

# The MDM2 antagonist nutlin-3 sensitizes p53-null neuroblastoma cells to doxorubicin via E2F1 and TAp73

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**Abstract.** Neuroblastoma (NB) is a primitive neuroectodermal tumor and the second most common solid tumor in children. NB exhibits heterogeneous behavior and spontaneous regression can occur in patients under 12 months of age. Response to treatment is both age- and stage-specific; however, patients over 1 year of age are generally considered high risk. NB tumors from these patients are often characterized by alterations in p53 expression and murine double minute (MDM2) activity with concomitant resistance to chemotherapy. We evaluated the ability of nutlin-3 to sensitize a p53-null and doxorubicin-resistant NB cell line, LA155N, to doxorubicin. Nutlin-3 treatment upregulated TAp73 and E2F1 protein levels. It potentiated the ability of doxorubicin to block cell proliferation and activate apoptosis and TAp73 knockdown resulted in a reduction of this sensitization. Additionally, PUMA expression was induced by the combination treatment, but reduced by knockdown of either TAp73 or E2F1. We conclude that, following nutlin-3 treatment, TAp73 and E2F1 are released from MDM2 and activated by doxorubicin to induce PUMA and apoptosis. This study addresses p53-independent mechanisms of nutlin-3 action in chemoresistant NB, especially in combination with chemotherapeutics. We believe that this model has strong clinical relevance for chemoresistant and p53 dysfunctional NB.

## Introduction

Neuroblastoma (NB) is a childhood solid tumor of the peripheral nervous system arising from neural crest progenitor cells and the most common extracranial pediatric solid tumor (1,2). Although high-risk NB is generally responsive to initial chemotherapy, children with advanced Stage 3 and 4 NB are

particularly at risk for relapse and chemoresistant disease, with a 5-year overall survival of ~25%. Alterations in p53 expression from the wild-type phenotype are rare in NB at diagnosis. However, inactivation or elimination of p53 is common in relapsed NB, one likely mechanism of resistance to therapy (3,4). The development of therapeutics that target p53-independent mechanisms for the treatment of chemoresistant NB is therefore of keen clinical interest.

MDM2 (murine double minute) is an E3 ubiquitin ligase and a nuclear phosphoprotein, which induces the nuclear export of p53 and promotes its ubiquitination and proteosomal degradation. The deregulation of MDM2 expression and activity occurs commonly in NB, and is thought to be, at least in part, responsible for the cytoplasmic sequestration of p53 (3-6). The loss of p14<sup>ARF</sup>, an inhibitor of MDM2 ubiquitin ligase activity, has been demonstrated in NB cell lines established at relapse (3) and MDM2 is a transcriptional target of N-Myc, amplification of which is strongly correlated with poor clinical outcome (5,11). Thus, there are multiple mechanisms of p53 inactivation in NB involving mutations that arise following extended drug therapy and overexpression of MDM2.

Nutlin-3, a selective small-molecule antagonist of MDM2, acts by binding the p53 pocket of MDM2, releasing p53 from negative control (reviewed in ref. 7). In this study, we investigated the ability of nutlin-3 to induce apoptosis in a p53-null and doxorubicin-resistant NB cell line, LA155N. LA155N contains p73, a homologue of p53 which occurs in multiple isoforms and is required for some NB cell differentiation (9,10,12). It is known that the p73 gene has at least two promoters and the resulting protein products have distinct and opposing effects. The full-length TAp73 form promotes apoptosis, while the amino-deleted  $\Delta$ Np73 form is transactivation deficient (13,15); it is thought that the relative expression of each determine the ultimate apoptotic outcome, as the  $\Delta$ Np73 form functions in a dominant-negative manner (12-15). Thus, the full-length, tumor-suppressive form functionally substitutes for p53. TAp73 induces cell cycle arrest and apoptosis by transactivating p53-responsive genes and shares many regions of protein homology with p53 (15,16). Importantly, both p53 and p73 have been shown to bind a hydrophobic pocket within the N-terminus of MDM2, a region which also binds HIF1 $\alpha$  and E2F1 (8,16).

E2F1, a member of the E2F transcription factor family and a functional oncogene, has been shown to activate apoptosis in

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the absence of functional p53 by inducing p73 transcription (17-19,21) and by the upregulation of PUMA, a proapoptotic Bcl-2 family member (20). It has been demonstrated that nutlin-3 functions dually to enhance TAp73 transcription and to stabilize the TAp73 protein (21). Both cisplatin and doxorubicin are known to activate E2F1 in some p53-inactive cancer cells (18).

