

MYCN-Directed Centrosome Amplification Requires MDM2-Mediated Suppression of p53 Activity in Neuroblastoma Cells

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Abstract

The *MYC* family oncogenes cause transformation and tumor progression by corrupting multiple cellular pathways, altering cell cycle progression, apoptosis, and genomic instability. Several recent studies show that MYCC (c-Myc) expression alters DNA repair mechanisms, cell cycle checkpoints, and karyotypic stability, and this is likely partially due to alterations in centrosome replication control. In neuroblastoma cell lines, MYCN (N-Myc) expression induces centrosome amplification in response to ionizing radiation. Centrosomes are cytoplasmic domains that critically regulate cytokinesis, and aberrations in their number or structure are linked to mitotic defects and karyotypic instability. Whereas centrosome replication is linked to p53 and Rb/E2F-mediated cell cycle progression, the mechanisms downstream of MYCN that generate centrosome amplification are incompletely characterized. We hypothesized that MDM2, a direct transcriptional target of MYCN with central inhibitory effects on p53, plays a role in MYC-mediated genomic instability by altering p53 responses to DNA damage, facilitating centrosome amplification. Herein we show that MYCN mediates centrosome amplification in a p53-dependent manner. Accordingly, inhibition of the p53-MDM2 interaction with Nutlin 3A (which activates p53) completely ablates the MYCN-dependent contribution to centrosome amplification after ionizing radiation. We further show that modulating MDM2 expression levels by overexpression or RNA interference-mediated posttranscriptional inhibition dramatically affects centrosome amplification in MYCN-induced cells, indicating that MDM2 is a necessary and sufficient mediator of MYCN-mediated centrosome amplification. Finally, we show a significant correlation between centrosome amplification and MYCN amplification in primary neuroblastoma tumors. These data support the hypothesis that elevated MDM2 levels contribute to MYCN-induced genomic instability through altered regulation of centrosome replication in neuroblastoma. [Cancer Res 2007;67(6):2448–55]

Introduction

The centrosome is a cytoplasmic domain that directs microtubule assembly and spindle formation during mitosis (1–3). Normal cells must replicate centrosomes exactly once per cell cycle in coordination with DNA replication during S-phase (4). Centrosome amplification is associated with abnormal spindle

pole formation and aberrant chromosomal segregation (5). The combination of DNA damage and oncogene overexpression is a particularly potent stimulus of centrosome amplification (6). Centrosome amplification has been shown to correlate with abnormal spindle assembly, multipolarity, development of aneuploidy, and elevated transformation rates in cell lines and in embryonic fibroblasts (7–12). A high incidence of centrosome amplification is found in many malignancies, suggesting that centrosome amplification may contribute directly to tumorigenesis and tumor progression *in vivo* (7, 13–15). Recently, the accumulation of supernumerary centrosomes persisting through mitosis in diploid cells has been identified as a precursor to aneuploidy, linking centrosome amplification and genomic instability (12).

MYC oncogenes also contribute to genomic instability during transformation. *c-Myc* can induce genomic instability by generating karyotypic alterations (structural or numerical changes in chromosome content) as well as smaller scale changes due to chromosomal rearrangements, aberrant repair, and locus-specific gene amplifications (16, 17). Transient expression of *c-Myc* (via a *Myc*-ER construct) can induce karyotypic abnormalities and gene amplification in cultured cells (18). *c-Myc* expression alters responses to ionizing radiation, DNA damage, and apoptotic stimuli contributing to genomic instability through multiple mechanisms (19).

Studies in transgenic mice show that neural crest-specific expression of MYCN causes neuroblast transformation and the development of neuroblastoma (20). In humans, MYCN-driven neuroblastoma has a highly variable karyotype and a high rate of aneuploidy and chromosomal alterations (21). A recent study shows that MYCN overexpression dramatically alters the centrosome complement of neuroblastoma cells exposed to ionizing radiation (22), suggesting that MYCN disrupts the regulation of centrosome replication, which is typically inhibited in the presence of DNA damage and cell cycle arrest (23).

Whereas centrosome amplification is clearly associated with increased tumorigenesis and malignant progression, the mechanisms controlling centrosome duplication are incompletely understood. Previous work has shown that this process is dependent on intact p53 signaling pathways and is linked to DNA replication via Rb/E2F signaling in normal cells (12, 24, 25). We recently showed that MDM2 is a transcriptional target of MYCN in neuroblastoma (26). Because MDM2 is a central negative regulator of p53 activity, we hypothesized that MDM2 may participate in the deregulation of centrosome amplification downstream of MYCN in these cells. We show here that p53-mediated suppression of centrosome amplification is markedly impaired in cells with constitutive MYCN expression. Inhibition of MDM2 at the posttranscriptional or protein-protein interaction level dramatically reduces MYCN-mediated centrosome amplification after ionizing radiation. Conversely, elevated MDM2 expression can substitute for MYCN in mediating centrosome amplification. Furthermore, we observe a significant correlation between amplification of *MYCN* and

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centrosome amplification in human neuroblastoma tumors. We propose that transcriptional activation of MDM2 in conjunction with cellular stress/DNA damage leads to centrosome amplification and may be a key effector of MYCN-driven genomic instability in neuroblastoma cells.

Materials and Methods

Cell culture and drug treatment. MYCN3 cells were constructed as described elsewhere (26) and maintained in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine). MYCN expression was induced by addition of 1 μ g/mL doxycycline. p53^{-/-} and p53^{+/+} HCT-116 cells were cultured in complete McCoy's 5A medium (supplemented as described above). For the centrosome amplification assays, cells were plated on coverslips or in T25 flasks at subconfluent density in the presence or absence of doxycycline (for MYCN3 cells). The next day, cells were irradiated with 10 Gy using a Gamma Cell 3000 Elan ¹³⁷Cs source γ -irradiator (MDS Nordion, Ottawa, Canada). In some cases, cells were treated continuously with 0.5 or 2.0 μ mol/L Nutlin 3A or 3B (gift from Dr. L. Vassilev, Hoffman-La Roche, Nutley, NJ) after irradiation. Cells on coverslips were harvested at various time points after irradiation for analysis.

