[Cell Cycle 6:10, 1249-1256, 15 May 2007]; ©2007 Landes Bioscience

Report

Cell Cycle Regulation Targets of MYCN Identified by Gene Expression Microarrays

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Original manuscript submitted: 03/07/07
Manuscript accepted: 04/01/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=4222

KEY WORDS

G_1 arrest, MYCN, neuroblastoma

ACKNOWLEDGEMENTS

We would like to thank the following for providing cell lines: Sue Cohn (NBLW, NBL-S), Ursula Kees (PER-108), Penny Lovat (IMR-32), John Maris (NB69), Mirco Ponzoni (GIMEN), Patrick Reynolds (LAN-6, SMSKCNR, CHLA136), Barbara Spengler (SKNBE2c), Manfred Schwab (Tet21N), and Naohiko Ikegaki for providing the MYCN 100 antibody. This work was supported by Cancer Research UK and the UK Department of Health.

ABSTRACT

Background: We have previously shown that MYCN knockdown causes a G_1 arrest in *MYCN* amplified (*MNA*), p53 wild type (wt) and p53 mutant *MNA* neuroblastoma cell lines, with increases in $p21^{WAF1}$ and hypo RB in p53 wt cell lines.[1]

Hypothesis: MYCN acts by inhibiting $p21^{WAF1}$, and also by $p21^{WAF1}$ independent mechanisms to override the G_1 checkpoint in exponentially growing cells.

Methods: Genes potentially regulated by MYCN were identified using gene expression microarrays in p53 wt *MNA* IMR-32 and p53 mutant *MNA* SKNBE(2c) neuroblastoma cell lines treated with MYCN or scrambled siRNA. Results were validated using qRT-PCR and confirmed using the regulatable MYCN expression system (SHEP Tet21N).

Results: MYCN knockdown altered the expression of several cell cycle related genes. SKP2 was down regulated in both cell lines, and up regulated in MYCN+ Tet21N cells. Expression of the WNT antagonist DKK3 increased in both cell lines and decreased in MYCN+ Tet21N cells. Expression of CDKN1C ($p57^{cip2}$) and TP53INP1 also increased after MYCN knockdown.

Conclusions: MYCN may override the G_1 checkpoint through down-regulation of SKP2 and TP53INP1 resulting in reduced $p21^{WAF1}$ expression in p53 wt cell lines, and in addition may act through the WNT signaling pathway in a p53 independent manner.

INTRODUCTION

Neuroblastoma is a childhood cancer derived from cells of the migrating neural crest. A number of genetic aberrations occur in aggressive neuroblastoma including *MYCN* amplification (*MNA*), chromosome 1p deletion and unbalanced gain of chromosome 17q. *MNA* occurs in ~20–25% of neuroblastoma cases and there is a strong correlation between *MNA* and poor prognosis.[2] *MYCN*, one of the MYC family of oncogenes structurally related to *MYCC* and *MYCL*, maps to chromosome 2p24. Ectopically regulated expression of MYCN has been shown to have similar effects transcriptionally and on the cell cycle as MYCC: increasing the expression of α-prothymosin and ornithine decarboxylase (ODC), and increasing the proliferation rate in the presence of growth factors.[3]

p53 plays a key role in maintaining genetic integrity in response to DNA damage. Activated p53 increases the expression of target genes including *CDKN1A* (*$p21^{WAF1}$*) to elicit a G_1 arrest, or *BAX* to activate apoptosis. p53 also up-regulates the expression of MDM2, a protein responsible for targeting p53 for degradation. This forms an auto regulatory loop to tightly regulate p53 levels in the cell.[4] p53 mutations in neuroblastoma are rare at diagnosis, however they do develop in response to cytotoxic therapy.[5] Other aberrations in the p53 pathway have also been reported in cell lines established from relapsed tumors, such as $p14^{ARF}$ deletion and methylation, and *MDM2* amplification.[6]

