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Report

The Role of *MYCN* in the Failure of MYCN Amplified Neuroblastoma Cell Lines to G_1 Arrest After DNA Damage

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ABSTRACT

We previously reported that 3 p53 wild type (wt) *MYCN* amplified (*MNA*) neuroblastoma cell lines failed to G_1 arrest after DNA damage despite induction of p53, $p21^{WAF1}$ and MDM2. We hypothesised that this was due to high MYCN expression. p53 responses to DNA damage were examined in an additional 13 p53 wt neuroblastoma cell lines. *MNA* was significantly associated with a failure to G_1 arrest after DNA damage ($p < 0.001$) and higher levels of apoptosis after irradiation ($p < 0.05$). $p21^{WAF1}$ and hypophosphorylated (hypo) RB accumulation post irradiation were significantly lower in cell lines that failed to G_1 arrest ($p < 0.05$). Conditional MYCN expression in non-*MNA* SHEP Tet21N cells did not affect the G_1 arrest after irradiation. MYCN knockdown using siRNA in 3 p53 wt *MNA* cell lines did not restore a G_1 arrest after irradiation, but increased the baseline G_1 population, $p21^{WAF1}$ and hypo RB expression. MYCN siRNA also caused a G_1 arrest in a p53 mutant *MNA* cell line. This study is the first to determine that *MNA* correlates with a failure to G_1 arrest and attenuated $p21^{WAF1}$ induction; however MYCN expression alone is not causally responsible.

INTRODUCTION

The *MYCN* oncogene is amplified in 25% of neuroblastoma and is strongly associated with a poor prognosis.[1] *MYCN* is located on chromosome 2p24 and encodes a 60–63 kDa transcription factor that is a member of the MYC family. MYC transcription factors have a conserved structure which includes a transcriptional activation domain in the N-terminus and a C-terminus basic helix-loop-helix/leucine zipper domain that plays a role in protein dimerisation, sequence specific DNA binding and the regulation of transcription.[2] MYCN heterodimerises with MAX and binds to E-box DNA sequences in promoters of MYCN target genes. MYCN expression is limited to central and peripheral nervous systems, kidney, lungs and spleen during embryonic development, unlike its counterpart MYC that is expressed in multiple tissues at all stages of development.[3]

A number of studies have characterised the role of MYCN in tumor progression and tumorigenicity. A transgenic mouse model, in which high levels of MYCN were expressed in neuroectodermal cells, developed neuroblastoma with chromosomal losses and gains in regions orthologous to those found in human neuroblastoma.[4,5] Reduction of MYCN expression using MYCN antisense oligonucleotides in the same model decreased proliferation and induced neuronal differentiation of neuroblastoma cells.[6] Conditional expression of MYCN in a non-*MNA* neuroblastoma cell line has been shown to shorten the time taken to progress through the cell cycle, increase proliferation and decrease attachment of cells to the extra-cellular matrix.[7] Although *MNA* is a poor prognostic indicator, the clinical role of MYCN expression has not been as clearly defined. A recent report has suggested that high levels of MYCN expression in tumors lacking amplification is associated with a favorable prognosis, and induced expression of MYCN in p53 mutant SK-N-AS cells slows growth by increasing levels of apoptosis and increasing the expression of favorable genes.[8]

p53 has been described as 'the guardian of the genome'.[9] After DNA damage, p53 binds DNA in a sequence specific manner and activates the transcription of a number of genes including *MDM2*, *BAX* and *$p21^{WAF1}$*. MDM2 forms an auto-regulatory feedback loop with p53. The induction of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor, induces a G_1 arrest until DNA has been repaired or apoptosis has been initiated.[10] p53 has been described as the most frequently mutated gene in human cancer and is particularly prevalent in adult malignancies; however several studies have found that *p53* mutations in both neuroblastoma tumors and cell lines are rare.[11,12] When *p53* mutations do occur they

are in progressive and relapsed neuroblastoma, suggesting that *p53* mutations occur as a mechanism for resistance to cytotoxic drugs that target the p53 pathway.[11] Other aberrations in the p53 pathway have been described to occur at relapse, such as *MDM2* amplification, and *p14^{ARF}* deletion and methylation.[13] MDM2 has been shown to be a direct transcriptional target of MYCN, with MYCN binding to the E-box in the P2 promoter region of *MDM2*. Slack et al propose that MYCN expression decreases the stability of p53 through increasing levels of MDM2 expression, consequently inhibiting MYCN driven apoptosis.[14]

An attenuated G_1 arrest in response to DNA damage has been observed in some wt p53 neuroblastoma cell lines despite induction of p53, MDM2 and p21^{WAF1}.[15-18] It has previously been reported that the *MNA*, *p53* wt cell lines SK-N-BE(1n), IMR-32 and NGP all fail to undergo a G_1 arrest in response to DNA damage and the non-*MNA* cell lines SHEP, SKNSH and SH-SY-5Y all undergo a G_1 arrest post irradiation.[17,18] This suggests that a failure to G_1 arrest is linked to *MNA*. In this study, the p53 responses to DNA damage were investigated in a further seven *MNA* and six non-*MNA* p53 wt cell lines to extend the above observations. To test the hypothesis that high MYCN expression is causally linked to a failure to undergo a G_1 arrest in response to DNA damage, MYCN expression was manipulated in the non-*MNA* SHEP cell line with a tetracycline regulated Tet21N MYCN expression vector. In addition MYCN was knocked down using siRNA in four *MNA* cell lines to determine if reduction of MYCN would restore a G_1 arrest in response to DNA damage.

Table 1 **Densitometry results for Western blots of ten *MNA* and seven non-*MNA* neuroblastoma cell lines after irradiation showing maximal p53, p21^{WAF1}, MDM2 and hypo RB induction with times of maximal induction**

Cell Line	MYCN Status	p53	MDM2	p21^{WAF1}	Hypo Rb
GIMEN	Non-*MNA*	1.2 2 h	12.8 6 h	3.0 24 h	1.2 24 h
NB-69	Non-*MNA*	1.5 6 h	1.9 6 h	0.3 6 h	0.8 6 h
SJNB-1	Non-*MNA*	0.8 6 h	0.8 6 h	1.8 6 h	1.2 6 h
NBL-S	Non-*MNA*	0.6 6 h	1.2 6 h	0.8 6 h	1.8 6 h
LAN-6	Non-*MNA*	0.7 6 h	0.8 6 h	2.1 24 h	1.0 6 h
SKNRA	Non-*MNA*	1.1 6 h	1.0 6 h	2.7 24 h	2.1 24 h
SH-SY-5Y	Non-*MNA*	1.0 2 h	4.4 6 h	1.5 24 h	3.7 24 h
PER-108	*MNA*	1.7 6 h	1.2 6 h	1.2 24 h	1.6 6 h
SKNBE(1n)	*MNA*	2.1 6 h	0.4 6 h	0.1 6 h	0.7 6 h
CHP902R	*MNA*	0.6 6 h	0.6 6 h	0.6 24 h	1.2 24 h
IMR-32	*MNA*	1.7 2 h	1.6 24 h	0.2 24 h	0.6 2 h
NGP	*MNA*	0.4 6 h	3.1 6 h	0.5 24 h	0.6 6 h
SMSKCNR	*MNA*	0.8 6 h	0.8 6 h	0.3 24 h	0.6 6 h
CHLA-136	*MNA*	1.5 6 h	0.5 6 h	0.6 24 h	0.3 6 h
NBLW	*MNA*	1.1 6 h	1.0 6 h	0.2 6 h	0.4 2 h
LS	*MNA*	0.3 24 h	3.9 24 h	0.1 6 h	0.6 6 h
TR14	*MNA*	0.1 6 h	3.1 24 h	0.2 6 h	0.9 2 h

30 μg protein was loaded for each sample and all densitometry results were normalised to a positive control (6 h irradiated SKNRA).