In this study, we combined nutlin-3 treatment with doxorubicin, an inhibitor of DNA topoisomerase II action and an inducer of DNA damage. Based on findings from other labs, we reasoned that a combination treatment of doxorubicin with nutlin-3 would cooperatively activate E2F1 (17,20), upregulate TAp73 transcription and increase its stability (21,22,27-29), promoting apoptosis in doxorubicin-resistant NB cells. Barbieri *et al* have found that MDM2 inhibition increases the cytotoxicity of genotoxic drugs in p53-wt NB cells (25), while Ambrosini *et al* have found that nutlin-3 enhances doxorubicin-induced cytotoxicity in a prostate cancer cell line containing mutant p53 (18). We have extended these findings to a clinically challenging, chemoresistant p53-null neuroblastoma cell line, LA155N. Our results demonstrate, for the first time, that nutlin-3 enhances the cytotoxicity of doxorubicin in p53-null neuroblastoma cells by the combined upregulation of TAp73 and activation of E2F1.

## Materials and methods

**Reagents and primers.** Nutlin-3 (cat. no. 10004372) was purchased from Cayman Chemical (Ann Arbor, MI), and represents an equimolar mix of the active (nutlin-3a) and inactive (nutlin-3b) isomers. In this study, all molar concentrations are expressed in terms of nutlin-3a. Doxorubicin was obtained from the pharmacy of Eggleston Hospital of Emory University. TAp73-specific forward and reverse primers were a gift from Dr C. Castellino (Emory University); the forward and reverse primer sequences are: 5'-ccggcgtggggaagatggc-3'; and 5'-ttgaactgggcatgacagatg-3'. The forward and reverse primer sequences for E2F1 are: 5'-acgtgtcaggacctctgtagcatt-3'; 5'-tcgatcgggacctgtgttctctta-3'; and for PUMA, forward: 5'-gacc tcaacgcacagta-3'; reverse: 5'-ctaattgggctccatct-3'. The forward and reverse sequences for GAPDH are: 5'-tttaactctggtaaagt gga-3'; reverse: 5'-gaatcatattggaacatgta-3'.

**Cell lines, light microscopy and siRNA silencing.** LA155N (p53-null), SH-SY5Y (p53 wild-type) and SKNB2 (p53 mutant) cells were maintained in RPMI medium + 10% FBS. Cells were photographed with an Olympus IX50. siRNA (Stealth Prevalidated cat. no. 106203; Invitrogen Life Technologies, Carlsbad, CA) was used for TAp73 knock-down; three pairs of siRNA constructs were tested for knockdown efficacy using transient transfections and were compared. The first targeted a region within exon 13; the second targeted a region within exon 7. The third, 5'-gaacgg auccagcaugagacgucuu-3' (#110915) targeted a region within exon 3 and was found to be most effective. The Stealth Negative Control medium GC content (cat. no. 12935) was also purchased from Invitrogen Life Technologies. siRNA against E2F1 (On-Target plus Smart Pool, L-003259) was purchased from Dharmacon RNA Technologies (Chicago, IL) and consisted of the following sequences: 5'-ucggagaacuuuu

cagauu-3', 5'-gagaagucacgaaugaga-3', 5'-gagcagaugguuuau guga-3', and 5'-gaacagggccacugacucu-3'. Cells were plated 24 h before transfection at a concentration of  $2 \times 10^4$  per 1 mm<sup>2</sup> well (96-well plate) or  $3 \times 10^5$  per 35 mm<sup>2</sup> well; siRNA or the negative control were transfected using Oligofectamine (Invitrogen; cat. no. 12252) at 100 nM, per manufacturer's instructions. Cells were assayed at 24, 48 and 72 h post transfection.

**Western blotting, antibodies and coimmunoprecipitation.** For direct Western blotting, cells were lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glyceraldehyde, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM PMSF. RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, 1 mM PMSF) was used to lyse cells for coimmunoprecipitation (IP). GE (Uppsala, Sweden) Protein G Sepharose Fast Flow beads were used for immune complex binding, following an overnight 4°C incubation of 500  $\mu$ g of cell lysate with a combined 2  $\mu$ g pool of anti-MDM2 mouse monoclonal antibodies in RIPA buffer. Beads were washed three times with RIPA buffer and once in low salt buffer (1% NP-40, 50 mM Tris, 1 mM PMSF). Cleared lysate (30-50  $\mu$ g/well) or the total volume of IP were run on 10 or 12.5% Tris/glycine PAGE gels and transferred to NC under standard conditions. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): PUMA $\alpha/\beta$  FL-193 (sc-28226), p73 E-4 (sc-17823), E2F-1 C-20 (sc-193), Bax 2D2 (sc-20067), MDM2 H-221 (sc-7918), p53 FL-393 (sc-6243). Anti-MDM2 (Ab-1, OP-46) was purchased from Calbiochem. Anti-p73 $\alpha/\beta$  Ab-2 was purchased from Thermo Scientific (Fremont, CA). Anti-MDM2 (SMP-14) and anti- $\beta$ -actin (AC-74) were both purchased from Sigma (St. Louis, MO). All primary antibodies were used at manufacturer's suggested concentrations for the designated purposes. Goat anti-mouse or rabbit HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at 1:2000.