After DNA damage *MNA* wild type neuroblastoma cell lines have lower levels of $p21^{WAF1}$ induction than non-MNA ones, and fail to undergo a G_1 arrest despite having wt p53.[1,7,8] $p21^{WAF1}$ causes a G_1 arrest by inhibiting the activity of cyclin E/cdk2 complexes, preventing the phosphorylation of RB and release of the E2F transcription factor.[9] $p21^{WAF1}$ levels are regulated at the transcriptional level by epigenetic silencing and mRNA stability. At the protein level $p21^{WAF1}$ is regulated by ubiquitin dependent and independent protein degradation pathways.[10] MYCC has been shown to repress $p21^{WAF1}$ expression at the transcriptional level through direct binding to the *CDKN1A* promoter after recruitment by Miz-1,[11] or by the sequestration of the SP1/SP3 *CDKN1A* transcriptional activator proteins.[12]

We recently reported ectopic expression of MYCN in a non-MNA neuroblastoma cell line did not affect $p21^{WAF1}$ induction or the G_1 arrest in response to DNA damage, and

MYCN knockdown in *MNA* cell lines did not restore a G_1 arrest after DNA damage, indicating that high levels of MYCN alone are not responsible for the failure to G_1 arrest after DNA damage.[1] Knockdown of MYCN expression by RNAi in 3 p53 wt *MNA* cell lines caused an accumulation of cells in G_1 alongside increases in $p21^{WAF1}$ and hypophosphorylated (hypo) RB, and MYCN knockdown in a p53 mutant cell line caused a G_1 arrest with no increase in $p21^{WAF1}$ or hypo RB.[1] This suggests that MYCN acts through p53 to decrease levels of $p21^{WAF1}$, and via a p53 independent mechanism to affect the G_1 checkpoint. In this study we investigated how MYCN acts on the G_1 checkpoint under normal growth conditions, by identifying potential downstream MYCN targets using MYCN siRNA in *MNA* neuroblastoma cell lines and gene expression microarray. In addition we investigated the effect of MYCN knockdown on levels of $p21^{WAF1}$ after irradiation.

MATERIALS AND METHODS

Cell lines. The p53 wt IMR-32[13] and p53 mutant SKNBE(2c)[7] cell lines were used for gene expression microarray experiments. GIMEN, NB69, LAN-6, NBL-S, PER108, SMSKCNR, NBLW, CHLA-136 and NGP cells were used as described previously.[1] All cell lines were grown in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% Fetal Calf Serum (FCS) (Gibco). Cells were grown at 37°C, in a 5% CO_2 humidified incubator.

DNA damage using 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 4Gy 310kV X-rays from a RS320 irradiation system (Gulmay Medical, Surrey, UK). Cells were harvested 2, 6 and 24 hours post irradiation for Western blotting. Experiments were carried out at least twice.

Western blotting. Whole cell extracts were prepared, and 30μg protein/sample separated by gel electrophoresis, and Western blotting performed using methods described previously.[8] Densitometry was performed using a Fuji-Las camera and the AIDA image analyser programme (Raytek, Sheffield, UK) used to quantify band intensities. Primary antibodies used were mouse monoclonal p53 DO7 at 1:1000 (Novocastra, Newcastle, UK), MDM2 at 1:100, $p21^{WAF1}$ at 1:100 (Calbiochem, Cambridge, MA), hypophosphorylated retinoblastoma protein (hypo RB) at 1:100 (BD Biosciences, Oxford UK), rabbit polyclonal SKP2 antibody (Sc7164) at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal MYCN 100 antibody at 1:100 dilution (gift from N. Igekaki). β-actin rabbit polyclonal antibody was used as a protein loading and transfer control at 1:1000 (Sigma, St. Louis, MO). The secondary goat anti-mouse and goat anti-rabbit antibodies were used at 1:1000 (DAKO, Glostrup, Denmark).

MYCN knockdown using siRNA. MYCN siRNA was performed using the methods previously described.[1] 40nM of MYCN or scrambled (SCR) siRNA was transfected into IMR-32 cells, and 25nM siRNA was transfected into p53 mutant SKNBE(2c) cells. A time course was carried out and maximal reduction in MYCN protein expression was observed in both cell lines 14 hours after transfection of MYCN siRNA. Cells were harvested 16 hours post transfection to look for early changes in gene expression and 48 hours later to look for later changes. Experiments were carried out in duplicate.

MYCN up regulation using the SHEP Tet21N system. The SHEP Tet21N cell line[3] 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, 10μg/ml tetracycline (Sigma) was added to growth media at least 24 hours prior to experiments.