MATERIALS AND METHODS

Tissue culture. Seventeen p53 wt human Mycoplasma-free neuroblastoma cell lines were studied.[13] The non-*MNA* cell lines were: SJNB-1,[19] NBL-S,[20] SKNRA,[12] SHSY5Y,[21] LAN-6,[22] NB69[23] and GIMEN[24] and the *MNA* neuroblastoma cell lines were IMR-32, NGP,[25] PER-108,[26] CHLA-136, SMSKCNR[12] TR14,[27] LS,[28] CHP902R,[29] SKNBE(1n)[18] and NBLW.[30] The *MNA* p53 mutant cell line SKNBE(2c) with a missense mutation in exon 5 codon 135: TGC (cysteine) to TTC (phenylalanine)[18] was used for siRNA experiments. All cell lines were grown in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco). Cells were grown at 37°C, in a humidified incubator with 5% CO_2.

DNA damage by γ-irradiation. Cells were plated in 28 cm^2 tissue culture dishes (Nunc, Naperville, IL) at 5 x 10^5 cells per plate and irradiated when ~70% confluent with 4 Gy 310 kV X-rays from a RS320 irradiation system (Gulmay Medical, Surrey, UK). Cells were harvested 2, 6 and 24 hors post irradiation for Western blotting and 24 hours post irradiation for cell cycle analysis. Experiments were carried out at least n = 2.

Protein lysate preparation and Western blotting. Whole cell extracts were prepared, and 30 μg protein/sample separated by gel electrophoresis and Western blotting performed using methods previously described.[17] Densitometry was performed using a Fuji-Las camera and the AIDA image analyser program (Raytek, Sheffield, UK) used to quantify band intensities.

Antibodies. Primary antibodies used were mouse monoclonal p53 DO7 at 1:1000 dilution (Novocastra, Newcastle, UK), MDM2 at 1:100, p21^{WAF1} at 1:100 (Calbiochem, Cambridge, MA), hypophosphorylated retinoblastoma protein (Hypo RB) at 1:100 and total retinoblastoma protein at 1:500, both from BD biosciences (Oxford UK) and the mouse monoclonal MYCN 100 antibody at 1:10 (gift from N. Igekaki). β-actin rabbit polyclonal used as a protein loading and transfer control at 1:1000 (Sigma, St Louis, MO). The secondary goat anti-mouse IgG and goat-anti rabbit IgG antibodies were both HRP conjugated and used at 1:1000 (DAKO, Glostrup, Denmark).

SHEP Tet21N MYCN expression system. The SHEP Tet21N cell line[7] was grown in RPMI 1640 supplemented with 10% tetracycline-free FCS (Clontech, Mountain View, CA) G418 and Hygromycin (Promega, Southampton, UK). SHEP Tet21N cells express MYCN in the absence of tetracycline. To switch off MYCN expression, 10 ng/ml Tetracycline (Sigma) was added to the growth media at least 24 hours prior to experiments.

Flow cytometry. Adherent cells (~2 x 10^6) were harvested with trypsin 24 hours post irradiation, fixed in ice cold 70% Ethanol 30% PBS solution and stored at 4°C. Cells were resuspended in fresh PBS with 40 μg/ml propidium iodide and 0.1 mg/ml RNAse A (Sigma) and incubated at 37°C for 30 minutes. Measurements were performed using a FACScan (Becton Dickinson Oxford, UK) and analysed using Cell Quest software (Beckton Dickinson) and WinMDI software (TSRI, La Jolla, USA). Cell cycle experiments were performed in triplicate.

Apoptosis measurements. Apoptosis measurements using Hoechst and FACs were carried out as described previously.[17] Cells were harvested 24, 48, 72 and 96 hours post irradiation, and apoptosis experiments for both methods were carried out in triplicate.

MYCN expression measured by quantitative reverse transcriptase PCR. RNA extraction, reverse transcriptase PCR and real time PCR was carried out as described in Carr et al.[13] Primers and probes for both the MYCN and the β-Actin control were Taqman assays from Applied Biosystems (Foster City, CA). Each of the samples were run in triplicate on a 384 well plate using a ABI prism 7900 detection kit (Applied Biosystems). Each experiment was carried out in triplicate.

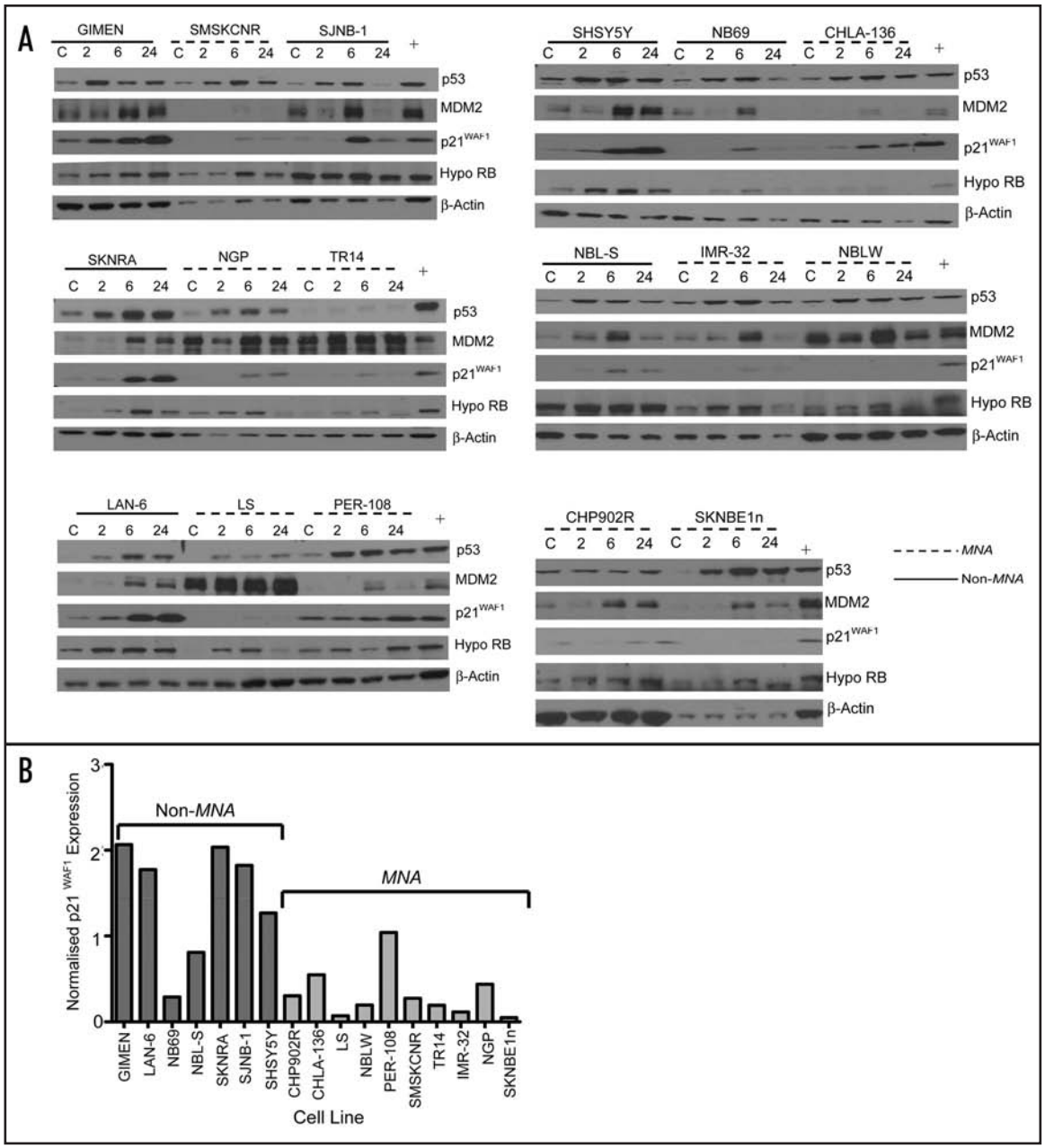

Figure 1. (A) Western blots for p53, MDM2, $p21^{WAF1}$, Hypo RB and β-actin in 17 cell lines 2, 6 and 24 hours post irradiation. The positive control is the 6h irradiated SKNRA sample. (B) Bar graph showing the normalised expression of $p21^{WAF1}$ at 6 hours post irradiation for all 17 cell lines tested. Levels of $p21^{WAF1}$ protein expression were significantly lower in *MNA* cell lines compared with non *MNA* cell lines ($p < 0.01$). Cell lines were irradiated n = 2.