**Real-time RT-PCR.** A two step real-time RT-PCR procedure was used. All reagents were purchased from Qiagen (Valencia, CA) and all methods used were those recommended by the manufacturer. Total RNA was isolated from cells using QIAshredders (cat. no. 79654) and RNeasy (cat. no. 74104). For RT, samples were incubated at 42°C using Qiagen QuantiTect RT reagents. SYBR-Green reagents (cat. no. 204143) were used for the 40 cycle real-time PCR and reactions were run on an Applied Biosystems 7500 real-time PCR machine. GAPDH was used to normalize levels of mRNA for the relative quantification method of analysis.

**Cell proliferation and apoptosis.** The tetrazolium-based colorimetric assay, WST-1 (cat. no.11644807, Roche Applied Science, Mannheim, Germany), was used for the quantification of cell proliferation. Cells were plated at  $2 \times 10^4$  cells/well of a 96-well plate and treatments were performed in triplicate. Measurements were made at 450 nm with a reference wavelength of 600 nm. The Cell Death Detection ELISA<sup>PLUS</sup> kit (cat. no.11774425, Roche Applied Science) was used to quantitate levels of mono- and oligonucleosomes in the

Table I. Percent cells in cell cycle phase, as determined by the Dean-Jenn-Fox model.<sup>a</sup>

Treatment	subG1	G1	S	G2/M
NT	2.6	23.6	28.7	19.7
Nut-4 $\mu$ M	2.0	21.9	30.7	20.8
Dox-1 $\mu$ M	4.6	10.7	41.4	8.7
Nut/Dox	8.6	19.9	33.3	11.9

<sup>a</sup>Cell cycle analysis of effects of nutlin-3 and doxorubicin treatments on LA155N cells. Refer to Fig. 1D. Nut/Dox, 4  $\mu$ M nutlin-3 plus 1  $\mu$ M doxorubicin.

cytoplasm of apoptotic cells after treatments. The manufacturer's instructions were followed, with the following modification:  $10^3$  cells were used per well of a 96-well plate. Measurements were made at 405 nm with a reference wavelength of 490 nm.

**Flow cytometry.** Cells were plated 24 h before treatments. Nutlin-3 (4  $\mu$ M), doxorubicin (1  $\mu$ M) or the combination were added to exponentially growing cells. At 24 h after treatment,  $1-2 \times 10^6$  cells from each treatment were fixed in 80% ethanol at  $-20^\circ\text{C}$  for at least 24 h. Fixed cells were stained with 20  $\mu\text{g/ml}$  propidium iodide in 0.1% Triton X-100, 200  $\mu\text{g/ml}$  RNase A and DPBS. Cells were run on a BD FACs LSR II and analyzed using FlowJo (TreeStar, Inc.) software.

**Statistical analyses.** All experiments were repeated independently at least three times. Where shown, results are present as mean  $\pm$  SE. Statistical analyses were conducted using student's t-test. Group differences resulting in P-values of  $<0.05$  were considered to be statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Results

**Nutlin-3 and doxorubicin induce apoptosis and inhibit proliferation synergistically.** To gain an initial understanding of the interactive effects of nutlin-3 and doxorubicin on p53-null cells, we evaluated the responses to these agents using proliferation and apoptotic assays. We found the  $\text{IC}_{50}$  for nutlin-3 to be  $\sim 25 \mu\text{M}$ , well above the pharmacologically relevant limit of  $16 \mu\text{M}$  (data not shown). Previous study in our lab had determined the  $\text{IC}_{50}$  for doxorubicin in LA155N cells to be  $4.9 \mu\text{M}$  (unpublished data). Nutlin-3 and doxorubicin interacted synergistically; applying the isobologram method of Loewe (24), at 4  $\mu\text{M}$  nutlin-3 we found the doxorubicin  $\text{IC}_{50}$  to be  $\sim 1.0 \mu\text{M}$ , an almost 5-fold decrease (Fig. 1a). Similarly, the combination significantly increased apoptosis (as measured by a histone-cleavage assay) relative to either treatment alone (Fig. 1b, top). Nutlin-3 treatment had no effect on cell cycle parameters, but doxorubicin induced an S-phase block. The combination treatment increased the number of cells in sub-G1 more than additively and reduced

the number of cells in G2/M (Fig. 1b, bottom, Panels A-D; Table I). These treatments were reflected morphologically as well (Fig. 1c). Untreated LA155N cells (panel A) and cells treated with 4  $\mu\text{M}$  nutlin-3 (panel B) were similar in appearance. In contrast, treatment with 1  $\mu\text{M}$  doxorubicin (panel C) resulted in much larger and markedly rounder cells, consistent with an S phase block (arrows). The combination treatment (panel D) with 4  $\mu\text{M}$  nutlin-3 + 1  $\mu\text{M}$  doxorubicin induced both detachment (as indicated by refractile cells) and pronounced neuron-like outgrowth (arrows).