Gene expression microarray. Cells were harvested 16 and 48 hours post siRNA transfection for microarray analysis. Total RNA was extracted using the RNeasy Midi kit (Qiagen, Ontario, Canada) and RNA integrity measured using a Bioanalyser (Agilent, Palo Alto, CA). Total cDNA was synthesized using a T7-Oligo dT Promoter Primer Kit (Affymetrix, Santa Clara, CA). In vitro transcription using biotinylated dUTP and dCTP was carried out using the Bioarray high yield transcript labelling kit (Enzo Diagnostics, Farmingdale, NY). Resulting cRNA was hybridized to a test array to assess the quality of the labelled nucleic acid before hybridising to U133 plus 2.0 Affymetrix arrays. Arrays were scanned using Microarray suite 5.0, and data was exported as CEL files. The cRNA synthesis, microarray hybridization and signal scanning steps were undertaken at the Cancer Research UK facility (Paterson Institute, Manchester, UK). Gene assignments to probe sets and candidate genes were analysed using Genespring GX version 7.1 (Agilent). Microarrays were carried out in duplicate for each condition (biological replicates). The array data for one of the replicates of the 48 h SCR treated SKNBE(2c) cells was omitted from the analysis because hierarchical clustering and principal components analysis on all of the genes in the array found that this replicate did not cluster with the other SKNBE(2c) arrays, suggesting unreliable data.

A comparison of differentially expressed genes was carried out for both cell lines at each time point. The cell cycle, cell growth and signal transduction gene lists (GO:0007049, GO:0016049 and GO:0030154) from the Gene Ontology database were compared between MYCN and SCR siRNA treated cell lines at 16 and 48 hours post siRNA transfection. There were fewer cell cycle related genes differentially regulated at 16 hours, so all genes with a ≥2 fold change in expression level were included. A larger number of cell cycle genes were differentially expressed at 48 hours and only genes with a ≥3 fold change were included.

Validation of gene expression microarray results using quantitative reverse transcriptase PCR. For selected genes of interest, microarray results were validated using quantitative reverse transcriptase PCR (qRT-PCR). RNA extraction, reverse transcriptase PCR and qPCR was carried out as described previously.[6] Primers and probes for Dickkopf 3 (DKK3), p53 inducible protein 1 (TP53INP1), S-phase associated kinase (SKP2), cyclin dependent kinase inhibitor 1C (CDKN1C), ODC and the control β-Actin were Taqman assays (Applied Biosystems, Foster City, CA). Each sample was run in triplicate on a 384 well plate using an ABI prism 7900 detection kit and results were analysed using SDS 2.2 (both from Applied Biosystems). Each experiment was repeated three times.

RESULTS

MYCN RNA and protein levels after MYCN knockdown. The amount of MYCN knockdown achieved in IMR-32 and SKNBE(2c) cells is shown in Figure 1A and B. MYCN protein knockdown was 36–71% in IMR-32 cells and 30–62% in SKNBE(2c) cells. The extent of MYCN knockdown in MYCN siRNA treated cells compared to SCR controls was not as high at the transcript level in both IMR-32 and SKNBE(2c) cells (Fig. 1A and B). 58.5% (mean) knockdown was observed in IMR-32 cells at 16 hours recovering to normal transcript levels at 48 hours post transfection. 49.5% (mean) knockdown was observed in the SKNBE(2c) cells at 16 hours