RNA Interference of MYCN. Small interfering RNA (siRNA) oligonucleotides targeted against exon 2 of *MYCN* were synthesised by Eurogentec (Seraing, Belgium). The sense strand of the *MYCN* siRNA sequence was: UGAUCUGCAAGAACCCAGA. A scrambled sequence of the MYCN siRNA was used as a negative control. The scrambled (SCR) control sequence was: AUGUUAUCUGGCCCGUGUA. A BLAST™ database search was carried out against the human genome (nucleotide database) to ensure that MYCN and SCR sequences had no cross reactivity with other gene transcripts. MYCN siRNA was transiently transfected into cells at final concentrations of either 25 nM for NGP and SKNBE(2c) or 40 nM for IMR-32 and NBLW using Lipofectamine (Invitrogen, Paisley, UK) for 24 hours according to manufacturer's instructions. Knock down of MYCN using siRNA was monitored by Western blot analysis and performed in 4 *MNA* neuroblastoma cell lines: p53 wt IMR-32, NBLW, NGP and the p53 mutant cell line SK-N-BE(2c). The results for FACs cell cycle and Western blot densitometry displayed in the figures were for the concentration of siRNA that gave the most consistent cell cycle results and percentage MYCN knockdown. Optimal MYCN knockdown was at 16 hours and was measured 48 hours later before an increase in MYCN returned.

Statistics. For statistical tests, some data sets did not have a normal distribution, (determined using an Anderson Darling Normality test) so a Mann Whitney test was used throughout with the level of statistical significance taken as $p < 0.05$.

RESULTS

p53 transcriptional function in response to DNA damage. The p53 functional response to DNA damage was investigated using Western blotting for p53, MDM2, $p21^{WAF1}$ and hypo RB in 17 p53 wt neuroblastoma cell lines, 7 non-*MNA* and 10 *MNA* cell lines (Table 1 and Fig. 1). Each sample was compared to a positive control (the 6 hours post irradiation SKNRA sample). The non-*MNA* SKNRA cell line has previously been reported to have an intact p53 response to DNA damage.[12]

The non-*MNA* cell lines: GIMEN, SJNB-1, SKNRA, LAN-6, NBL-S, SHSY5Y all had higher levels of $p21^{WAF1}$ induction after DNA damage compared with *MNA* cell lines (Fig. 1A and B). The NB69 non-*MNA* cell line had relatively low levels of $p21^{WAF1}$ and hypo RB after irradiation (Fig. 1 and Table 1). Lower levels of $p21^{WAF1}$ and hypo RB induction were observed in 9/10 *MNA* cell lines: SMSKCNR, CHLA-136, IMR-32, NBLW, NGP, TR14, LS, SKNBE1n and CHP902R (Fig. 1 and Table 1). The *MNA* PER-108 cell line had higher levels of $p21^{WAF1}$ and hypo RB induction compared with other *MNA* cell lines, similar to levels in non-*MNA* cell lines (Fig. 1). Levels of $p21^{WAF1}$ induction 6 and 24 hours post irradiation were significantly lower in *MNA* compared with non-*MNA* cell lines ($p < 0.01$ and $p < 0.05$) (Fig. 1B) and levels of hypo RB were significantly lower 6 and 24 hours post irradiation ($p < 0.01$). Comparison of induced p53 and MDM2 levels showed no significant difference between *MNA* and non-*MNA* cell lines (Table 1).

Cell cycle arrest after irradiation of cell lines. The SKNRA cell line shown in Figure 2A as an example of a non-*MNA* cell line, underwent a significant G_1 arrest 24 hours after irradiation ($p < 0.001$). The proportion of cells in G_1 increased from 78.4 ± 1.2% to 88.0 ± 1.4% and the proportion in S-phase decreased from 9.8 ± 0.5% to 1.6 ± 0.1%. The G_1:S ratio increased from 8.3 ± 0.5 to 60.8 ± 8.7 post irradiation (Fig. 2A and C). All six non-*MNA* cell lines underwent a G_1 arrest in response to DNA damage (Fig. 2A and C). Cell cycle data for the non-*MNA* NBL-S cell line is consistent with a previous report showing a significant G_1 arrest after irradiation in this cell line.[31]

The *MNA* NBLW cell line failed to undergo a G_1 arrest in response to DNA damage (Fig. 2B and C). The G_1:S ratio did not change significantly between the control (2.8 ± 0.1) and 24 hours post irradiation samples (3.3 ± 0.7). The proportion of cells in G_1 decreased from 60.6 ± 0.8% to 43.8 ± 0.8% after irradiation and the proportion of S-phase cells decreased from 21.3 ± 0.7% to 17.5 ± 2.7% and cells accumulated in G_2 increasing from 18.4 ± 0.8% to 39.0 ± 2.1% (Fig. 2B). Six of the seven *MNA* cell lines NBLW, SMSKCNR, CHLA-136, CHP902R, TR14, and LS failed to G_1 arrest after irradiation. The G_1:S ratios decreased or remained the same after irradiation (Fig. 2B and C). The G_1:S ratio of the *MNA* CHP902R cell line increased after irradiation, however this was not statistically significant ($p = 0.13$). The only *MNA* cell line that did G_1 arrest was the PER-108 cell line, which also has higher levels of $p21^{WAF1}$ and hypo RB induction after irradiation (Table 1 and Fig. 1).

Cell cycle data from the 13 cell lines analysed in the current study was combined with existing data on NGP, IMR-32, SK-N-BE(1n), SHEP, SK-N-SH and SH-SY-5Y cell lines from[17] and[18] in Figure 2D. *MNA* cell lines had significantly lower increases in G_1:S ratio after irradiation when compared with non-*MNA* neuroblastoma cell lines ($p < 0.001$).

Apoptosis after irradiation induced DNA damage. The apoptotic response to irradiation was compared in five non-*MNA* and eight