**Effects of nutlin-3 and doxorubicin on TAp73 and E2F1 in NB p53-null cells.** We initially evaluated protein expression in response to nutlin-3 treatment in NB cells representing different p53 phenotypes. We found that nutlin-3 upregulated TAp73 expression in the p53-null cells, but not in the p53 wild-type (wt) or mutant cells. Conversely, nutlin-3 upregulated p53 in the p53 mutant and wt cells. The downstream effector Bax was upregulated in the p53 wt cells, but was not expressed at detectable levels in the p53 mutant or null cells (Fig. 2A). We next asked if doxorubicin, or the combination of doxorubicin and nutlin-3, would result in an enhanced upregulation relative to the nutlin-3 treatment alone in the p53-null cell line. Although doxorubicin increased TAp73 expression, the combination did not (Fig. 2B). E2F1 has been implicated in nutlin-3-mediated apoptosis (17-19). We found that E2F1 expression was upregulated in response to nutlin-3, unchanged in response to doxorubicin treatment and at intermediate levels following treatment with the combination (Fig. 2B).

**Nutlin-3 treatment inhibits TAp73 binding to MDM2 in p53-null NB cells.** We used IP to evaluate and confirm the effects of nutlin-3 on the p73-MDM2 interaction. We found that in untreated p53-null cells, TAp73 binds to MDM2 and that 24 h nutlin-3 treatment largely inhibits this binding (Fig. 3).

**TAp73 knockdown reverses nutlin-3-mediated inhibition of proliferation in p53-null NB cells.** To evaluate the role of TAp73 in the nutlin-3-mediated anti-proliferative response, we used siRNA to knock down expression of TAp73. The chosen construct (#15) targeted a region in exon 3, and was most effective at reducing the full-length pro-apoptotic TAp73 and truncated forms over a period of 24-72 h (Fig. 4a). This effect was also evident at the mRNA level; mRNA levels were decreased by  $>70\%$  (data not shown). siRNA knockdown of TAp73 significantly reversed the anti-proliferative effect seen with the nutlin-3/doxorubicin combination treatment. Interestingly, nutlin-3 treatment following TAp73 knockdown promoted proliferation to  $\sim 130\%$  of the control (Fig. 4b, top panel). Morphological differences were also evident (Fig. 4b, bottom panel). In the presence of 8  $\mu\text{M}$  nutlin-3 and 1  $\mu\text{M}$  doxorubicin combination treatment, cells transfected with the negative control siRNA displayed apoptotic features (A); the TAp73 knockdown did not (B).

**Expression of BH3-only proapoptotic PUMA is dependent on TAp73 and E2F1.** To determine the roles of TAp73 and E2F1 on the expression of proapoptotic BH3-only PUMA, we used