Immunofluorescence. Direct immunofluorescent staining of fixed cells using antibodies directed to γ -tubulin or γ -H2AX identifies the centrosome complement within each cell and the extent of DNA damage resulting from irradiation, respectively, and was quantified by microscopic inspection. Briefly, cells were fixed on coverslips in ice-cold 100% methanol for 20 min at -20°C . Coverslips were blocked for 30 min in PBS/1 mg/mL bovine serum albumin (BSA) at room temperature. Anti- γ -tubulin monoclonal antibody (Sigma, St. Louis, MO) or anti- γ -H2AX (Cell Signaling, Danvers, MA) was then added to coverslips in PBS/10 mg/mL BSA at a dilution of 1:400 for 90 min at 37°C . The cells were twice washed in PBS/0.1% Tween at room temperature, then in PBS, and again blocked as above and incubated with Alexa Fluor-conjugated antimouse secondary antibody (1:200; Molecular Probes, Carlsbad, CA) for 30 min at 37°C . Finally, the cells were washed as described above and mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing medium (Vector Laboratories, Burlingame, CA). At least 300 cells were examined for each coverslip. Only mononuclear cells were considered for analysis.

For analysis of primary neuroblastoma centrosome amplification, 8- μ m sections were cut from formalin-fixed, paraffin-embedded tumors and incubated for 4 h on glass slides. Slides were deparaffinized in xylenes and dehydrated in graded ethanol washes. Antigen retrieval was accomplished by heating the slides in $1\times$ SSC for 8 min. Slides were then blocked in normal serum before incubation with anti- γ -tubulin monoclonal antibody (1:100 in PBS/5% BSA/0.5% Tween) overnight at 4°C . The next day, slides were washed and then incubated with fluorescent secondary antibody as described above and mounted with coverslips and DAPI mounting medium. Slides were examined and quantified by J.H., blinded to the MYCN amplification status of the slides.

RNA interference and overexpression constructs and transduction. Posttranscriptional gene silencing was achieved by retroviral transduction of the host cell using pSuper plasmid vectors encoding target-specific short hairpin RNAs designed to be cleaved into mature small interfering RNAs (siRNA). Complementary oligo pairs (59-mer) containing effective 19-mer target sequences were annealed and introduced into the pSuper.puro retroviral construct according to the manufacturer's recommendations (OligoEngine, Seattle, WA). Target sequences used were as follows: MDM2-sequence, 5'-gccattgctttgaagtta; nontargeting sequence, 5'-tctccgaacctgtcagct. Overexpression was achieved by transduction of cells with pBabe MDM2 and pBabe MYCN. The complete coding sequences of MDM2 and MYCN were amplified using the following primers, respectively: MDM2 BamHI fwd, ccgggatccatgtgcaataccaacatgtctgtac; MDM2 EcoRI rev, ccggaattcctaggggaaataagtagcacaatc; MYCN EcoRI fwd, ccggaattcatccgagctgtccacgtc; and MYCN EcoRI rev, ccggaattcctagcaagtcgagctgtgt. The resulting products were introduced into pBabe Puro. Digestion of these PCR

products with BamHI and EcoRI (pBabe MDM2) or EcoRI alone (pBabe MYCN) generated a fragment suitable for ligation into pBabe puro. Retroviral supernatants were generated in the 293T cell line by simultaneous transfection of retroviral constructs, pRDF (encoding the RD114 amphotropic envelope) and pEQPAM3-E (encoding Gag Pol; ref. 27; both plasmids are kind gifts of Dr. Gianpietro Dotti, Baylor College of Medicine, Houston, TX). Cells were transduced by tandem incubations 24 h apart with appropriate supernatants including 4 μ g/mL polybrene. Subsequent selection with 2 μ g/mL puromycin generated stable pools of drug-resistant cells integrating the provirus. Freshly transduced cells were used for all experiments described.

MDM2 promoter luciferase reporter analysis. The MDM2 luc wild-type luciferase reporter plasmid was previously described (26). The E-box element at -481 bp was mutated from CACGTG to CAGG using overlapping primer mutagenesis to construct the MDM2 luc Mut reporter plasmid. MYCN pcDNA 3.1 D/V5-His was constructed by TOPO cloning (Invitrogen, Carlsbad, CA). The coding region of MYCN (codons 1-464) was amplified using Pfx polymerase (Invitrogen) and the following oligonucleotides: forward, 5'-caccatgccgagctgc; reverse, 5'-gcaagtcgagctgttcaatt. The plasmid pcDNA 3.1 D/V5-His/lacZ (Invitrogen) was used as transfection control.

Western blot analysis. Techniques used for Western blot analysis have previously been described (26). Antibodies used included anti-MDM2 (Calbiochem, San Diego, CA), anti-cyclin-dependent kinase (CDK)-2, anti-p53, anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-CDK2(Thr¹⁶⁰) (Cell Signaling Technology, Danvers, MA), and anti- β -actin (Sigma).