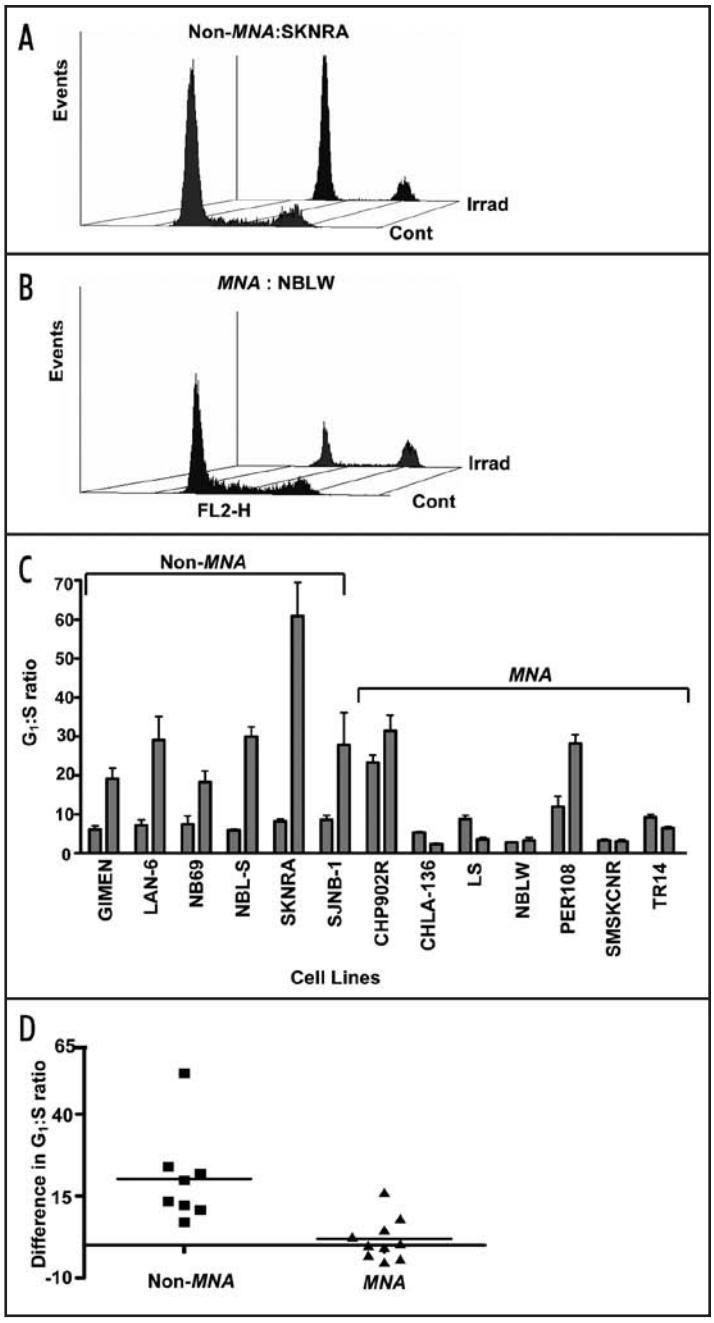

Figure 2. Cell cycle data. a) Cell cycle histograms for non-*MNA* SKNRA and b) *MNA* NBLW cell lines. Cont = non-irradiated cells and Irrad = 24 hour post irradiated samples. c) Summary of cell cycle data, displayed as G_1:S ratios for the panel of 13 neuroblastoma cell lines tested. d) Comparison of the differences in G_1:S ratio between non-*MNA* and *MNA* cell lines ($p < 0.005$ Mann Whitney test). This figure includes data for IMR-32, SKNBE1n, SHSY5Y and NGP cells from references 17 and 18. Cell cycle experiments were carried out n = 3.

MNA neuroblastoma cell lines including four previously studied cell lines[17,18] (Fig. 3). The non-*MNA* LAN-6, SJNB-1, GIMEN, SHSY5Y and SKNRA cell lines underwent lower levels of apoptosis measured by Hoechst staining following irradiation compared with PER-108, NBLW, CHLA-136, LS and SMSKCNR *MNA* cell lines. Maximal levels of apoptosis at 96 hours were 5-24% by Hoechst and 16–36% by FACs for the above non-*MNA* cell lines compared with 42–70% apoptosis for the above *MNA* cell lines (Fig. 3A–C). The

Table 2 ***MNA* cell lines treated with siRNA. MYCN knockdown is the % knockdown of MYCN expression in siRNA treated cells compared to the SCR control**

Cell Line	MYCN Knock Down	p53	$p21^{WAF1}$	Hypo RB	Total RB	SCR siRNA G_1:S	MYCN siRNA G_1:S	SCR Versus MYCN siRNA
IMR-32	36-71%	1.2 ± 0.2	14.6 ± 8.1	4.5 ± 2	1.2 ± 0.1	2.6 ± 0.3	5.0 ± 0.5	$p < 0.005$
NBLW	40-58%	0.8 ± 0.2	1.7 ± 0.3	1.9 ± 0.7	0.8 ± 0.09	2.4 ± 0.1	4.0 ± 0.2	$p < 0.001$
NGP	66-71%	0.6 ± 0.1	3.7 ± 1.1	2.0 ± 0.5	0.5 ± 0.03	2.8 ± 0.2	7.2 ± 0.3	$p < 0.005$
SKNBE(2c)	30-62%	0.6 ± 0.2	0.5 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	4.4 ± 0.3	7.3 ± 0.8	$p < 0.01$

Fold increases for p53, $p21^{WAF1}$, Hypo RB and total RB are relative to the SCR control. Cell cycle data for the siRNA treated MNA cell lines is expressed as G_1:S ratios. The differences in the mean G_1:S ratio between MYCN siRNA treated and SCR controls were compared using a Mann Whitney test. siRNA experiments were carried out n = 3.

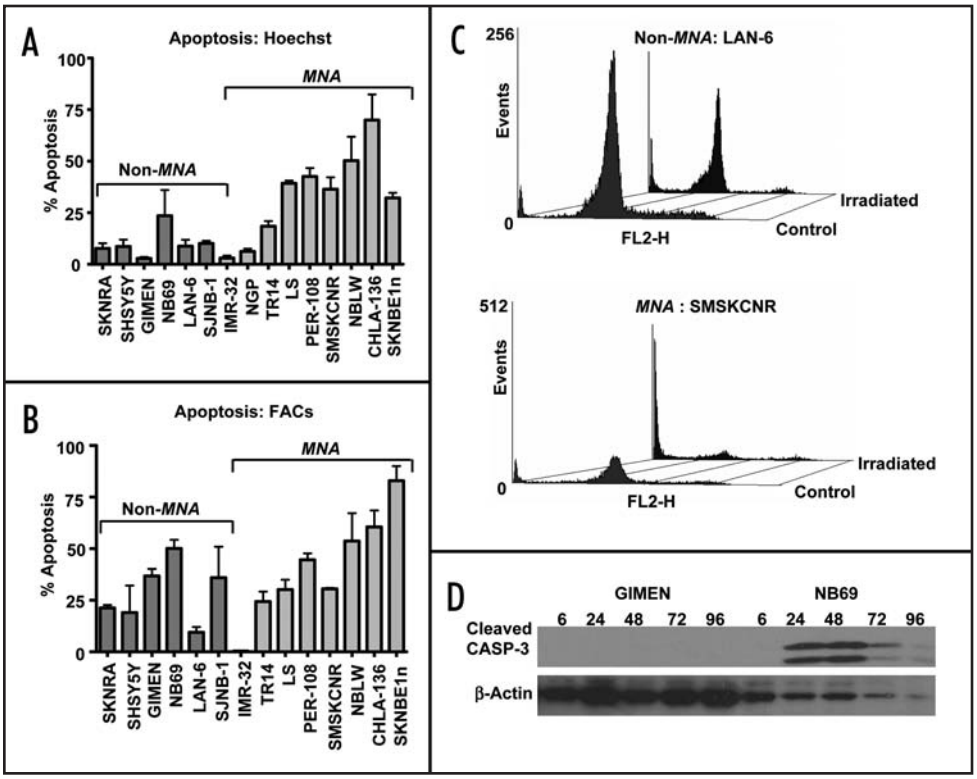

Figure 3. (a) % apoptosis 96 hours post irradiation determined by Hoechst staining (b) % apoptosis determined by FACs. (c) Cell cycle histograms for the non-*MNA* LAN-6 and *MNA* NBLW cell lines. Apoptotic cells were present in the sub G_1 peak on FL2-H cell cycle histograms (d) cleaved caspase -3 for GIMEN and NB69 cells 24-96 hours post irradiation. Apoptosis experiments were carried out n=3.

non-*MNA* NB69 cell line was more sensitive with maximal levels of apoptosis of 55% and 50% at 48 hours measured by FACs and Hoechst respectively and the *MYCN* and *MDM2* amplified TR14 cell line was more resistant with maximal apoptosis being 24% by FACs and 18% by Hoechst. Levels of apoptosis were generally higher measured by FACs compared with Hoechst, but particularly so for GIMEN cells (Fig. 3A and B). There was no cleaved caspase-3 after irradiation in GIMEN cells (Fig. 3D) compared with NB69 cells, where cleaved caspase-3 was present from 24 hours after irradiation. This confirmed the apoptosis levels measured by Hoechst and suggested FACs analysis had over-estimated apoptosis. There was no significant difference in levels of apoptosis 96 h post irradiation in *MNA* compared with non-*MNA* cell lines as determined by FACs. However, levels of apoptosis measured by Hoechst were significantly higher in *MNA* cell lines than non-*MNA* cell lines ($p < 0.05$).

Effect of induced MYCN expression in SHEP Tet21N cells. The p53 downstream response to DNA damage was intact in both Tet21N $MYCN^+$ and Tet21N $MYCN^-$ cells (Fig. 4A). p53 levels increased after irradiation, although maximal levels were observed at different times; 6 hours post irradiation (Tet21N $MYCN^+$) and 2 hours post irradiation (Tet21N $MYCN^-$). MDM2 levels in both Tet21N $MYCN^+$ and Tet21N $MYCN^-$ cells were maximal at 6 hours post irradiation, reaching higher levels in the Tet21N $MYCN^+$ cells. $p21^{WAF1}$ increased in both Tet21N $MYCN^+$ and Tet21N $MYCN^-$ cells 24 hours post irradiation. The baseline level hypo RB was higher in the Tet21N $MYCN^-$ compared to Tet21N $MYCN^+$ cells. After irradiation hypo RB increased in the Tet21N $MYCN^+$ cells to maximal levels at 24 hours comparable to those in the Tet21N $MYCN^-$ cells (Fig. 4A). Levels of total RB remained constant (data not shown).

