.

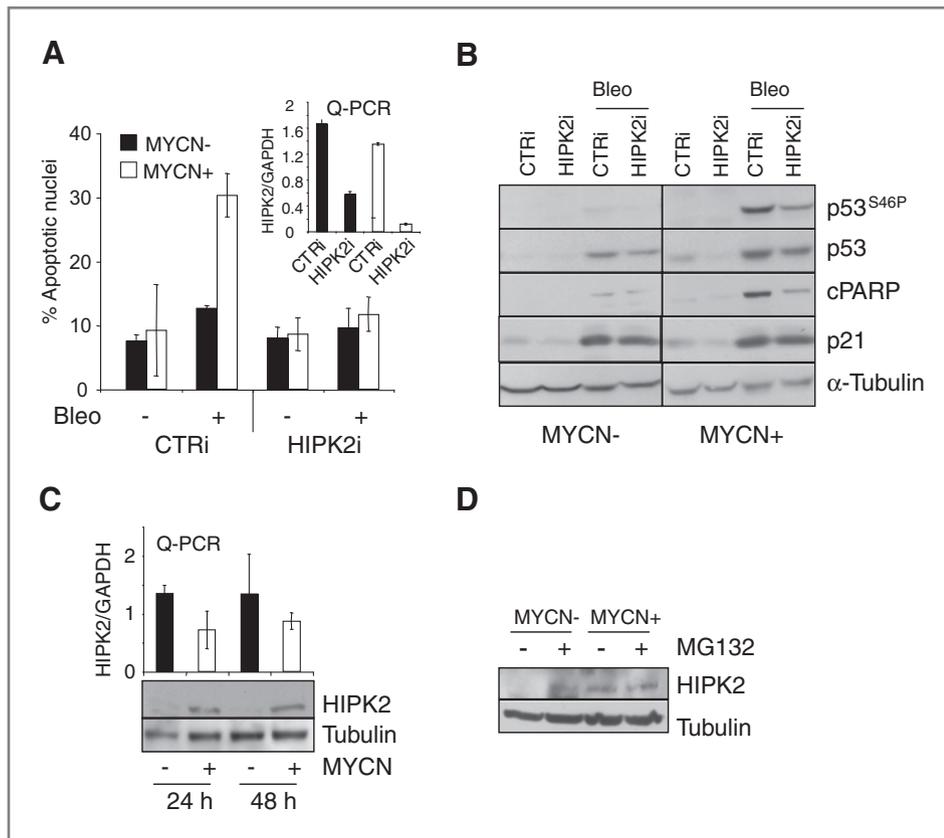


Figure 3. HIPK2 is required for MYCN-dependent apoptosis and is regulated by MYCN at the protein level. A, HIPK2 knockdown was achieved by transient transfection with sh-RNAi and was measured by Q-RT-PCR (inset). Count of the apoptotic nuclei shows that HIPK2 specific RNAi (HIPK2i), but not control RNAi (CTRi), impaired bleomycin-induced apoptosis occurring in MYCN⁺ cells. B, immunoblot showing the expression of the indicated proteins in HIPK2 RNAi transfected and bleomycin-treated cell extracts. C, immunoblot (bottom) and Q-RT-PCR analysis (top) showing HIPK2 expression in MYCN⁺ and MYCN⁻ cells at the protein and transcript level, respectively. D, immunoblot of MYCN⁺ and MYCN⁻ cell extracts showing HIPK2 expression on MG132 (50 μ mol/L) treatment.

HIPK2 is expressed and required for bleomycin-induced apoptosis in MNA human neuroblastomas

Although it is clearly established that wtp53 is preserved in virtually all human primary neuroblastomas, HIPK2 expression has never been assessed in human neuroblastic tumors. Therefore, to verify whether MNA neuroblastomas expressed HIPK2, we examined 13 MNA primary human neuroblastoma samples by immunohistochemistry. Indeed, we found readily detectable HIPK2 expression in approximately 50% (6 out of 13) of the primary MNA neuroblastomas with a variable level of nuclear/cytoplasmic localization (Fig. 5A), suggesting that the HIPK2-p53 pathway is potentially maintained in a large fraction of MNA human neuroblastomas.