Results

Centrosome amplification correlates significantly with amplification of MYCN in primary neuroblastoma tumors. A recent report described high levels of centrosome amplification following DNA damage in the SH-EP neuroblastoma cell line constitutively overexpressing MYCN (22). By comparison, a control SH-EP cell line expressing little or no endogenous MYCN exhibited very little centrosome amplification. To test the relevance of the above findings in primary human tumors, we assessed the frequency of centrosome amplification in a series of neuroblastoma tumors with known MYCN amplification status resected before therapy. Immunofluorescent centrosome staining of 20 neuroblastomas revealed a significant correlation ($P < 0.02$) between centrosome amplification and amplification of the MYCN locus (Fig. 1). Three of four MYCN-amplified sections exhibited centrosome amplification frequencies $>20\%$, whereas only 1 of 16 MYCN-nonamplified sections exceeded 20%. The majority of nonamplified tumor specimens had $<5\%$ of cells with evidence of centrosome amplification. These data generated using archived tumor specimens suggest that *in vivo* expression of MYCN is associated with markedly increased centrosome amplification. Because MYCN is strongly linked to a poor prognosis and an aggressive cellular phenotype, an attractive hypothesis is that aberrant centrosome replication downstream of MYCN contributes to the development of these traits. Further studies will more rigorously test the hypothesis that centrosome amplification and MYCN amplification are consistently correlated in larger sample groups.

Inducible MYCN expression results in centrosome amplification following DNA damage. To further examine this apparent MYCN-regulated effect, we first constructed the MYCN3 cell line derived from SH-EP, but with MYCN expression under the control of a doxycycline inducible (Tet-On) promoter (BD Biosciences, San Jose, CA; previously described in ref. 26). This method enabled experiments to be done in a single cell line rather than in two separate transduced pools or clones, eliminating artifacts associated with independent selection of constitutive expression cell lines.

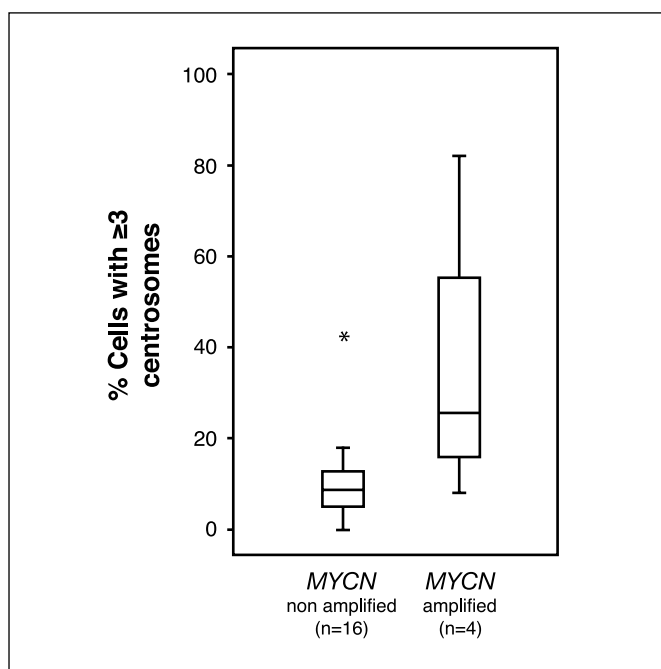


Figure 1. Centrosome amplification correlates significantly with amplification of *MYCN* in primary neuroblastoma tumors. Observed percentage of centrosome amplification in *MYCN*-nonamplified ($N = 16$) versus *MYCN*-amplified ($N = 4$) primary neuroblastomas represented as box plots. *MYCN* status was determined by fluorescent *in situ* hybridization at the time of diagnosis as part of patient risk evaluation at approved Children's Oncology Group core laboratories. Amplification is defined as >10 copies of *MYCN* (either on double minutes or homologous staining regions). The box boundaries span the interquartile range with median values (*horizontal line within the box*). Values more than three interquartile ranges from the end of a box are considered extreme outliers (*asterisk*). Whiskers above and below extend to the most distant points not deemed outliers or extreme outliers. There is a strong association between elevated centrosome amplification and *MYCN* amplification status. $P < 0.02$, paired sample *t* test.

As seen in Fig. 2, centrosome amplification results similar to those of Sugihara et al. were obtained in our *MYCN* inducible neuroblastoma cell line upon irradiation. Figure 2A shows representative $\times 100$ images of *MYCN3* cells stained for γ -tubulin, a structural component of centrosomes. Nonirradiated cells with either one or two centrosomes (*left*) and irradiated mononuclear cells with centrosome amplification (three or more centrosomes; *right*) are shown. As seen in Fig. 2B, *MYCN* induction stimulates a marked time-dependent increase in centrosome amplification relative to the low basal level of amplification observed in response to irradiation alone. The highest level of centrosome amplification observed was $\sim 30\%$ for the *MYCN*-induced line versus $\sim 15\%$ for the noninduced line at 72 h postirradiation. Sugihara et al. (22) observe no centrosome amplification in response to *MYCN* overexpression in the absence of a DNA damage stimulus such as irradiation or aphidicolin, results consistent with our own (data not shown). In Fig. 2C, Western blot analysis reveals increased p53 and MDM2 expression levels in the *MYCN3* cell line (26) preirradiation ($t = 0$) and 24 and 48 h postirradiation. p53 levels rapidly increase after DNA damage in both induced and non-induced cells. Predictably, high p53 levels result in increased MDM2 expression in both induced and noninduced cells (Fig. 2C).