After irradiation both Tet21N $MYCN^-$ and Tet21N $MYCN^+$ cells underwent a G_1 arrest. The G_1:S ratio in the Tet21N $MYCN^-$ cells increased significantly from 12.7 ± 1.8 in the control to 28.9 ± 4.5, after irradiation ($p < 0.005$). The G_1:S ratio of the Tet21N $MYCN^+$ cells increased significantly from 7.7 ± 0.7 in the control to 20.9 ± 2.0 after irradiation ($p < 0.005$) (Fig. 4B). The G_1 population was higher in Tet21N $MYCN^-$ cells under normal growth conditions: 79.5 ± 2.0% compared with 70.4 ± 1.8% in Tet21N $MYCN^+$ cells (Fig. 4B). Growth curve assays revealed that Tet21N $MYCN^+$ cells had a faster doubling time of 78.5 ± 1.4 hours, compared with Tet21N $MYCN^-$ cells (90.4 ± 1.2 hours) (data not shown). Tet21N $MYCN^+$ cells underwent more apoptosis after irradiation than Tet21N $MYCN^-$ cells 18.8 ± 3.9 % apoptosis compared with 4 ± 1.1 % 72 hours post irradiation respectively (data not shown).

The level of Tet21N MYCN expression in the Tet21N SHEP cells were compared with the other cell lines at the mRNA and protein level (Fig. 4C and D). MYCN mRNA levels in the Tet21N $MYCN^+$ cells were similar to the *MNA* cell lines IMR-32, CHLA-136 and PER-108. MYCN protein expression in the Tet21N $MYCN^+$ cells

were comparable to MYCN levels observed in *MNA* cell lines (Fig. 4D). Surprisingly, the CHP902R cell line had no MYCN mRNA or protein expression despite being *MNA*. The non-*MNA* LAN-6, NBL-S and SJNB-1 have all been reported to have higher levels of MYCN expression compared with other non-*MNA* cell lines,[16,20,22] which was confirmed here at the RNA and protein level (Fig. 4C and D).

Reduction of MYCN levels using siRNA and the effect on the cell cycle. MYCN knockdown using siRNA in 3 *MNA* p53 wt neuroblastoma cell lines: IMR-32, NBLW and NGP caused a significant G_1 arrest in all 3 cell lines (Table 2 and Fig. 5). Under normal growth conditions there was a significant increase in the G_1:S ratio between the SCR and MYCN siRNA treated IMR-32 control samples ($p < 0.001$) (Figs. 5b, c and Table 2). The G_1 population increased from 51.6 ± 1.0 % to 65.5 ± 0.8 % when treated with MYCN siRNA, accompanied by a small decrease of 6.9 ± 0.7% in the S-phase (Fig. 5B and C). MYCN knockdown in the NGP, NBLW and IMR-32 cell lines increased the levels of $p21^{WAF1}$ and hypo RB without affecting p53 protein levels (Table 2 and Fig. 5A). MDM2 levels also remained unchanged (data not shown). The smaller increases in $p21^{WAF1}$ and hypo RB in NBLW cells may be due to the lower levels of MYCN knockdown (Table 2).

MYCN knockdown in the p53 mutant, *MNA* cell line SKNBE(2c) (Fig. 6 and Table 2) also induced a significant G_1 arrest ($p < 0.01$). MYCN siRNA treatment increased the G_1 population by 8.1 ± 0.9% compared with the SCR controls. The S-phase also decreased by 3.9 ± 0.7% (Fig. 6B and C). There were no changes in the levels of CDK inhibitors $p21^{WAF1}$, $p27^{cip/kip}$ or hypo RB after MYCN knockdown (Fig. 6A).

The effect of MYCN knockdown on the G_1 checkpoint in response to irradiation in *MNA* cell lines. MYCN knockdown in *MNA* cell lines did not restore a G_1 arrest in response to DNA damage. None of the p53 wt *MNA* cell lines showed a statistically significant increase in G_1:S phase ratio after irradiation. The G_1:S ratio of MYCN siRNA treated IMR-32 cells decreased from 5.0 ± 0.5 to 3.1 ± 0.3 after irradiation (Fig. 5B). The G_1:S ratio of the IMR-32 cells treated with SCR siRNA remained constant at 2.5 ± 0.3 and 2.6 ± 0.3 in the non-irradiated and irradiated samples respectively. The G_1:S ratio of NGP and NBLW cells both decreased after irradiation and the cells failed to undergo a G_1 arrest regardless of MYCN knockdown (data not shown).

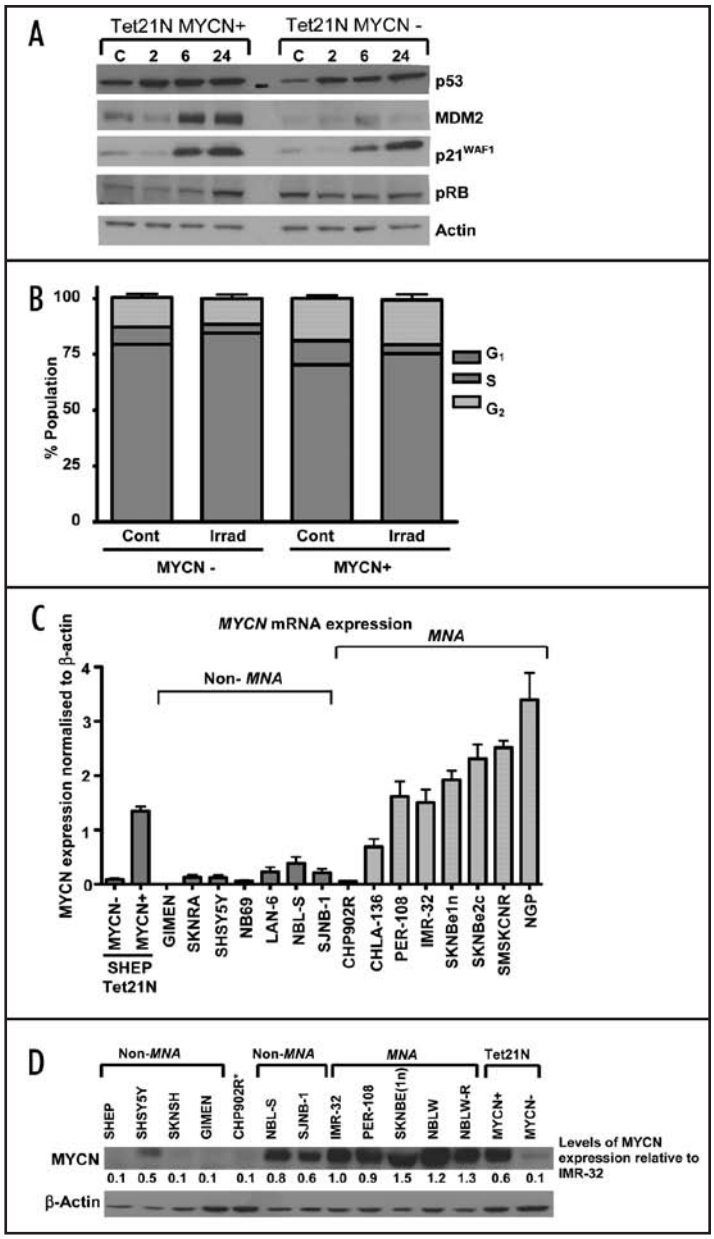

Figure 4. (A) SHEP Tet21N $MYCN^-$ and $MYCN^+$ Western blots for control and samples harvested 2, 6 and 24 hours post irradiation (n = 3). (B) Bar chart for cell cycle data for Tet21N $MYCN^-$ and $MYCN^+$ cells in control and 24 hours post irradiation samples (n = 3). (C) Levels of MYCN mRNA expression by real time reverse transcriptase PCR normalised to β-Actin (n = 4). (D) Levels of MYCN protein by Western blot for a panel of *MNA* and non-*MNA* neuroblastoma cell lines.