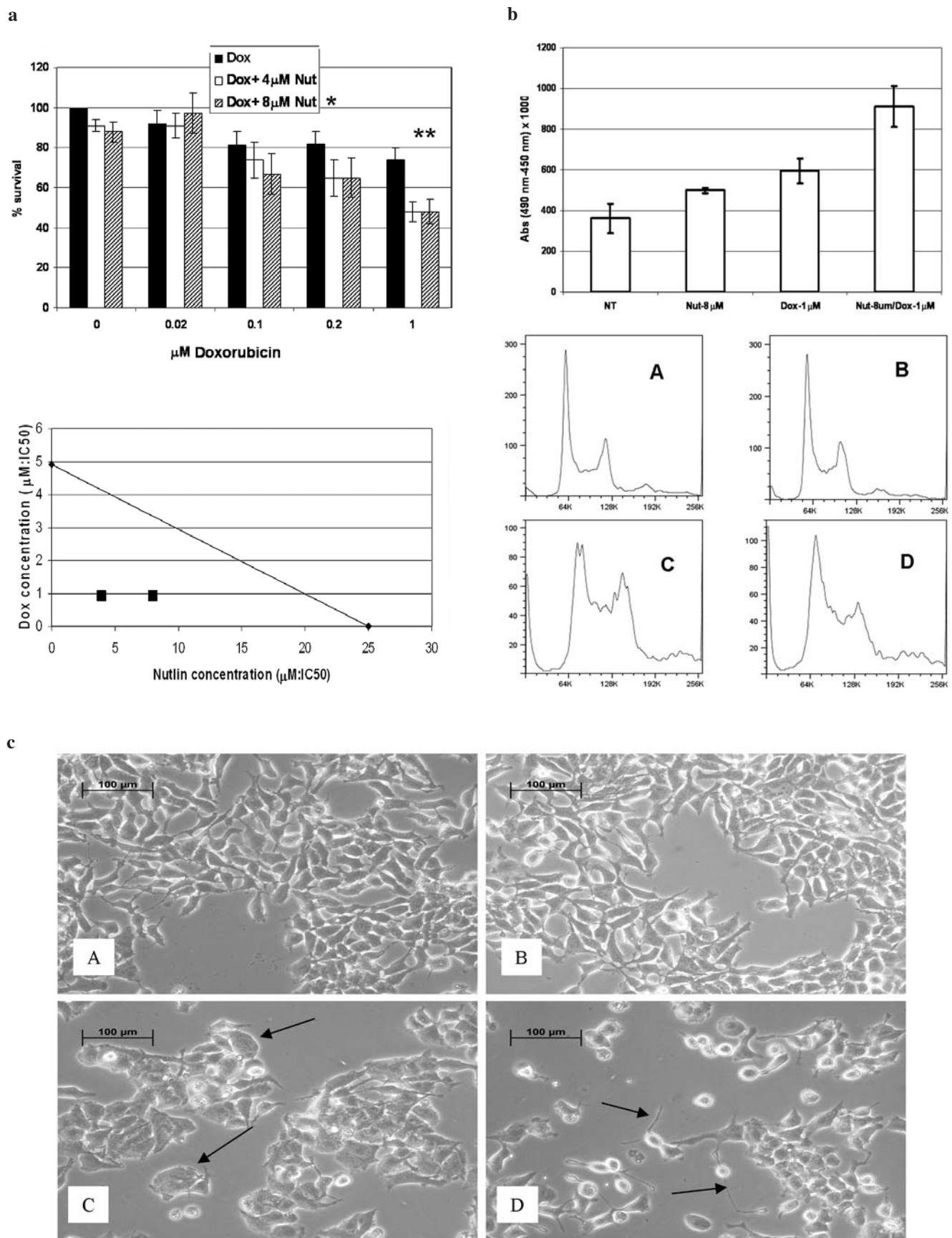


Figure 1. Nutlin-3 and doxorubicin induce apoptosis and inhibit proliferation synergistically. (a) Top panel, dose response for single and combination treatments, as measured by 24 h WST-1 assay. Results represent three separate experiments performed in triplicate. Bottom panel, isobologram demonstrating synergistic interaction between doxorubicin and nutlin-3 (4 and 8  $\mu$ M) in reducing cell proliferation. (b) Top panel, ELISA apoptosis assay using quantification of mono- and oligonucleosomes in cytoplasm as measure of apoptosis. Results are representative and show one experiment performed in triplicate. Bottom panel, representative cell cycle profiles using the Dean-Jenn-Fox model. (b,A) No treatment; (b,B) 4  $\mu$ M nutlin-3; (b,C) 1  $\mu$ M doxorubicin; (b,D) 4  $\mu$ M nutlin-3 + 1  $\mu$ M doxorubicin. (c) LA155N cells following: (c,A) No treatment; (c,B) treatment with 4  $\mu$ M nutlin-3; (c,C) treatment with 1  $\mu$ M doxorubicin; (c,D) treatment with 4  $\mu$ M nutlin-3 + 1  $\mu$ M doxorubicin (x400).

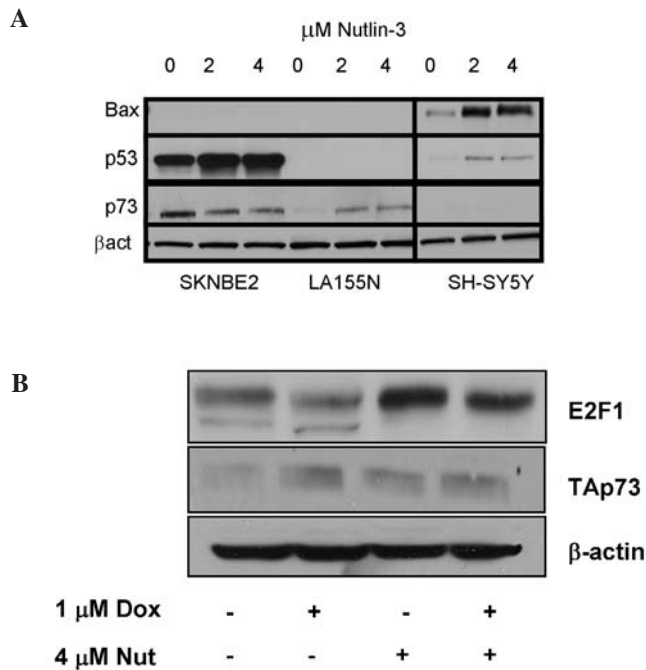


Figure 2. Effects of nutlin-3 and doxorubicin on TAp73 and E2F1 in p53 wt, p53 mutant and p53-null NB cells. (A) Levels of Bax, p53 and TAp73 were evaluated by Western blot analysis following 24 h treatment with 2 and 4 μM nutlin-3 in a p53 mutant cell line (SKNBE2), a p53 null cell line (LA155N) and a p53 wild-type cell line (SH-SY5Y). (B) Levels of E2F1 and TAp73 in LA155N cells evaluated by Western blot analysis following 24 h treatment with 4 μM nutlin-3 and 1 μM doxorubicin, singly and in combination.