As a means of standardizing DNA damage inflicted by irradiation, we stained cells with an anti- γ -H2AX antibody 24 h after exposure to irradiation. Double-strand breaks result in rapid phosphorylation and recruitment of histone variant H2AX, which is detected by

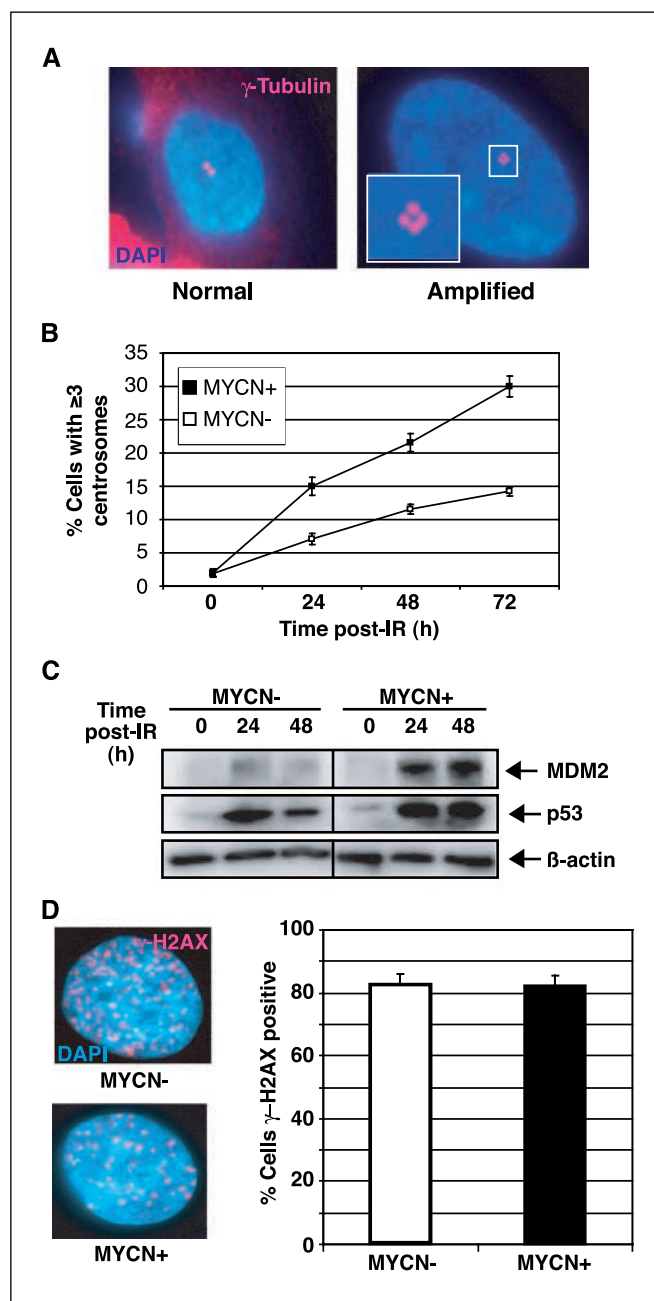


Figure 2. Induction of *MYCN* promotes centrosome amplification in response to DNA damage. *A*, immunofluorescent staining for γ -tubulin. *Left*, a nonirradiated cell with a normal complement of two (or one) centrosome(s). *Right*, a cell at 72 h after exposure to 10-Gy γ -irradiation with an abnormal centrosome complement of three or more. Merged images show signals for γ -tubulin (red) and nuclear DNA (blue) in a single field. *B*, analysis of centrosome amplification kinetics in the *MYCN3* cell line in the presence (■) or absence (□) of *MYCN* overexpression induced by the addition of doxycycline. The percentage of cells with three or more centrosomes (amplified) is quantified at 24, 48, and 72 h after irradiation. *Points*, mean of three independent observations; *bars*, SD. For each observation, 300 cells were examined. The experiment was repeated thrice with similar results. *, $P < 0.005$, ANOVA comparison of line slopes derived from linear regression analysis. *C*, Western blot analysis of *MYCN3* cells at various times postirradiation. Blots were probed for MDM2 and p53. β -Actin served as a loading control. *D*, *left*, immunofluorescent staining for γ -H2AX. Similar levels of γ -H2AX positivity in cells noninduced (*MYCN*-) or induced (*MYCN*+) for *MYCN* expression. *Right*, quantification of results for γ -H2AX staining 24 h postirradiation (10 Gy) in the presence (*closed column*) or absence (*open column*) of *MYCN* overexpression induced by the addition of doxycycline. For each observation, 300 cells were examined. *Columns*, mean; *bars*, SE. The experiment was repeated thrice with similar results.

anti- γ -H2AX as punctate nuclear staining following irradiation. Representative $\times 100$ images for MYCN-induced and noninduced γ -H2AX-stained cells are seen in Fig. 2D (left). As shown in Fig. 2D (right), we find no MYCN-dependent alterations in the frequency of γ -H2AX-positive cells 24 h after irradiation, a result consistent with previous findings (22) indicating that MYCN-dependent centrosome amplification must not be due to increased sensitivity of the cells to insult.

Inhibition of p53 is critical for MYCN-mediated centrosome amplification. We have previously shown the transcriptional activation of MDM2 by MYCN via a consensus E-box element in the first exon promoter region of *MDM2* (26). Given the critical role played by the MDM2-p53 pathway in the regulation of centrosome duplication, we hypothesized that the centrosome amplification described above and by Sugihara et al. (22) could be due to effects of MYCN on p53 function. To directly test this hypothesis, we made use of $p53^{+/+}$ and $p53^{-/-}$ somatic knockout colon cancer cell lines (28), which were transduced to constitutively express MYCN. Western blot analysis (Fig. 3A) of stably transduced pools shows both $p53^{+/+}$ and $p53^{-/-}$ cell lines with stable retroviral expression of MYCN or a control construct. These stable pools were then treated as described for the MYCN3 cell line; cells were grown on coverslips, subjected to 10-Gy irradiation, and, subsequently, centrosome amplification was monitored. In all cell lines, a low baseline level of centrosome amplification was observed in the nonirradiated cells (data not shown). Seventy-two hours after irradiation, a high level of centrosome amplification was observed in $p53^{-/-}$ cells in both the presence and absence of constitutive MYCN expression. In contrast, in $p53^{+/+}$ cells, a 2.5-fold increase in centrosome amplification accompanied only MYCN expression (Fig. 3B). These data suggest strongly that MYCN is upstream of p53 in the pathway responsible for directing centrosome amplification.