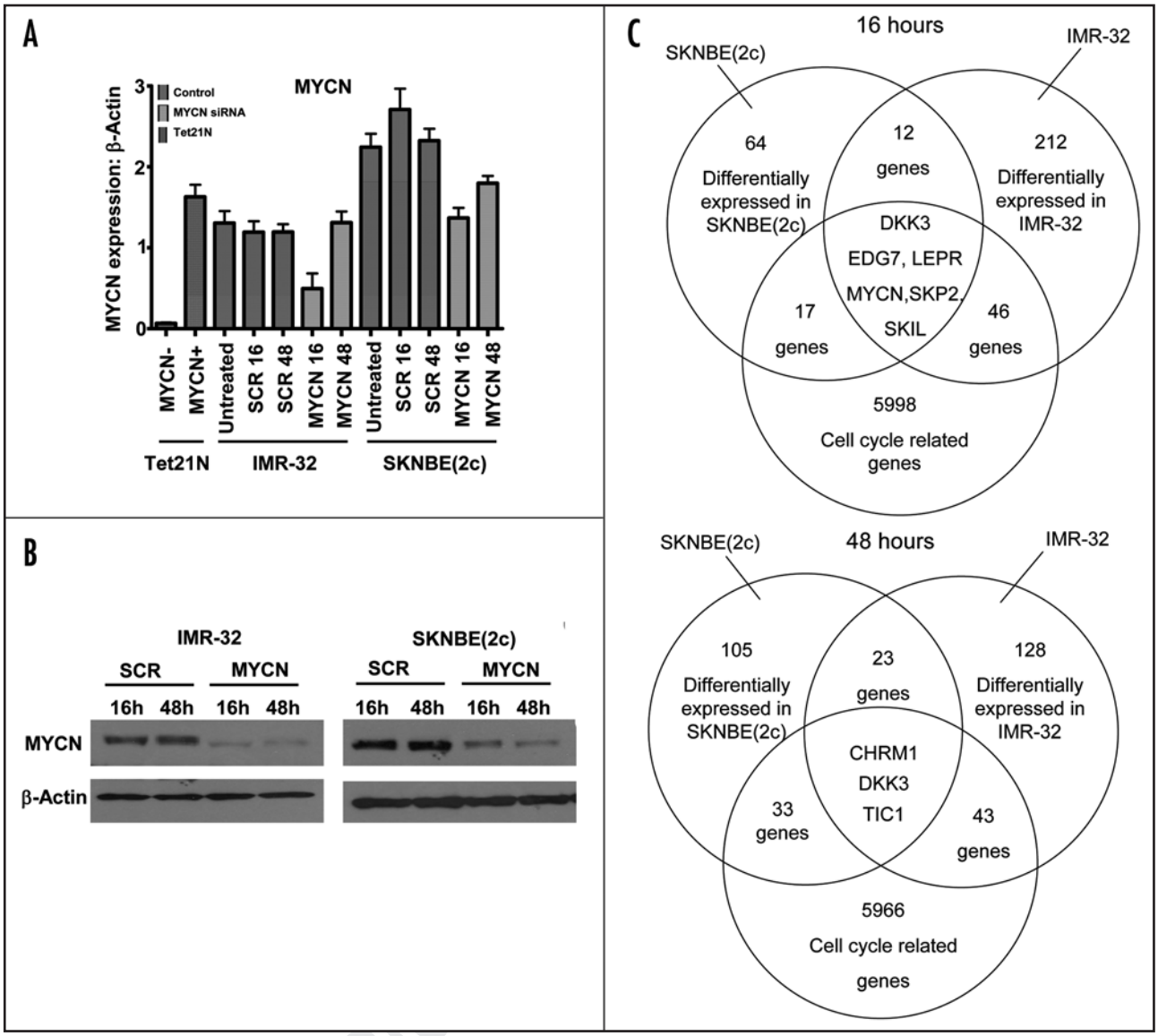

Figure 1. (A) Levels of MYCN transcripts using qRT-PCR 16 and 48 hours after transfection with MYCN or SCR siRNA alongside untreated controls. (B) Levels of MYCN protein after knockdown in IMR-32 and SKNBE(2c) cells. (C) Venn diagram showing cell cycle related genes differentially regulated 16 hours and 48 hours after transfection with MYCN siRNA in IMR-32 and SKNBE(2c) cells.

recovering to 22.5% knockdown at 48 hours. Protein knockdown remained unchanged between 16 and 48 hours post transfection in both cell lines (Fig. 1B).

Cell cycle related gene expression microarray results after MYCN siRNA knockdown. Venn diagrams showing cell cycle related genes differentially expressed at 16 and 48 hours after transfection in IMR-32 and SKNBE(2c) cells are shown in (Fig. 1C). More genes were up-regulated than down regulated after MYCN knockdown, suggesting that MYCN represses transcription of numerous cell cycle related genes. In IMR-32 cells MYCN mRNA knockdown was detected in both cell lines 16 hours after transfection (Fig. 2A and B and Table 1), but not at 48 hours, confirming the qRT-PCR results (Fig. 1A).