DISCUSSION

Lower levels of $p21^{WAF1}$ and hypo RB induction and a failure to G_1 arrest in *MNA* neuroblastoma cell lines after DNA damage. *MNA* is significantly associated with lower levels of $p21^{WAF1}$ induction and hypo RB accumulation and a failure to G_1 arrest after DNA damage (Table 3). It has previously been suggested that the abrogation of G_1 arrest in neuroblastoma is caused by dysfunctional $p21^{WAF1}$ unable to bind Cdk2 as immunoprecipitation revealed an absence of $p21^{WAF1}$-cdk2 complexes in NBL-S, SJNB-1 and NB1643 cells.[16] However, the current study has shown that NBL-S and SJNB-1 cells do G_1 arrest in response to DNA damage and the failure to G_1 arrest in *MNA* neuroblastoma cell lines is more likely to be dependent on lower levels of $p21^{WAF1}$ induction after irradiation, as opposed to dysfunctional $p21^{WAF1}$.

It is possible that the attenuated $p21^{WAF1}$ induction is due to MYCN repression of $p21^{WAF1}$ after irradiation. After MYCN knockdown p53 wt *MNA* cell lines showed increases in $p21^{WAF1}$ and hypo RB, with no change in p53 levels. This would be consistent with direct repression of *$p21^{WAF1}$* transcription by MYCN similar to *$p21^{WAF1}$* repression by c-MYC.[32,33] However, conditional MYCN expression in the Tet21N cells to levels comparable with those in *MNA* cell lines did not affect $p21^{WAF1}$ induction or the G_1 checkpoint after irradiation. In addition the MYCN over expressing cell lines NBL-S, SJNB-1 and LAN-6 all had high $p21^{WAF1}$ induction and underwent a G_1 arrest and the *MNA* CHP902R cell line has low MYCN expression and low levels of $p21^{WAF1}$ induction. Finally, MYCN RNAi in 3 wt p53 *MNA* cell lines did not restore a G_1 arrest in response to DNA damage. Taken together this data shows lower

Table 3 **Results summary**

	Low $p21^{WAF1}$ Induction	High $p21^{WAF1}$ Induction	Fail to G_1 Arrest	Do G_1 Arrest	Low % Apoptosis	High % Apoptosis
Non-*MNA* n = 7	NB69	GIMEN NBL-S SJNB-1 SHSY5Y SKNRA LAN-6		GIMEN NBL-S SJNB-1 SHSY5Y SKNRA LAN-6 NB69 PER108	GIMEN SJNB-1 SHSY5Y SKNRA LAN-6	NB69
n =6 for apoptosis						
MNA n = 10	SMSKCNR IMR-32 CHLA-136 CHP902R NBLW TR14 LS NGP SKNBE(1n)	PER108	SMSKCNR IMR-32 CHLA-136 CHP902R NBLW TR14 LS NGP SKNBE(1n)		IMR-32 TR14 NGP	PER108 SMSKCNR CHLA-136 NBLW LS SKNBE(1n)
n =9 for apoptosis						

Summary of all 17 cell lines tested for $p21^{WAF1}$ induction and G_1 arrest after irradiation and levels of apoptosis at 96 hours determined by Hoechst staining. High apoptosis > 24% apoptosis (median level of apoptosis across the cell lines) and Low apoptosis < 24%

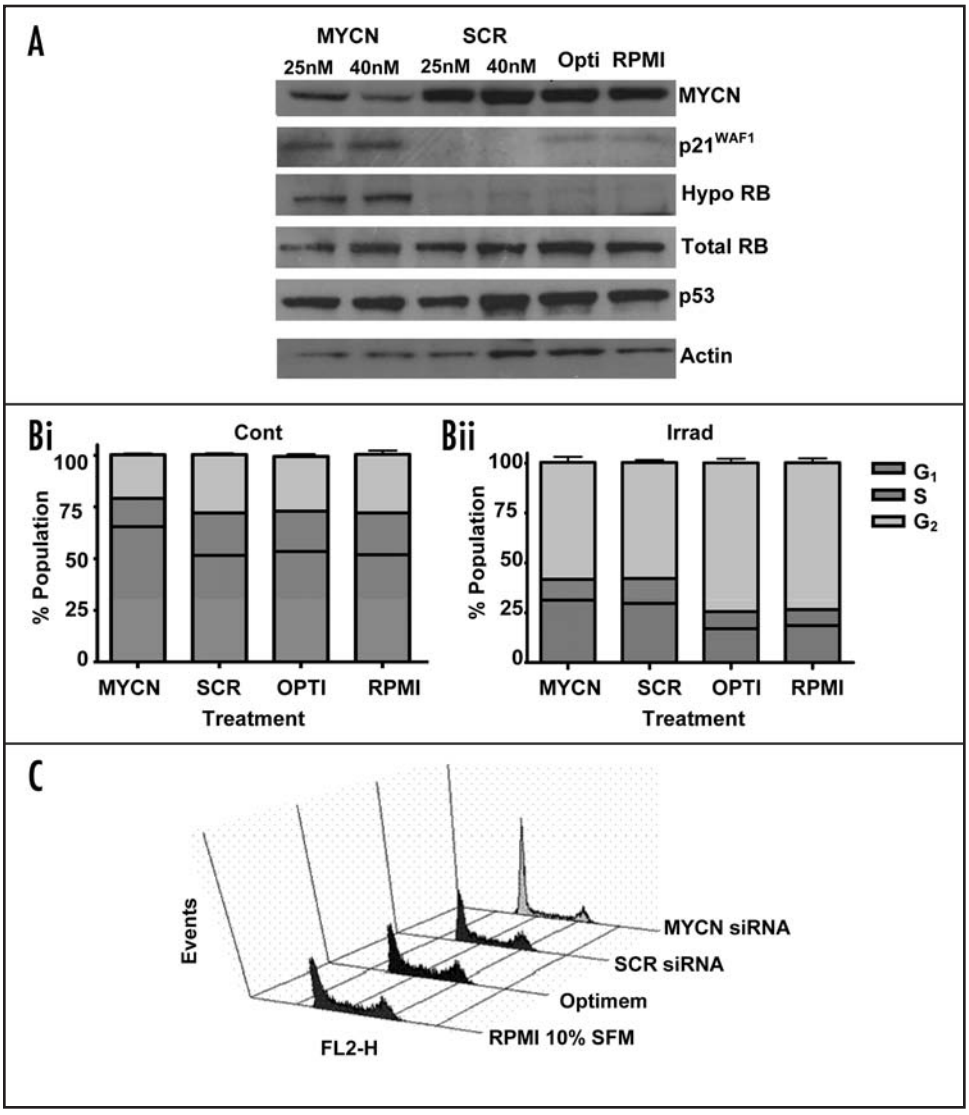

Figure 5. IMR-32 cells treated with siRNA. (A) Western blot of IMR-32 cells treated with 25 nM and 40 nM MYCN siRNA and SCR siRNA, Optimem serum free media control (Opti) and RPMI 10% FCS control. (B) Cell cycle distribution of IMR-32 cells treated with 40 nM siRNA (i) control non-irradiated and (ii) 24 hours post irradiation. (C) Histogram of nonirradiated IMR-32 cells. (siRNA experiments were carried out n = 3).

levels of $p21^{WAF1}$ induction and a failure to G_1 arrest in response to DNA damage are not due to high MYCN expression alone.

Another factor may be cooperating with MYCN in *MNA* cell lines to lower $p21^{WAF1}$ levels such as H-Twist. Levels of H-Twist expression have been found to correlate strongly with MYCN expression in both tumours and cell lines. In addition to being apoptotic, H-twist expression has been reported to attenuate p53 function after irradiation, lowering levels of $p21^{WAF1}$ induction. It is possible that H-Twist is responsible for the low levels of $p21^{WAF1}$ induction and subsequent failure to G_1 arrest observed in this paper. However the anti-apoptotic effects described in[34] were not demonstrated in the panel of cell lines tested, as *MNA* neuroblastoma cell lines were more likely to undergo apoptosis than non-*MNA* cell lines 96 hours post irradiation ($p < 0.05$).