To confirm that MYCN regulation of MDM2 is independent of p53-mediated transcription, we made use of an *MDM2* promoter luciferase reporter plasmid to test the responsiveness of the *MDM2* promoter (26) to MYCN in the absence of p53. As predicted, we show a 2.5-fold increase in *MDM2* reporter activity on introduction of MYCN into the Saos-2 (p53-deficient) cell line by transient transfection (Fig. 3C). This magnitude of response is comparable to that seen in other cell lines with functional p53 (26). The absence of a response to MYCN in an E-box deletion mutant of the *MDM2* reporter plasmid documents the specificity of the interaction between MYCN and the *MDM2* promoter.

MDM2 is a critical effector of MYCN-mediated centrosome amplification. To further test our hypothesis that MDM2 plays an essential role in MYCN-mediated centrosome amplification, we used a potent and selective small-molecule inhibitor of the MDM2-p53 interaction, Nutlin 3A (29). Nutlin 3A has previously been shown to inhibit the interaction of MDM2 and p53 by competitively binding to MDM2 at the p53 interacting site (29). Repeating the experiment described in Fig. 2, a familiar increase in centrosome amplification is observed in response to MYCN induction at 72 h postirradiation in MYCN3 cells treated with vehicle (Fig. 4A). As predicted, however, treatment of the cells with 0.5 $\mu\text{mol/L}$ Nutlin 3A immediately postirradiation was sufficient to abolish all MYCN-mediated centrosome amplification at 72 h. This Nutlin 3A dose is significantly lower than the previously established IC_{50} for growth of this cell line as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 72 h after drug treatment (30). Increasing the Nutlin 3A dosage to 2.0 $\mu\text{mol/L}$ led to a modest further reduction in MYCN-mediated

centrosome amplification. Importantly, results obtained using Nutlin 3B, an inactive enantiomer of Nutlin 3A (29), reveal that the observed effects on MYCN-mediated centrosome amplification depend on the ability of the drug to disrupt the MDM2-p53

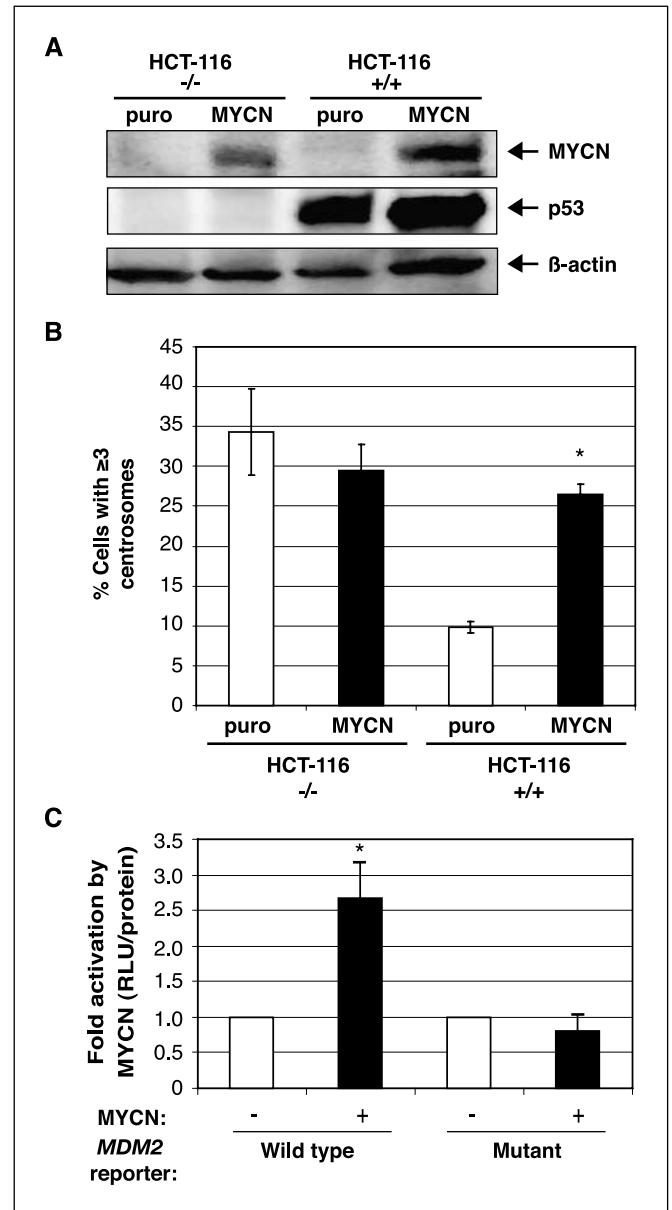


Figure 3. Inhibition of p53 is critical for MYCN-mediated centrosome amplification. **A**, Western blot analysis of $p53^{-/-}$ and $p53^{+/+}$ HCT-116 colon cancer cell lines stably transduced with retroviral supernatants to constitutively express MYCN or a control vector (puro) Blots were probed for MYCN and p53. β -Actin served as a loading control. **B**, analysis of centrosome amplification in $p53^{-/-}$ and $p53^{+/+}$ HCT-116 colon cancer cell lines transduced to constitutively express MYCN (closed columns) or puro (open columns). The percentage of cells with three or more centrosomes (amplified) is quantified at 72 h postirradiation (10 Gy) as described above. Columns, mean of three independent observations; bars, SD. For each observation, 300 cells were examined. *, $P < 0.01$, paired sample t tests with Bonferroni correction applied (two tests). **C**, luciferase activities of lysates prepared from transient transfection of the p53-deleted Saos-2 cell line with a MYCN expression plasmid (+) and an MDM2 promoter reporter plasmid. Control plasmid pCDNA 3.1 D/V5-His/lacZ (-) and an E-box mutated (*Mutant*) reporter plasmid served as controls. Columns, mean relative light units (RLU) standardized by total protein; bars, SE. All transfections were done in triplicate and repeated at least thrice with similar results. *, $P < 0.05$, paired sample t tests of the means with Bonferroni correction applied (two tests).