Some previously reported MYCN target genes e.g., *ODC* and α-*prothymyosin* did not change. qRT-PCR was carried out for ODC, and confirmed that the fold decrease at both time points for both cell lines was <2 fold (data not shown). Other previously identified MYCN targets were differentially regulated in MYCN and SCR treated IMR-32 cells: MYCC (Fig. 2A) and CDC42 (Fig. 2C). Cross regulation of MYCC by MYCN has previously been described.[14,15] *CDC42*, also repressed by MYCN,[16] was up-regulated in MYCN siRNA treated IMR-32 cells at 48 hours. CDC42 however was not up regulated in p53 mutant SKNBE(2c) cells, suggesting that p53 may be involved in the regulation of CDC42 by MYCN (Fig. 2C).

Early changes in cell cycle related gene expression after MYCN knockdown. Genes differentially expressed in IMR-32 cells alone at 16 hours are shown in Figure 2A. A greater number of cell cycle related genes were differentially expressed in IMR-32 cells at 16 hours (60 probes, 46 genes) compared with SKNBE(2c) (24 probes, 23 genes) (Fig. 2B). This suggests that MYCN might modulate many genes in a p53 dependent manner. In IMR-32 cells two cyclins: *cyclin A1* (CCNA1) and cyclin G2 (CCNG2) were upregulated alongside the cyclin dependent kinase inhibitor $p57^{kip2}$