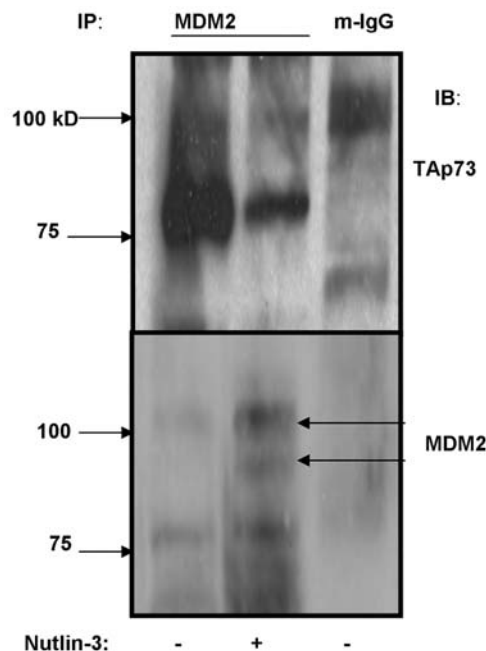


Figure 3. Nutlin-3 treatment inhibits TAp73 binding to MDM2 in p53-null NB cells. MDM2 was immunoprecipitated with 2 μg of pooled MDM2 monoclonal antibodies from 500 μg of LA155N cell lysate. After detection of TAp73, the blot was stripped for reprobing with MDM2. mIgG, normal mouse serum.

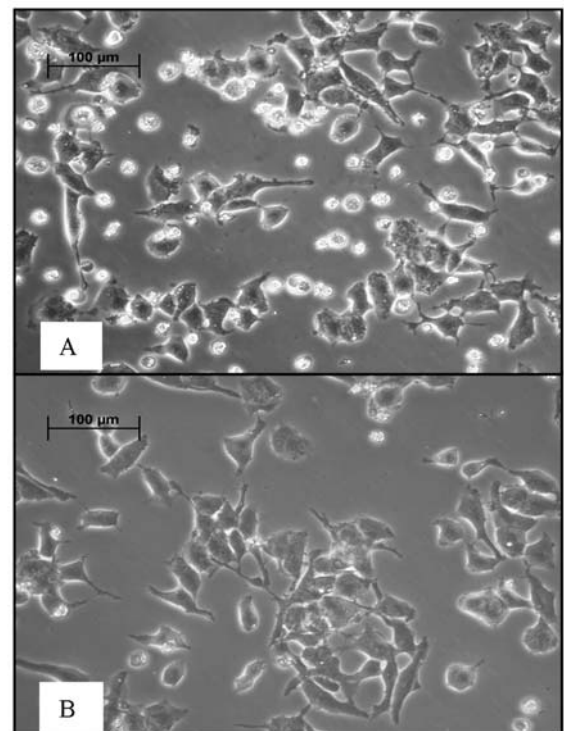
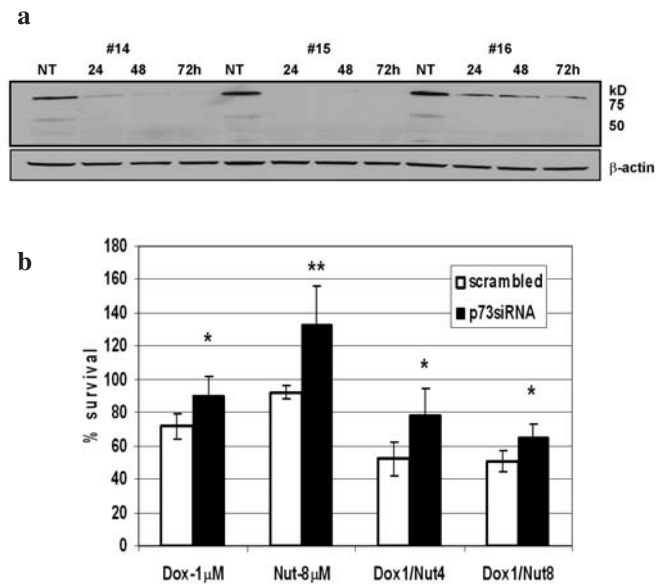


Figure 4. TAp73 knockdown reverses nutlin-3-mediated inhibition of proliferation in p53-null NB cells. (a) LA155N cells were transfected with 3 different constructs of TAp73 siRNA and a control construct at a concentration of 200 pmol per 35 mm<sup>2</sup> dish. Protein expression was evaluated at 24, 48 and 72 h. The p73 (Clone ER-15) monoclonal antibody detects the full-length TAp73 form (~80 kDa). (b) Top panel: cells were transfected with TAp73 siRNA or with the control siRNA for 24 h. Doxorubicin, nutlin-3 or the combination were added for an additional 24 h. Proliferation was measured by WST-1 assays relative to the untransfected control. Results are shown for pairwise experiments and represent three separate experiments performed in triplicate. Untreated, untransfected control, 100%. Bottom panel, LA155N cells transfected with: (b,A) negative control siRNA; and (b,B) TAp73 siRNA. Cells were then treated with the combination 8 μM nutlin-3 + 1 μM doxorubicin. (x400).