It is also possible that other genetic aberrations that coexist with *MYCN* amplification may be responsible for the failure to G_1 arrest in response to DNA damage. *MNA* neuroblastoma cells often have other

chromosomal gains and losses that occur in neuroblastoma including allelic loss of 1p and 17q gain.[35] Alternatively it is possible that the expression of coamplified genes on the *MYCN* amplicon may be involved in the failure to G_1 arrest, such as *DDX1*, *N-cym*, or *NAG*. Little is known of the functions of genes in the amplicon (reviewed in ref. 36).

***MNA* cell lines are more sensitive to apoptosis after irradiation.** In this study *MNA* neuroblastoma cell lines underwent significantly higher levels of apoptosis after DNA damage compared with non-*MNA* cell lines (Table 3). MYCN may switch the p53 response to DNA damage from cell cycle arrest to apoptosis through the transcription of apoptosis mediators including PUMA and PIG3 in a similar manner to that described for c-MYC.[33] The non-*MNA* NB69 cell line which had lower levels of $p21^{WAF1}$ induction than other non-*MNA* cell lines underwent high levels of apoptosis after irradiation and is c-MYC overexpressing.[37] Interestingly, MYCN expression in the Tet21N cell line increased apoptosis after irradiation (data not shown) however the cells still underwent a G_1 arrest with high levels of $p21^{WAF1}$ after irradiation (Fig. 4). The *MYCN* and *MDM2* amplified cell lines TR14 and NGP underwent less apoptosis than other *MNA* cell lines (Table 3), and in addition to lower levels of $p21^{WAF1}$ and hypo RB after irradiation, there was also less induction of p53, as MDM2 attenuates the function of p53. Lower levels of p53 function would prevent apoptotic pathways from being activated.[38] The *MNA* IMR-32 cell line also has lower levels of apoptosis compared with other *MNA* cell lines (Fig. 3 and Table 3), which is possibly because it is methylated for caspase-8.[39]

MYCN siRNA alters the proportion of cells in G_1. MYCN influences control of cell cycle progression, as reduction of MYCN expression in 4 *MNA* cell lines caused a significant increase of cells in G_1 compared with the SCR siRNA (Table 2). Cell cycle data from the SHEP Tet21N experiments supports these findings as Tet21N MYCN- cells had a higher G_1:S ratio than Tet21N $MYCN^+$ cells under normal growth conditions, and levels of hypo RB were higher in control Tet21N $MYCN^-$ cells than Tet21N $MYCN^+$ cells, indicating that MYCN expression decreased the proportion of cells in G_1, as previously reported.[7] Studies using protein-nucleic acids to selectively inhibit MYCN activity in neuroblastoma cell lines have also reported an increase in the G_1 population in IMR-32 cells when MYCN activity was inhibited.[40] In the current study, knockdown of MYCN in the 3 p53 *MNA* wt neuroblastoma cell lines increased levels of $p21^{WAF1}$ and hypo RB without an alteration in p53 levels. MYCN knockdown causes a baseline G_1 arrest, but knock down of MYCN is not sufficient to restore a further increase in G_1 arrest after DNA damage in *MNA* cell lines.

MYCN knockdown in the SKNBE(2c) p53 mutant cell line induced a significant G_1 arrest without accumulation of $p21^{WAF1}$ or hypo RB, indicating that although induction of $p21^{WAF1}$ after MYCN knockdown plays a role in causing a G_1 arrest in p53 wt neuroblastoma cell lines, p53 mutant cell lines can undergo a G_1 arrest independent of p53, $p21^{WAF1}$ and $p27^{kip1}$. In the absence of p53 function, MYCN may act downstream of RB to affect the G_1 checkpoint, perhaps by a direct effect on E2F1 as has been suggested for c-MYC.[41] Knocking down MYCN or inhibiting its activity to induce growth arrest may be of benefit clinically in neuroblastomas with functional inactivation of the p53 pathway, as is sometimes found at relapse.

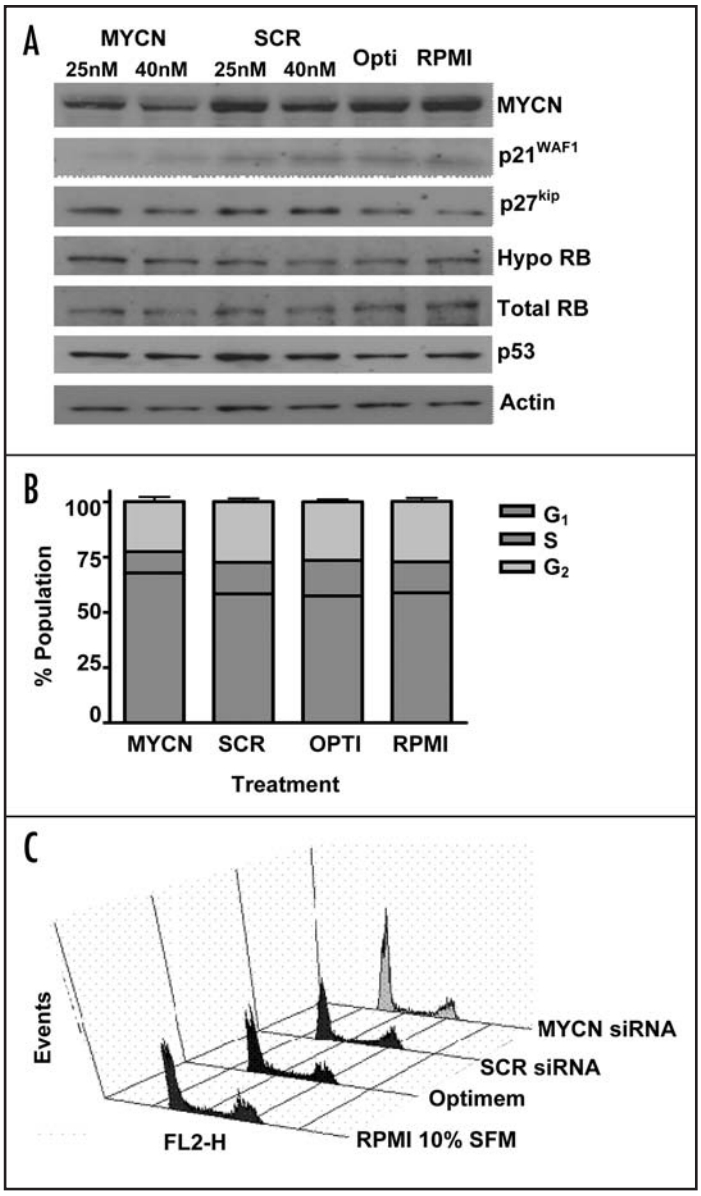

Figure 6. SKNBE(2c) cells treated with siRNA. (A) Western blot of SKNBE(2c) cells treated with 25 nM and 40 nM MYCN siRNA and SCR siRNA, Optimem serum free media control (Opti) and RPMI 10%FCS control. (B) Cell cycle distribution of SKNBE(2c) cells treated with 25nM siRNA in control non-irradiated cells. (C) Histogram of non-irradiated IMR-32 cells (siRNA experiments were carried out (n = 3).

CONCLUSIONS

This study is the first to show that *MNA* is significantly associated with lower levels of $p21^{WAF1}$ induction, hypo RB accumulation and a failure to G_1 arrest after DNA damage in neuroblastoma cell lines. Neuroblastoma cell lines with low levels of $p21^{WAF1}$ and hypo RB induction undergo more apoptosis than those with higher levels of $p21^{WAF1}$ and hypo RB induction. Modulating MYCN expression either by conditional expression or MYCN knockdown using siRNA in *MNA* cell lines did not influence the G_1 arrest in response to DNA damage. We conclude that neuroblastoma cell lines that fail to G_1 arrest have lower levels of $p21^{WAF1}$ induction and hypo RB accumulation after DNA damage; however the MYCN oncoprotein alone is not causally responsible.