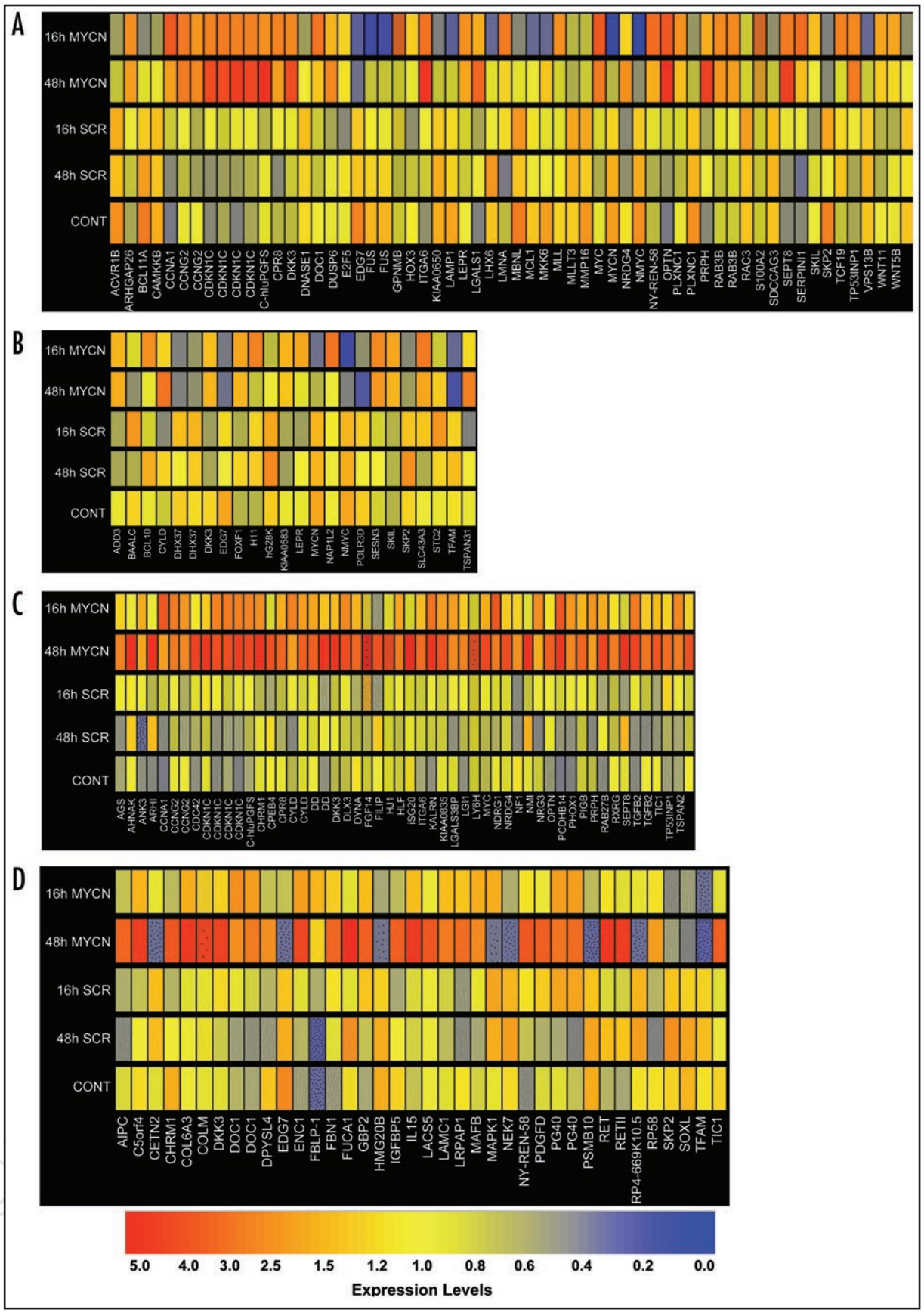

Figure 2. Cell cycle related genes >2 fold differentially regulated in (A) IMR-32 cells and (B) SKNBE(2c) 16 hours after MYCN knockdown. Cell cycle related genes >3 fold differentially regulated in (C) IMR-32 and (D) SKNBE(2c) cells 48 hours after MYCN knockdown.

Table 1 **List of cell cycle related genes that are differentially regulated 16 and/or 48 hours after MYCN siRNA treatment in both IMR-32 and SKNBE(2c) cells***

Gene	Description	Mean Fold Change			
		16h IMR-32	48h IMR-32	16h SKNBE2c	48h SKNBE2c
CHRM1	Brn25 Homo sapiens cDNA clone	1.3 ± 0.8	3.3 ± 1.4	1.0 ± 0.3	3.6 ± 0.2
DKK3	Dickkopf (X. laevis) homolog 3	2.2 ± 1.0	4.5 ± 0.8	2.0 ± 0.1	3.8 ± 0.5
EDG7	Lysophosphatidic acid receptor	0.3 ± 0.004	0.4 ± 0.001	0.5 ± 0.001	1.4 ± 0.001
LEPR	Leptin receptor	2.2 ± 0.001	1.9 ± 0.01	2.42 ± 0.01	0.9 ± 0.01
NMYC (1)	v-myc myelocytomatosis viral related oncogene	0.1 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	1.3 ± 0.8
NMYC (2)	v-myc myelocytomatosis viral related oncogene	0.1 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.8 ± 0.03
SKIL	Ski-like oncogene	2.2 ± 0.05	1.09 ± 0.02	2.16 ± 0.4	1.8 ± 0.001
SKP2	S-phase kinase-associated protein 2 (p45)	0.3 ± 0.2	0.5 ± 0.2	0.4 ± 0.4	1.2 ± 0.6
TIC1	Testican-1	1.0 ± 0.002	3.8 ± 0.1	1.0 ± 0.01	3.1 ± 2.7

*The fold differences relative to the SCR control are shown at 16 and 48 hours for both cell lines. Note: The genes in bold were validated using qRT-PCR.

(*CDKN1C*) gene (Fig. 2A). Changes in expression of six genes were common to both IMR-32 and SKNBE(2c) cells: *DKK3*, *EDG7*, *LEPR*, *MYCN*, *SKP2* and *SKIL*. The up regulated genes included DKK3, an antagonist of WNT signaling[17] and SKIL (Ski-like oncogene), which represses Smad transcription factors involved in TGFβ signaling.[18] SKP2, a component of the Skp1-Cullin-F-box-skp2. Roc1 ubiquitin ligase complex (SCF^{skp2}.Roc1)[19] was down regulated (Fig. 1C, 2A, 2B and Table 1).

Late changes in cell cycle related gene expression after MYCN knockdown. Cell cycle related genes differentially expressed 48 hours after MYCN knockdown compared with SCR siRNA controls are shown in Figure 2C and D. Forty-six genes and 53 probes were differentially regulated in the IMR-32 cells (Fig. 2C). All genes identified in IMR-32 cells had increased expression after MYCN knockdown. The gene with the greatest fold increase in IMR-32 cells was *c-hluPGFS* (*AKR1C3*) a member of the aldoketose reductase family. *AKR1C3* had increased expression in IMR-32 cells alone (Fig. 2C). Two genes involved in transforming growth factor (TGF) signaling were up-regulated: *TGFβ-II* and *CDKN1C* (Fig. 2C). The expression of a number of tumor suppressor genes was increased in IMR-32 cells when MYCN was knocked down: *NDRG1*, *ARHI*, *LGI1* and *TP53INP1* (Fig. 2C). Interestingly, low expression of *ARHI*, which is located at 1p31, was found to be associated with a poor outcome in neuroblastoma.[20] *ARHI* is a maternally imprinted gene, and its loss of expression has also been implicated in both ovarian and breast cancer.[21] Thirty-five genes (37 probes) were differentially regulated in SKNBE(2c) cells, these include the up regulation of the mitotic progression protein *NEK7* and the *RET* oncogene (Fig. 2D). Genes increased in expression in both cell lines at 48 hours included the WNT antagonist *DKK3*, *CHRM1* (muscarinic receptor M1) and *Testican-1* (Table 1). The following genes were chosen for validation by qRT-PCR: *DKK3*, *SKP2*, *CDKN1C* and *TP53INP1*.