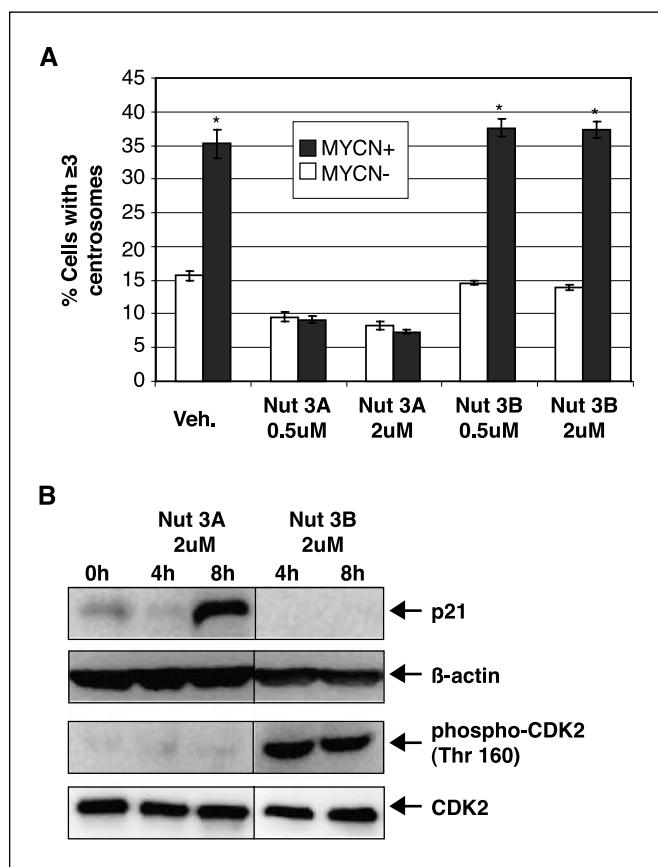


Figure 4. MDM2 is a critical effector of MYCN-mediated centrosome amplification. *A*, analysis of centrosome amplification kinetics in the MYCN3 cell line in the presence (*closed columns*) or absence (*open columns*) of MYCN overexpression, following exposure to 0.5 or 2.0 $\mu\text{mol/L}$ Nutlin 3A or Nutlin 3B. The percentage of cells with three or more centrosomes (amplified) is quantified at 72 h postirradiation (10 Gy). *Columns*, mean of three independent observations; *bars*, SD. For each observation, 300 cells were examined. The experiment was repeated thrice with similar results. *, $P \leq 0.005$, paired sample *t* tests with Bonferroni correction applied (five tests). *B*, Western blot analysis of MYCN3 cells following 4 and 8 h of treatment with 2.0 $\mu\text{mol/L}$ Nutlin 3A or Nutlin 3B. Blots were probed for p21 and phospho-CDK2(Thr¹⁶⁰). β -Actin and total CDK2 served as loading controls.

interaction (Fig. 4A). Under the conditions of the experiment, no measurable cell death occurred on Nutlin 3A treatment (data not shown).

Previous reports have described critical roles for p21 and CDK2 in the normal and pathologic regulation of centrosome duplication. As with p53, p21 is essential for maintenance of normal centrosome complements in mouse embryonic fibroblast cells (25) and is thought to exert its negative regulatory influence by inhibiting the activation of CDK2/cyclin A or CDK2/cyclin E complexes (31). Consistent with these observations, centrosome duplication cannot occur in the absence of CDK2 (32). We show in Fig. 4B that even brief treatment of MYCN3 cells with 2.0 $\mu\text{mol/L}$ Nutlin 3A is sufficient to induce p21 expression and to diminish phosphorylation levels of CDK2 at the Thr¹⁶⁰ site, which is critical for CDK activity. Treatment of cells with the inactive enantiomer Nutlin 3B again results in no comparable changes in p21 expression or CDK2 phosphorylation.

MDM2 is necessary and sufficient for MYCN-mediated centrosome amplification. To extend and confirm the results described above validating MDM2 as a critical effector in

mediating MYCN-dependent centrosome amplification, we modulated MDM2 expression with both viral transduction of a cytomegalovirus-driven expression construct and siRNA-mediated transcriptional inhibition. MYCN3 cells were transduced with the pSuper RNA interference retroviral vector containing a previously published sequence targeting MDM2 (33) or a pBabe vector constitutively expressing MDM2. We did Western blot analysis to confirm changes in MDM2 and p53 protein levels (Fig. 5A). A dramatic decrease in MDM2 expression is observed in the RNA interference-transduced cells relative to cells transduced with a nontargeting sequence or the parent cell line. A modest increase in MDM2 expression is observed in the pBabe MDM2-transduced cell line that constitutively expresses MDM2. As expected, there is evidence of p53 stabilization in the MDM2 knockdown cell line but not in any of the other transduced cell lines described. As shown in Fig. 4B, the cells transduced with the nontargeting vector are sensitive to MYCN-mediated centrosome amplification as are the parent (nontransduced) cells. In contrast, cells with MDM2 knockdown, as shown in Fig. 5B, are insensitive to centrosome amplification mediated by MYCN overexpression. These results are consistent with the results obtained from cells treated with Nutlin 3A.