siRNA against TAp73 or E2F1. Knockdown of either TAp73 or E2F1 resulted in a reduction of PUMA in cells treated with nutlin-3/doxorubicin. Conversely, the combination treatment

increased levels of PUMA in cells transfected with control siRNA (Fig. 5A). Using Q-RT-PCR, we evaluated levels of E2F1, TAp73 and PUMA mRNA following E2F1 knock-

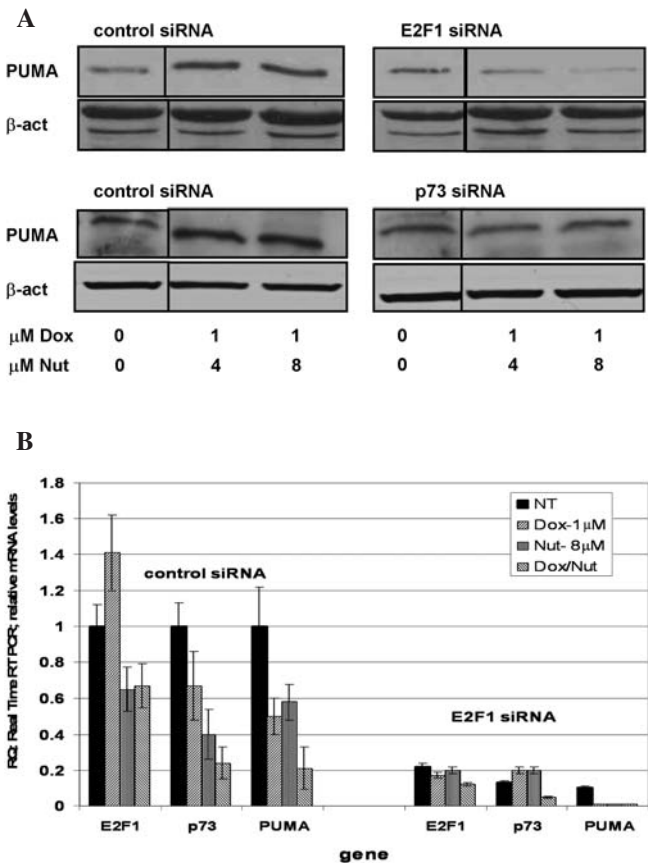


Figure 5. Expression of BH3-only proapoptotic PUMA is dependent on TAp73 and E2F1. (A) Cells were transfected with TAp73 siRNA, E2F1 siRNA, or with the control siRNA. At 24 h post transfection, cells were treated with 1 μM doxorubicin in combination with nutlin-3 for an additional 24 h. (B) Total RNA was isolated for use in real RT-PCR analysis from E2F1 transfected cells treated for an additional 24 h with 1 μM doxorubicin singly or in combination with nutlin-3. Data were calibrated against untreated samples from the control siRNA.

down. The reduction of E2F1 mRNA virtually abrogated TAp73 and PUMA mRNA both in untreated and treated cells (Fig. 5A).

Discussion

In this study, we evaluated the interaction between nutlin-3 and doxorubicin in inhibiting proliferation and inducing apoptosis in doxorubicin-resistant NB p53-null cells. Previously, nutlin-3 has been shown to disrupt the p53-MDM2 interaction in p53 wt cells, thereby stabilizing p53 and promoting apoptosis through induction of G1 cell cycle arrest (7,11,26). However, studies of p53 dysfunctional cells have yielded conflicting results; initial study suggested that these cells are nutlin-3-resistant (26), but more recent studies have demonstrated sensitivity via the disruption of p73-MDM2 binding (19,23). Our results indicate that TAp73 plays a critical role in response to nutlin-3 in p53-null NB cells and that cells are sensitized to nutlin-3 via the addition of a genotoxic agent. Thus, genotoxic damage is critical to potentiate the effects of nutlin-3 in inducing TAp73 and E2F1 activity.

It is known that p73 binds to the N-terminal hydrophobic pocket of MDM2 and Lau *et al* (23) recently provided evidence that nutlin-3 blocks p73-MDM2 interaction in a p53-mutant NB cell line (SKNBE2). Similarly, we found that nutlin-3 reduces TAp73 binding to MDM2 in p53-null NB cells (Fig. 3). MDM2 inhibits p73 transcriptional activity (27) and nutlin-3 treatment enhances p73 transcription in the absence of p53 (23). Although we saw an increase in TAp73 protein expression following 24 h nutlin-3 treatment (Fig. 2A and B), mRNA levels were reduced at this later timepoint (Fig. 5B). This may reflect stabilization of the TAp73 protein in the absence of ongoing transcription.