Validation of microarray results. Changes in expression of selected genes were validated in IMR-32 and SKNBE(2c) cells, and in MYCN+ and MYCN- SHEP Tet21N cells, to determine if increasing MYCN expression had the opposite effect to MYCN knockdown.

SKP2. Levels of SKP2 down regulation by qRT-PCR were smaller than by microarray. A ~2 fold reduction in SKP2 mRNA was observed in both cell lines 16 hours after transfection (Fig. 3A). Reduction in SKP2 expression in MYCN siRNA treated IMR-32 cells was confirmed using Western blotting 48 hours post transfection (Fig. 3B), showing that SKP2 protein levels were low despite restoration of transcript levels by this time. There was a two-fold increase in SKP2 transcripts in Tet21N $MYCN^+$ cells compared to $MYCN^-$ cells (Fig. 3A). However, the level of SKP2 protein did not correlate with MYCN expression or *MNA* status across a panel of neuroblastoma

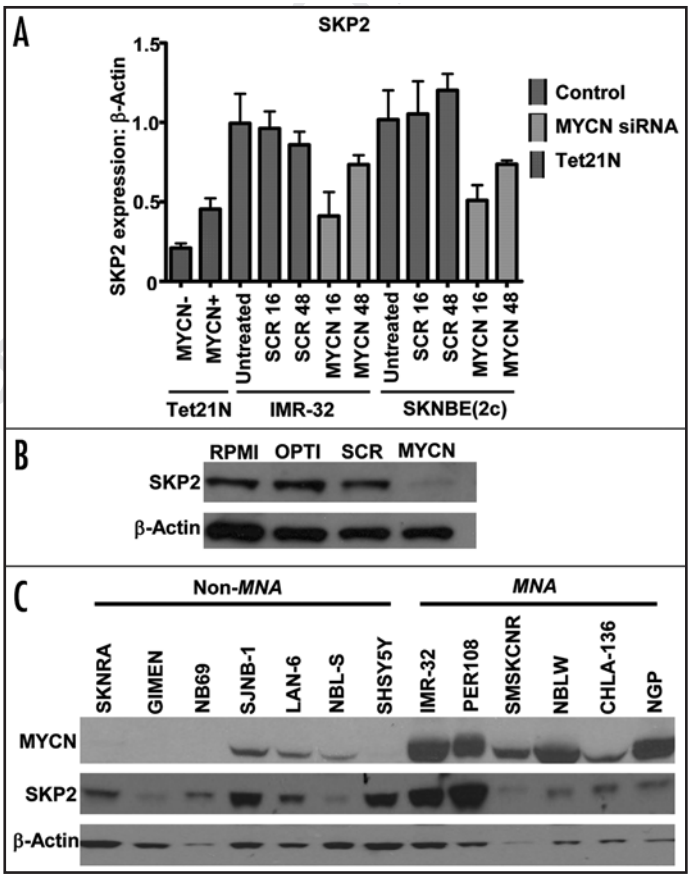

Figure 3. Validation of (A) SKP2 transcript levels after MYCN knockdown in IMR-32 and SKNBE(2c) cells. (B) SKP2 protein levels after MYCN knockdown. (C) Levels of SKP2 protein expression determined by WB in a panel of 13 neuroblastoma cell lines.