To further elucidate the function of MDM2 in MYCN-mediated centrosome amplification, MYCN3 cells constitutively expressing

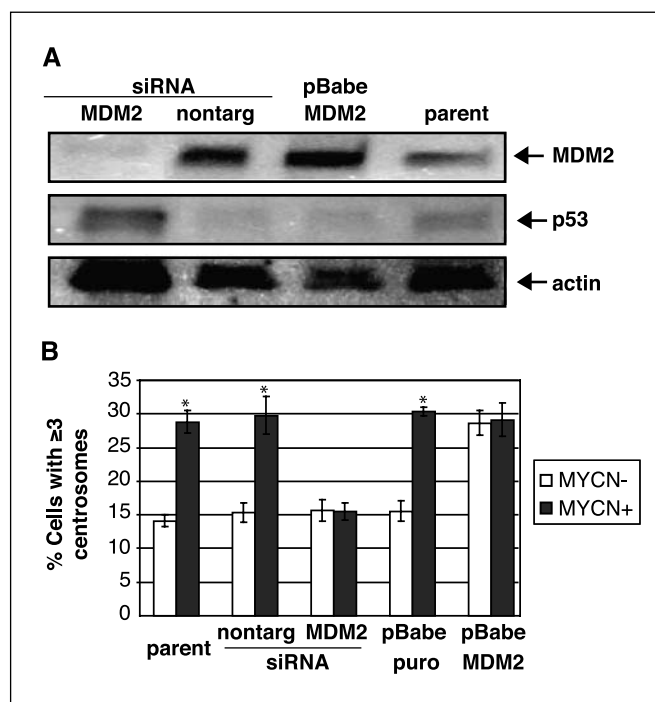
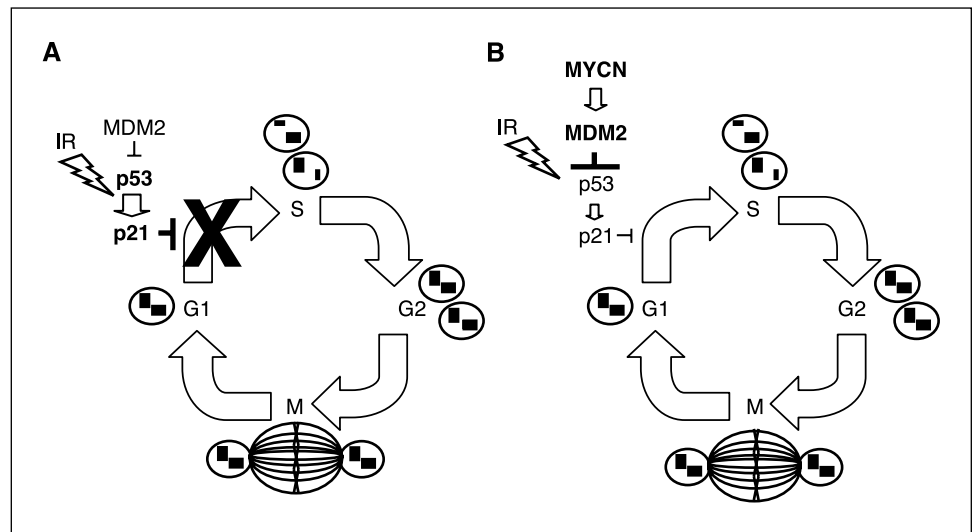


Figure 5. MDM2 knockdown abolishes MYCN-mediated centrosome amplification and constitutive MDM2 overexpression can substitute for MYCN. *A*, Western blot analysis of MYCN3 cells transduced with retroviral supernatants to express pSuper MDM2 siRNA (*MDM2*), pSuper nontargeting siRNA (*nontarg*), and constitutive MDM2 (*pBabe MDM2*). Nontransduced parental cells are included for comparison (*parent*). Blots were probed for MDM2 and p53. β -Actin served as a loading control. *B*, analysis of centrosome amplification kinetics in the MYCN3 cell line in the presence (*closed columns*) or absence (*open columns*) of MYCN overexpression following no transduction (*parent*) or transduction with pSuper nontargeting siRNA (*nontarg*), pSuper MDM2 siRNA (*MDM2*), pBabe puro, and pBabe MDM2. The percentage of cells with three or more centrosomes (amplified) is quantified at 72 h after irradiation (10 Gy). *Columns*, mean of three independent observations; *bars*, SD. For each observation, 300 cells were examined. *, $P < 0.005$, paired sample *t* tests with Bonferroni correction applied (five tests).

Figure 6. Model of the mechanism by which MYCN may mediate centrosome amplification. **A**, modest MDM2 expression levels are insufficient to circumvent p53 stabilization induced by irradiation and DNA damage checkpoint signals (*IR*). Accordingly, p53 efficiently arrests both the cell cycle and the centrosome duplication cycle at the G₁-S boundary. **B**, MYCN activity leads to elevated MDM2 levels, resulting in reduced stabilization of p53 despite the damage stimulus provided by irradiation. Diminished p53 signal results in a failure to arrest the centrosome duplication cycle and the amplification of centrosomes in the presence of general cell cycle arrest.



MDM2 (Fig. 5A) were also assayed. We observe similar levels of centrosome amplification in the induced and noninduced cells, indicating that MDM2 can substitute for MYCN in the stimulation of centrosome amplification.

Discussion

Heterogeneous chromosomal instability is one of the hallmarks of neuroblastoma (21). Evidence from a transgenic model of neuroblastoma shows that targeted MYCN overexpression is sufficient to promote neuroblastoma-like disease of similar histopathology and behavior to its human counterpart (20). Importantly, another feature of the tumors that arise in this model animal includes chromosomal alterations at regions syntenic to those observed in human neuroblastoma (20). Data herein and from a recent work (22) suggest that MYCN-mediated centrosome amplification may contribute to aneuploidy, genomic instability, and, therefore, neuroblastoma tumorigenesis. Evidence supporting the hypothesis that the development of aberrant centrosome number is an early critical event in promoting chromosomal instability and cellular transformation continues to accumulate (for review, see ref. 34). Centrosome amplification has been correlated with stage and grade in some cancers and has been proposed as a prognostic indicator (9, 35–38). *In vivo* carcinogenesis models have identified centrosome amplification as a precursor to oncogenic lesions (9, 10, 39).