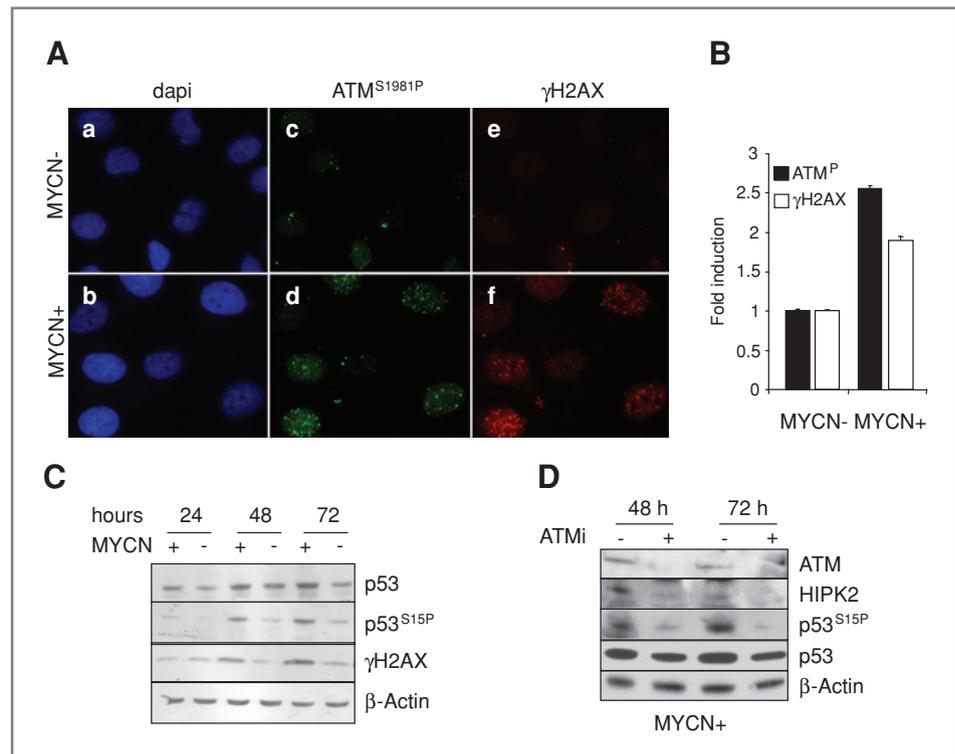
Next we evaluated whether the MYCN-induced HIPK2 accumulation observed in SHEP Tet21/N cells could be recapitulated in MNA neuroblastoma cell lines. Compared with MNSC, MNA neuroblastoma cells showed, on average, higher levels of HIPK2 protein (Fig. 5B) consistent with their higher sensitivity to bleomycin-induced apoptosis (see Fig. 1A). The MNA, but p53-mutant, SK-N-BE2c cells represented once again a sharp exception, whereas among the MNSC cells the highest HIPK2 expression was detected in SY5Y cells, which are known to express relatively high levels of MYCN (21). HIPK2 transcript levels did not directly correlate with MNA or with HIPK2 protein levels,

consistent with a nontranscriptional regulation of its expression by MYCN (not shown). To test HIPK2 functional role in MNA neuroblastoma cells we knocked down its expression by specific RNAi in LAN5 and KCNR cells. This led to inhibition of bleomycin-induced apoptosis in both cell lines (Fig. 5C) indicating that the MYCN-induced HIPK2-p53 pathway is recruitable for triggering apoptosis also in MNA neuroblastoma cells.