There is a growing consensus concerning the role of E2F1 in inducing apoptosis via p53-independent mechanisms following DNA damage. Although the E2F1 protein is best known as a transcription factor that enhances S-phase entry and cell proliferation, its proapoptotic properties have been well documented in cells lacking functional p53. Following DNA damage, both p73 and E2F1 are stabilized largely by the induced checkpoint kinases Chk1/Chk2; Chk2 phosphorylates E2F1, and both Chk1 and Chk2 play critical roles in p73 protein induction (22,28). It is also known that

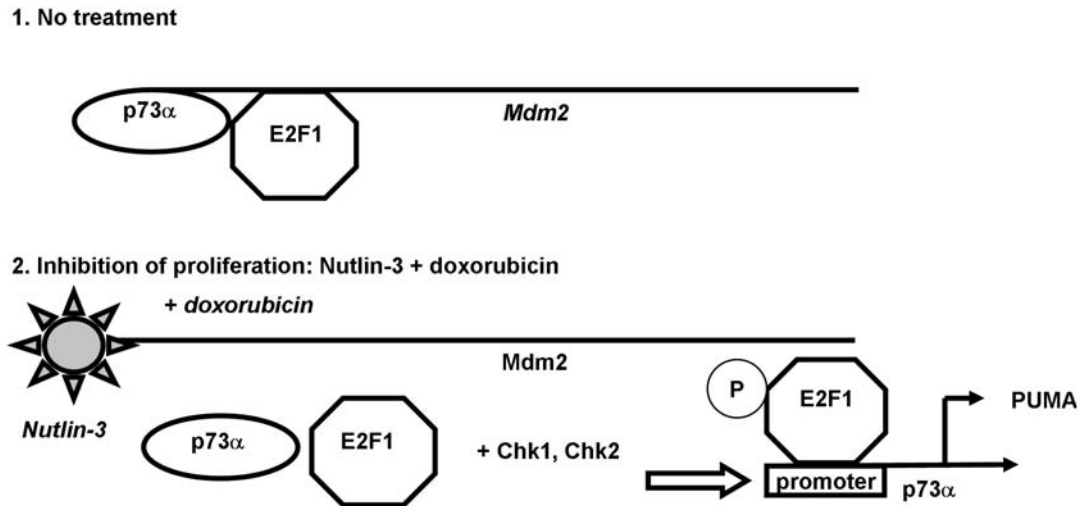


Figure 6. A schematic diagram for the proposed synergistic interaction of doxorubicin and nutlin-3 in the p53-null NB cell line, LA155N.



E2F1 transactivates p73 (29) and that p73 is required for E2F1-induced apoptosis (20). Another role for DNA damage may be to selectively degrade the anti-apoptotic  $\Delta$ Np73 form, increasing the TAp73 to  $\Delta$ Np73 ratio (30). Our data suggest that the increased apoptosis observed in p53-null NB cells treated with nutlin-3/doxorubicin is dependent on the induction of E2F1 and TAp73 in the presence of DNA damage. In contrast, we hypothesize that the strongly proliferative response to nutlin-3 in the absence of doxorubicin and TAp73 reflects the failure to upregulate Chk1/Chk2 (Fig. 4b).

Based on our results, we postulate a mechanism for the interaction of TAp73 and E2F1 in p53-null NB cells treated with combined nutlin-3 and doxorubicin (Fig. 6). In untreated cells, TAp73 and E2F1 are bound to MDM2. Combination treatment leads both to nutlin-3-mediated dislocation of E2F1/TAp73 from MDM2 and doxorubicin-mediated DNA damage. Without TAp73 dislocation from MDM2, induction of downstream apoptotic proteins will occur at only low levels. DNA damage induces Chk1/Chk2 which subsequently phosphorylate both E2F1 and p73, stabilize E2F1 and promote TAp73 transcription. Thus, without concurrent DNA damage, we hypothesize that p53-null tumors will be resistant to nutlin-3.

The net result of increased activation of E2F1 and TAp73 is the induction of downstream apoptotic activators. p73-induced apoptosis is mediated by a strong induction of PUMA (p53 upregulated modulator of apoptosis), a BH3-only protein, and by a much weaker Bax promoter transactivation (30). Subsequently, PUMA localizes to the mitochondria, where it interacts with Bcl-2 and induces cytochrome c release (31). Here, we demonstrate a clear induction of PUMA (Fig. 5A) and a correlation to apoptosis in response to the combination treatment (Fig. 1b and c). Our results suggest that PUMA, not Bax, is the major downstream apoptotic target. The significant reductions of TAp73 and PUMA mRNA following siRNA knockdown of E2F1 underscore the importance of transcriptional activation of E2F1 in TAp73 and PUMA expression (Fig. 5B).

Taken together, it is apparent that the synergistic interactions involved in driving apoptosis in chemoresistant, p53-null NB cells following combination treatment include: i) the activation and stabilization of E2F1; and ii) the enhancement of p73 function, indicated by the upregulation of PUMA. Thus, nutlin-3 may prove useful in the treatment of highly aggressive chemoresistant, p53-null NB tumors, especially in combination with genotoxic therapies.

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