References

1. Schwab M. *MYCN* in neuronal tumours. Cancer Lett 2004; 204:179-87.
2. Wenzel A, Schwab M. The mycN/max protein complex in neuroblastoma. Short review. Eur J Cancer 1995; 31A:516-9.
3. Galderisi U, Di Bernardo G, Cipollaro M, Peluso G, Cascino A, Cotrufo R, Melone MA. Differentiation and apoptosis of neuroblastoma cells: Role of *N-myc* gene product. J Cell Biochem 1999; 73:97-105.
4. Hackett CS, Hodgson JG, Law ME, Fridlyand J, Osoegawa K, de Jong PJ, Nowak NJ, Pinkel D, Albertson DG, Jain A, Jenkins R, Gray JW, Weiss WA. Genome-wide array CGH analysis of murine neuroblastoma reveals distinct genomic aberrations which parallel those in human tumors. Cancer Res 2003; 63:5266-73.
5. Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM. Targeted expression of MYCN causes neuroblastoma in transgenic mice. Embo J 1997; 16:2985-95.
6. Burkhart CA, Cheng AJ, Madafiglio J, Kavallaris M, Mili M, Marshall GM, Weiss WA, Khachigian LM, Norris MD, Haber M. Effects of MYCN antisense oligonucleotide administration on tumorigenesis in a murine model of neuroblastoma. J Natl Cancer Inst 2003; 95:1394-403.
7. Lutz WSM, Scurmann J, Wenzel A, Lohr A, Schwab M. Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 1996; 13:803-12.
8. Tang XX, Zhao H, Kung B, Kim DY, Hicks SL, Cohn SL, Cheung NK, Seeger RC, Evans AE, Ikegaki N. The MYCN enigma: Significance of MYCN expression in neuroblastoma. Cancer Res 2006; 66:2826-33.
9. Lane DP. Cancer: p53, guardian of the genome. Nature 1992; 358:15-6.
10. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408:307-10.
11. Tweddle DA, Pearson AD, Haber M, Norris MD, Xue C, Flemming C, Lunec J. The p53 pathway and its inactivation in neuroblastoma. Cancer Lett 2003; 197:93-8.
12. Keshelava N, Zuo JJ, Chen P, Waidyaratne SN, Luna MC, Gomer CJ, Triche TJ, Reynolds CP. Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. Cancer Res 2001; 61:6185-93.
13. Carr J, Bell E, Pearson AD, Kees UR, Beris H, Lunec J, Tweddle DA. Increased frequency of aberrations in the p53/MDM2/p14(ARF) pathway in neuroblastoma cell lines established at relapse. Cancer Res 2006; 66:2138-45.
14. Slack A, Chen Z, Tonelli R, Pule M, Hunt L, Pession A, Shohet JM. The *p53* regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. Proc Natl Acad Sci USA 2005; 102:731-6.
15. McKenzie PP, Guichard SM, Middlemas DS, Ashmun RA, Danks MK, Harris LC. Wild-type p53 can induce p21 and apoptosis in neuroblastoma cells but the DNA damage-induced G_1 checkpoint function is attenuated. Clin Cancer Res 1999; 5:4199-207.
16. McKenzie PP, Danks MK, Kriwacki RW, Harris LC. P21Waf1/Cip1 dysfunction in neuroblastoma: A novel mechanism of attenuating G_0-G_1 cell cycle arrest. Cancer Res 2003; 63:3840-4.
17. Tweddle DA, Malcolm AJ, Cole M, Pearson AD, Lunec J. p53 cellular localization and function in neuroblastoma: Evidence for defective G_1 arrest despite WAF1 induction in MYCN-amplified cells. Am J Pathol 2001; 158:2067-77.
18. Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J. Evidence for the development of *p53* mutations after cytotoxic therapy in a neuroblastoma cell line. Cancer Res 2001; 61:8-13.
19. McPake CR, Tillman DM, Poquette CA, George EO, Houghton JA, Harris LC. Bax is an important determinant of chemosensitivity in pediatric tumor cell lines independent of Bcl-2 expression and p53 status. Oncol Res 1998; 10:235-44.
20. Cohn SL, Salwen H, Quasney MW, Ikegaki N, Cowan JM, Herst CV, Sharon B, Kennett RH, Rosen ST. High levels of N-myc protein in a neuroblastoma cell line lacking N-myc amplification. Prog Clin Biol Res 1991; 366:21-7.
21. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 1978; 38:3751-7.
22. Wada RK, Seeger RC, Brodeur GM, Slamon DJ, Rayner SA, Tomayko M, Reynolds CP. Characterization of human neuroblastoma cell lines that lack *N-myc* gene amplification. Prog Clin Biol Res 1988; 271:57-69.
23. Feder MK, Gilbert F. Clonal evolution in a human neuroblastoma. J Natl Cancer Inst 1983; 70:1051-6.
24. Cornaglia-Ferraris P, Ponzoni M, Montaldo P, Mariottini GL, Donti E, Di Martino D, Tonini GP. A new human highly tumorigenic neuroblastoma cell line with undetectable expression of N-myc. Pediatr Res 1990; 27:1-6.
25. Brodeur GM, Sekhon G, Goldstein MN. Chromosomal aberrations in human neuroblastomas. Cancer 1977; 40:2256-63.
26. Kees UR, Ford J, Dawson VM, Ranford PR, Armstrong JA. Three neuroblastoma cell lines established from consecutive samples of one patient which show distinct morphologic features, MYCN amplification, and surface marker expression. Cancer Genet Cytogenet 1992; 59:119-27.
27. Cowell JK, Rupniak HT. Chromosome analysis of human neuroblastoma cell line TR14 showing double minutes and an aberration involving chromosome 1. Cancer Genet Cytogenet 1983; 9:273-80.
28. Rudolph G, Schilbach-Stuckle K, Handgretinger R, Kaiser P, Hameister H. Cytogenetic and molecular characterization of a newly established neuroblastoma cell line LS. Hum Genet 1991; 86:562-6.
29. Thompson PM, Maris JM, Hogarty MD, Seeger RC, Reynolds CP, Brodeur GM, White PS. Homozygous deletion of CDKN2A (p16INK4a/p14ARF)but not within 1p36 or at other tumour supressor loci in neuroblastoma. Cancer Res 2001; 61:679-86.
30. Foley J, Cohn SL, Salwen HR, Chagnovich D, Cowan J, Mason KL, Parysek LM. Differential expression of N-myc in phenotypically distinct subclones of a human neuroblastoma cell line. Cancer Res 1991; 51:6338-45.
31. Goldman SC, Chen CY, Lansing TJ, Gilmer TM, Kastan MB. The p53 signal transduction pathway is intact in human neuroblastoma despite cytoplasmic localization. Am J Pathol 1996; 148:1381-5.
32. Gartel ALYX, Goufman E, Shianov P, Nissim H, Najmabadi F, Tyner A. Myc represses the p21 (WAF1/CIP1) promoter and interacts with Sp1/Sp3. PNAS 2001; 98:4510-5.
33. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature 2002; 419:729-34.
34. Valsesia-Wittmann S, Magdeleine M, Dupasquier S, Garin E, Jallas AC, Combaret V, Krause A, Leissner P, Puisieux A. Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells. Cancer Cell 2004; 6:625-30.
35. van Noesel MM, Versteeg R. Pediatric neuroblastomas: Genetic and epigenetic 'danse macabre'. Gene 2004; 325:1-15.
36. Scott D, Elsden J, Pearson A, Lunec J. Genes coamplified with MYCN in neuroblastoma: Silent passengers or codeterminants of phenotype? Cancer Lett 2003; 197:81-6.
37. Ikegaki N, Kastumata M, Tsujimoto Y. The expression and modulation of proteins associated with physiological cell death in neuroblastoma cells. Prog Clin Biol Res 1994; 385:117-22.
38. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein *MDM2* conceals the activation domain of tumour suppressor *p53*. Nature 1993; 362:857-60.
39. van Noesel MM, van Bezouw S, Voute PA, Herman JG, Pieters R, Versteeg R. Clustering of Hypermethylated genes in neuroblastoma. Genes Chromosomes Cancer 2003; 38:226-33.
40. Tonelli R, Purgato S, Camerin C, Fronza R, Bologna F, Alboresi S, Franzoni M, Corradini R, Sforza S, Faccini A, Shohet JM, Marchelli R, Pession A. Anti-gene peptide nucleic acid specifically inhibits MYCN expression in human neuroblastoma cells leading to cell growth inhibition and apoptosis. Mol Cancer Ther 2005; 4:779-86.
41. Leone G, Degregory J, Sears R, Jakoi L, Nevins JR. MYC and Ras collaborate in inducing accumulation of active cyclin E/cdk2 and E2F. Nature 1997; 387:422-6.