The nongenotoxic compound Nutlin-3 induces p53 and HIPK2 accumulation to efficiently trigger apoptosis in MNA neuroblastomas

Due to the presence of wtp53 in virtually all neuroblastomas and having shown that HIPK2 is expressed in approximately 50% of MNA neuroblastomas and that the HIPK2-p53 pathway is critical for MYCN-dependent sensitization to apoptosis, we sought to investigate whether p53-reactivating compounds such as Nut-3 might regulate this pathway. Interestingly, Nut-3 strongly inhibited survival (Supplementary Fig. S4) and induced apoptosis more efficiently than, and cooperatively with, bleomycin in MNA neuroblastoma cells (Fig. 6A and B). At suboptimal doses, it dramatically sensitized these cells to cytotoxic drugs, including bleomycin, ADR, and CDDP, leading to death virtually the entire cell population (Supplementary Fig. S4). In solid tumors of non-neuroectodermal origin Nut-3 was shown to

Figure 4. MYCN inhibits HIPK2 degradation via an ATM-dependent DDR pathway. A, immunofluorescent analysis of ATM and H2AX phosphoepitopes in MYCN⁺ and MYCN⁻ cells. B, quantitation of the number of cells showing more than 3 large ATM^{S1981P} or γ H2AX foci. C, immunoblot showing the expression of the indicated proteins and epitopes in MYCN⁺ and MYCN⁻ cells. D, immunoblot showing the expression of the indicated proteins and epitopes in MYCN⁺ cells after 48 and 72 hours from ATM RNAi (ATMi) transfection.



promote accumulation of p53 with no posttranslational modifications to induce cell cycle arrest rather than apoptosis (42). In keeping with its ability to disrupt MDM2/p53 interaction, in MNA neuroblastoma cells Nut-3 induced p53 accumulation in the absence of relevant p53^{S15} phosphorylation (Fig. 6A). Surprisingly, however, it favored the accumulation of a S46-phosphorylated p53 by itself and strongly promoted bleomycin effect (Fig. 6A), consistent with the occurrence of apoptosis. To better understand the molecular mechanisms underlying this response, we evaluated the behavior of key factors in response to Nut-3 by comparing MNA neuroblastoma cells with the previously characterized U2OS osteosarcoma cells, where MDM2 induced by Nut-3 was shown to actively contribute to the inhibition of apoptosis through HIPK2 degradation (27). We found that, despite MDM2 increase in all cells, Nut-3 determined the expected reduction of HIPK2 expression and accumulation of nonphosphorylated p53 only in the U2OS cells undergoing growth inhibition. Conversely, HIPK2 was even further accumulated in the MNA KCNR and IMR32 cells undergoing apoptosis in response to Nut-3, consistent with the occurrence of p53^{S46} phosphorylation (Fig. 6B). Importantly, the strong HIPK2 expression detected in Nut-3-treated MNA neuroblastoma cells was relevant for their apoptotic response because HIPK2 depletion by RNAi reduced the rate of apoptosis in KCNR cells (Fig. 6C). Therefore, Nut-3 efficiently induces apoptosis in MNA neuroblastoma cells (and sensitizes them to the effect of DNA damaging drugs) via an unexpected and possibly

tumor-type specific regulation of the p53 proapoptotic activator HIPK2.

Discussion

Improving our understanding of the molecular mechanisms linking MYCN to apoptosis may contribute to designing more appropriate therapeutic tools for the high-risk subset of patients with MNA tumors. Here, we report that MYCN sensitizes neuroblastoma cells to apoptosis by upregulating the HIPK2-p53 pathway via an oncogene-dependent DDR and that this pathway is targeted by the p53-reactivating compound Nut-3 at different levels (i.e., p53 and HIPK2 activation) in MNA cells, making it suitable for therapeutic intervention.