Several studies have investigated the molecular processes regulating normal centrosome duplication. In particular, previous efforts have underlined a critical role for the p53 tumor suppressor in the regulation of centrosome duplication (40). Consistent with the highly elevated levels of genomic instability and cancer observed in *p53*^{-/-} mice, mouse embryonic fibroblasts derived from these mice are also characterized by high levels of centrosome amplification (25). MDM2 is the major negative regulator of p53 function (41), and this study as well as previous work shows that overexpression of MDM2, like p53 deletion, leads to centrosome amplification (42). Detailed analysis of the process has identified a strong candidate pathway downstream of p53 requiring p21 to inhibit cyclin A- and/or cyclin E-associated CDK2 activity to regulate the process of centrosome duplication (25, 43, 44). The consequence of deregulated centrosome duplication by impaired

p53 or p21 function or elevated expression of cyclin E is the accumulation of supernumerary centrosomes (45, 46).

Alternative and parallel pathways to p53 modulation regulating centrosome duplication continue to be revealed. Recently, a role for p27^{Kip1} in suppression of MYCN-mediated centrosome duplication was elucidated (47). These authors show that loss of the CDK inhibitor p27^{Kip1} leads to aberrant centrosome duplication and propose a model for p53-independent CDK-mediated suppression of centrosome replication upon DNA damage. In addition, an inhibitory role for p16^{INK4A} regulating centrosome duplication in human mammary fibroblasts was recently described (12). The authors show that p16^{INK4A} loss results in uncoupling of the centrosome duplication and DNA replication cycles, as well as an increase in aneuploidy. Consistent with the observations described above, the authors hypothesize that p16^{INK4A} could be inhibiting cyclin E transcription via Rb hypophosphorylation or liberation of p21 from the cyclin D/CDK4 complex to inhibit cyclin A/cyclin E/CDK2 activity directly (12). Our data presented herein describe an additional p53-dependent mechanism for centrosome amplification active in p53 wild-type neuroblastoma cell lines and tumors.

Recent work has described the direct positive transcriptional regulation of MDM2 by MYCN in neuroblastoma cells (26). Neuroblastoma is uniformly p53 wild-type at diagnosis (48) and requires a mechanism to inhibit p53-directed apoptosis in response to stress. MYCN-mediated regulation of MDM2 suggests a mechanism by which p53 function could be blunted by constitutively elevated MYCN activity in neuroblastoma (49). In this study, we tested the possibility of a function for MDM2 in promoting MYCN-mediated centrosome amplification via the negative regulation of p53 and downstream components of the p53 pathway. We predicted that MYCN would be unable to promote centrosome amplification if the expression of MDM2, or its interaction with p53, was disrupted. We also predicted that MDM2 overexpression could substitute for MYCN in the induction of centrosome amplification.

Indeed, we found that constitutive MYCN expression resulted in the failure of p53 wild-type HCT-116 cells to control centrosome amplification following DNA damage (Fig. 3B). In contrast, counterpart HCT-116 p53^{-/-} cells experienced centrosome amplification both in the presence and absence of MYCN expression. These data strongly suggest that MYCN may impair p53 function(s) that

normally regulates excessive or inappropriate duplication of centrosomes. We hypothesize that MDM2, as the major negative regulator of p53 function (41) and a transcriptional target of MYCN (26), may be a key effector in MYCN-mediated centrosome amplification.

Consistent with a hypothesis requiring MYCN-mediated transactivation of MDM2 to impair p53 function, we also describe the p53-independent activation of an MDM2 reporter construct (Fig. 3C). Nutlin 3A, which specifically inhibits the interaction of MDM2 with p53, impairs centrosome amplification promoted by conditional MYCN overexpression in a neuroblastoma cell line (Fig. 4A). This response is potent because MYCN-mediated centrosome amplification is completely ablated by a drug dose significantly lower than the IC₅₀ previously established for growth inhibition in this same cell line (30). Furthermore, the absence of any discernible effect on centrosome amplification using an inactive enantiomer of the same inhibitor molecule suggests that this is a highly specific drug effect.

Supporting these results, we also show a similar effect of posttranscriptional gene silencing of MDM2 by siRNA in the same cell system (Fig. 5). In contrast, overexpression of MDM2 is sufficient to promote centrosome amplification in the absence of MYCN induction, supporting the conclusion that MDM2 acts downstream of MYCN (Fig. 5). These findings are consistent with our initial strong correlative data supporting the hypothesis that MYCN amplification and centrosome amplification coexist in primary neuroblastoma tumors (Fig. 1). Taken together, these data argue that MDM2 is a necessary and sufficient effector of MYCN in promoting centrosome amplification. To our knowledge, this report is the first to indicate a direct role for MDM2 as a critical effector of a MYCN-mediated process that promotes genomic instability.

In summary, the data presented here support a model whereby MYCN-mediated transactivation of MDM2 results in impaired p53 function subsequent to induction of DNA damage, leaving the ability of the cell to regulate centrosome duplication severely compromised (Fig. 6). The accumulation of supernumerary centrosomes then results in aneuploidy and heritable genetic defects that ultimately favor tumorigenesis (34).

The study of centrosome amplification has gained momentum along with renewed interest in the role played by loss of genomic integrity in promoting tumorigenesis. Our ability to inhibit MYCN-driven centrosome amplification with MDM2 inhibitors may also have important therapeutic implications. Neuroblastomas (and many other tumors) accumulate additional genetic alterations leading to p53 inactivation and chemoresistance at relapse (50). Novel nongenotoxic therapeutic approaches, such as using MDM2 inhibitors to activate p53, may reduce genomic instability in the face of genotoxic chemotherapy, preventing mutation and escape from therapy. Elucidation of other requirements for MYCN-mediated centrosome amplification is likely to shed further light on the role played by MYCN in promoting genomic instability in neuroblastoma and suggest new therapeutic approaches.

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