The very seldom occurrence of mutations in the TP53 oncosuppressor gene in human neuroblastomas (43) initially led to the hypothesis of its irrelevance for neuroblastic carcinogenesis. However, intrinsic p53 activity is intact in neuroblastoma cells and might be recruited in neuroblastoma cell responses to DNA damaging drugs or Nut-3, suggesting that p53 may be functionally inactivated in neuroblastic tumors either due to its delocalization to the cytoplasm or as a consequence of molecular and functional alteration of the p14^{ARF}-MDM2 axis (reviewed in ref. 22). Our data indicate that an intact p53 is also crucial for the increased sensitivity to apoptosis of MYCN-overexpressing neuroblastomas and are consistent with p53 being required for chemotherapy-induced apoptosis in the MYCN trans-

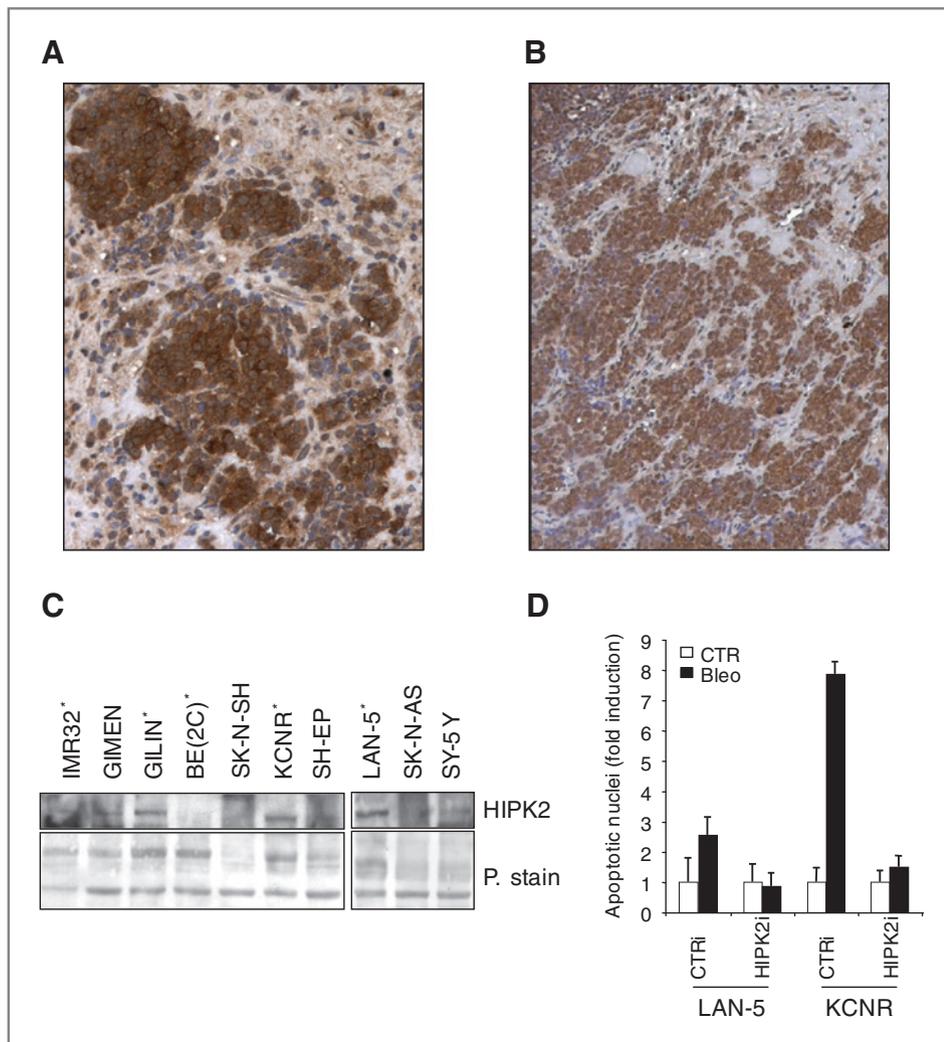


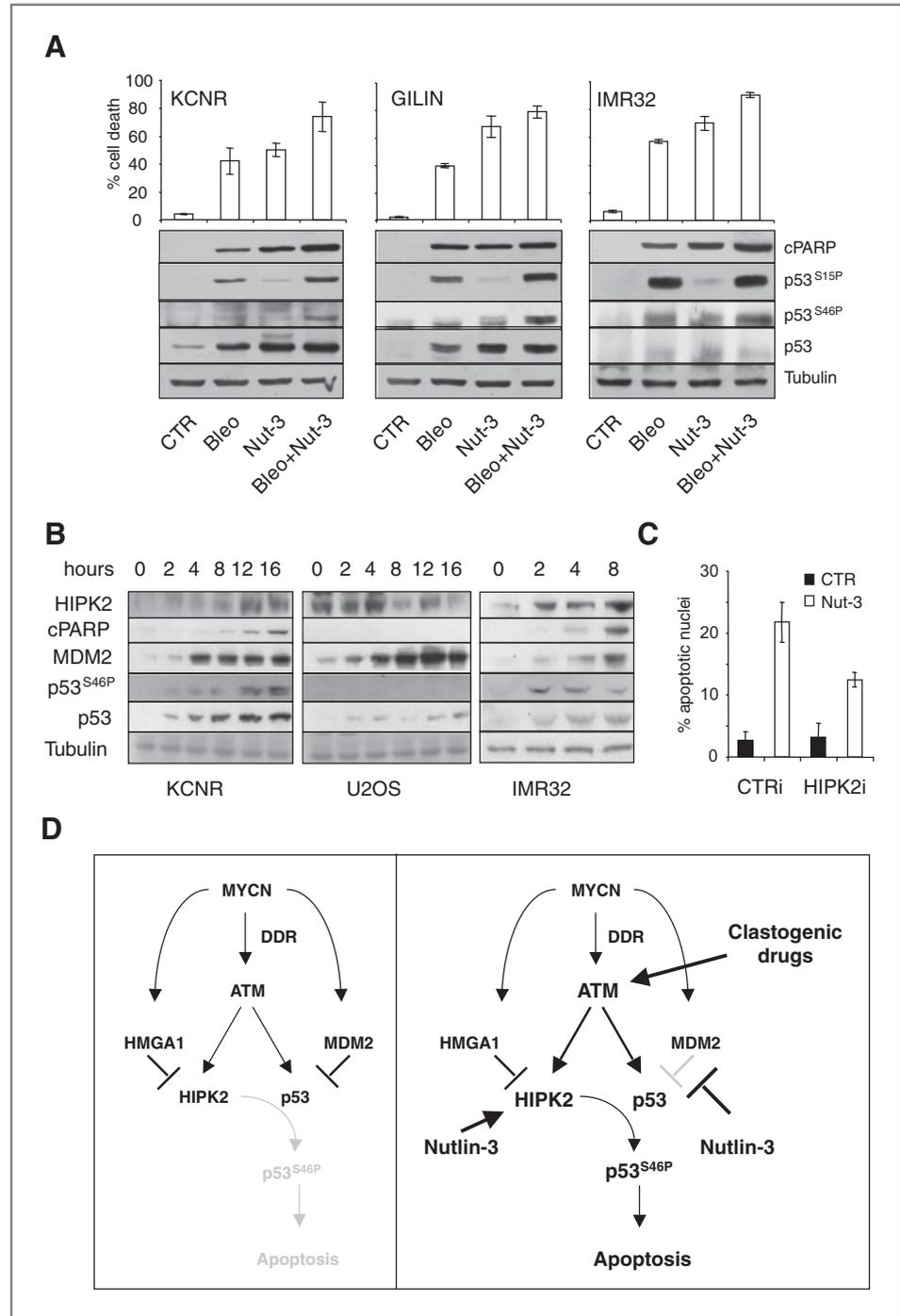
Figure 5. HIPK2 is expressed in primary human neuroblastomas and is required for bleomycin-induced apoptosis in MNA neuroblastoma cell lines. A and B, immunohistochemistry showing representative HIPK2 protein expression in 2 different MNA neuroblastoma specimens. C, immunoblot showing HIPK2 expression in a panel of neuroblastoma cell lines. *, MNA cells; loading is normalized on Ponceau staining of the blotted proteins. D, count of apoptotic nuclei shows that transient transfection with the HIPK2 specific RNAi, but not with the control RNAi, impaired bleomycin induced apoptosis in LAN-5 and KCNR MNA cell lines.

genic mice (44). In particular, we showed that this proapoptotic phenotype of MYCN-overexpressing cells also required p53 phosphorylation at serine 46 and were associated to a more pronounced activation of proapoptotic target genes compared with those involved in cell cycle arrest, such as the p21^{WAF1}, in keeping with the current understanding of p53 activity (32, 33). Among a few kinases able to phosphorylate p53^{S46}, we focused our attention on HIPK2, a protein required for apoptosis induced by neurotrophin deprivation in developing sympathetic neurons (45), whose role in p53^{S46} phosphorylation and p53-dependent apoptosis has clearly been established (26, 36). We showed that HIPK2 is largely expressed in human primary MNA neuroblastomas at transcript and protein levels. Its expression is increased by MYCN and its inactivation impairs apoptosis and p53^{S46} phosphorylation, in MYCN-overexpressing Tet21/N cells and in MNA cell lines. Although, at present, we cannot exclude the involvement of other kinases in p53^{S46} phosphorylation in neuro-

blastoma cells, our data show that HIPK2-mediated p53 activation is pivotal for the proapoptotic phenotype of MYCN-overexpressing neuroblastomas. Further HIPK2-regulated and p53-independent mechanisms, such as inhibition of TrkA and bcl-xL, or CtBP degradation (reviewed in ref. 46), might also contribute to the proapoptotic phenotype induced by MYCN, and this issue needs to be addressed in a following paper.

Remarkably, MYCN does not regulate HIPK2 transcript, but induces its protein accumulation. HIPK2 expression is tightly controlled via proteasomal degradation by several E3 ubiquitin ligases (37–40). DNA damage was shown to promote HIPK2 accumulation through an ATM/ATR-dependent inhibition of its degradation by Siah1 and/or WSB1 ubiquitin ligases (38, 40). Oncogene-dependent replication stress was also shown to induce DNA damage and ATM/ATR activation leading to p53-dependent apoptosis (or senescence), suggesting that DDR might represent an endogenous anticancer barrier contrasting transforma-

Figure 6. Nutlin-3 efficiently induces cell death and cooperates with clastogenic drugs by upregulating HIPK2 expression in MNA cells. A, cell death induced by either bleomycin, Nut-3 (10 μ mol/L) or both was measured by trypan blue exclusion test (top) in the indicated MNA neuroblastoma cell lines and the expression of the indicated proteins and phosphoepitopes was assessed by immunoblot (bottom). B, immunoblots showing the expression of the indicated proteins and phosphoepitopes on Nut-3 administration, in KCNR and IMR32 MNA cell lines undergoing apoptosis and in the growth arresting U2OS osteosarcoma cells. C, count of apoptotic nuclei shows that transient transfection with the HIPK2 specific RNAi, but not with the control RNAi, impaired Nut-3 induced apoptosis in KCNR MNA cells. D, schematic representation of the molecular pathway responsible for sensitization to apoptosis by MYCN in human neuroblastoma (see text for description).



tion induced by several oncogenes (41). Why p53 accumulation under these conditions should result in apoptosis (and/or senescence) instead of a transient growth arrest remained unexplained, thus far. Our data fit this model and extend it by providing evidence that the potent oncogene MYCN induces DDR and leads to stabilization of both p53 and HIPK2, as indicated by ATM and NBS1

RNAi experiments. Interestingly, both HIPK2 and p53^{S46} phosphorylation have also been involved in cellular senescence (47, 48) and mouse embryo fibroblasts bearing a human mutant knock-in p53 nonphosphorylatable at p53^{S46} escape from oncogene-induced senescence (49). Together with our present data, these observations support the speculation that oncogenes might induce apoptosis and/

or cell senescence due to the contemporary accumulation of both p53 and its proapoptotic/senescence activator HIPK2, via DDR.

In addition to these general and speculative hypotheses, our data indicate that accumulation of both p53 and HIPK2 is related to sensitization to apoptosis rather than apoptosis induction, in MYCN-overexpressing neuroblastoma cells. In this environment, p53 is functionally counteracted by the MYCN-induced deregulation of several other factors, such as TWIST1, MDM2, BMI1, CHD5, and PPM1S (ref. 50 and references therein). MYCN also induces increased expression of HMGA1 (51) that might contribute to restraining HIPK2 (ref. 29; Fig. 6D). Therefore, by increasing the level of both positive and negative regulators of the p14^{ARF}-MDM2-p53 pathway, MYCN might set a different equilibrium with a "lower threshold" for the induction of apoptosis by incoming modulators of the same pathway such as DNA damaging drugs and p53-reactivating compounds (Fig. 6D).

Restoration of p53 expression proved to be strikingly effective in inducing tumor regression in animal models (52–54) and its reactivation by small molecules is being tested in clinical trials for solid and hematologic malignancies (NLM Identifier: NCT00559533, NCT00623870, NCT00676910). The expression of the p53 proapoptotic activator HIPK2 in a large fraction of human neuroblastomas and the link we underscored between MYCN-dependent apoptosis and the HIPK2-p53 pathway further support the utilization of p53-reactivating compounds for the treatment of high-risk patients with MNA neuroblastoma, but highlight the need for a better characterization of their biological and biochemical effects in MNA neuroblastoma models. Indeed, we found that p53 induced by Nut-3 in MNA neuroblastoma cells is phosphorylated at S⁴⁶. These results are rather unexpected because Nut-3 is known to preferentially promote cell growth inhibition and

accumulation of an unmodified p53 in other types of solid tumors (42), possibly due to the MDM2-dependent degradation of HIPK2 (27). To our surprise, Nut-3 further increased HIPK2 protein accumulation, providing a potential explanation to the strong apoptotic response Nut-3 induced either alone or in combination with clastogenic agents in MNA neuroblastomas, that we and others observed (22, 55). HIPK2 knockdown experiments further support this hypothesis and indicate the unusually efficient apoptotic response to Nut-3 in MNA neuroblastoma cells might be directly linked to a particular and perhaps tumor-type specific regulation of the HIPK2-p53 pathway.

In conclusion, we have shown that MYCN sensitizes neuroblastoma cells to apoptosis via a DDR-dependent induction of p53 and its kinase HIPK2, which in turn phosphorylates p53 at serine 46 in response to DNA damaging drugs and/or Nut-3, committing it toward apoptosis. This pathway, conserved in MNA cell lines and in a large fraction of MNA neuroblastoma tumors might be exploited for therapeutic purposes in the most difficult-to-treat subset of neuroblastoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was partially supported by grants from Associazione Italiana per la Ricerca sul Cancro, Ministry of Health (Progetto Integrato Oncologia), National Research Council (CNR), Ministry of University and Research, the Pasteur Institute, and Cenci-Bolognietti Foundation.

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Received May 25, 2010; revised November 04, 2010; accepted November 22, 2010; published OnlineFirst December 20, 2010.

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Mol Cancer Res 2011;9:67-77. Published OnlineFirst December 20, 2010.

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