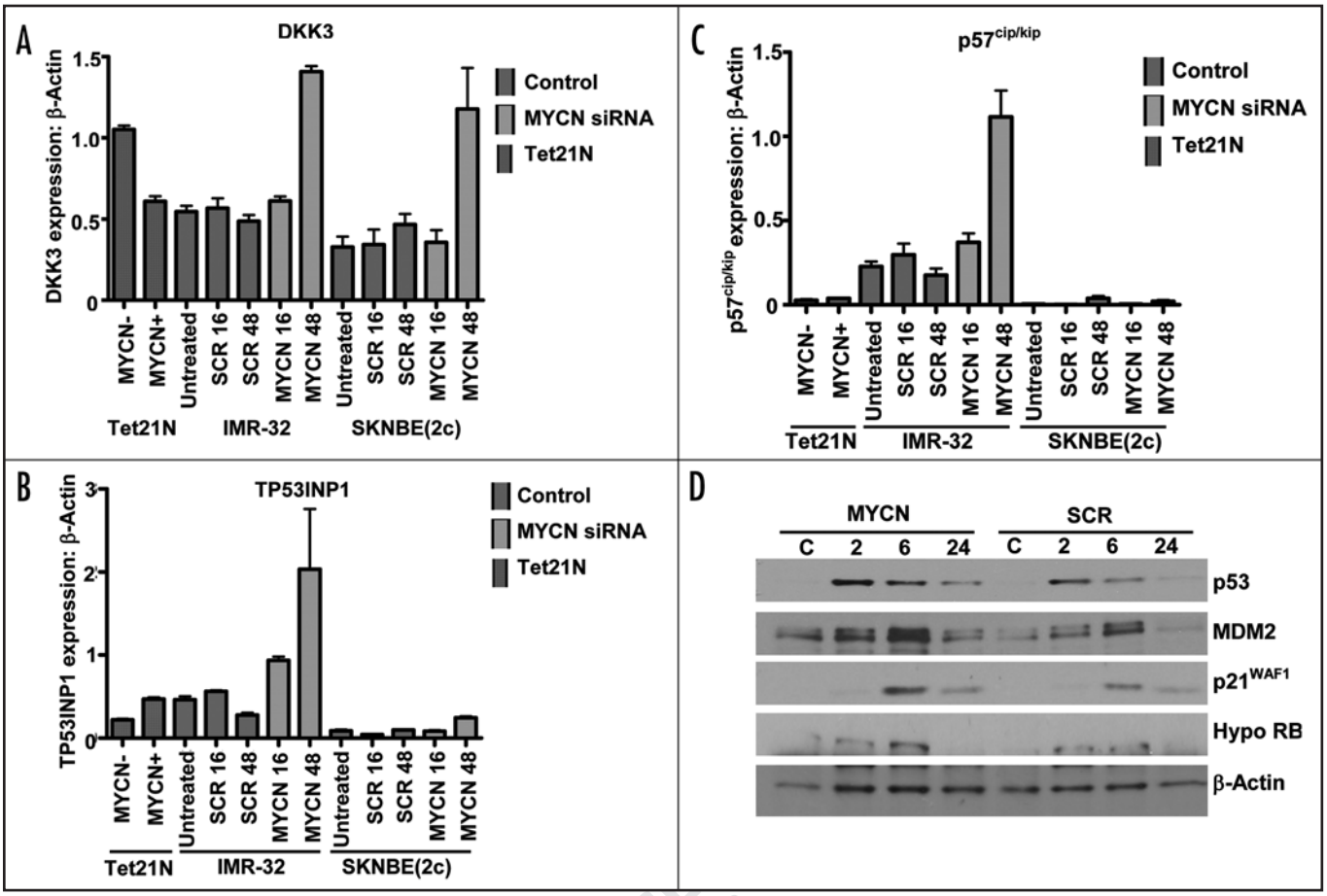

Figure 4. Validation of transcript levels of cell cycle genes after MYCN knockdown in IMR-32, SKNBE(2c) and Tet21N MYCN+ and MYCN- cells: (A) DKK3 (B) TP53INP1 (C) CDKN1C/p57kip d) The p53 response to 4Gy γ-irradiation induced DNA damage after MYCN knockdown in IMR-32 cells, compared to SCR siRNA treated cells.

cell lines (Fig. 3C). The non-*MNA* SKNRA, SJNB-1, LAN-6, SHSY5Y and the *MNA* PER-108 and IMR-32 cell lines all had high levels of SKP2 expression.

DKK3. qRT-PCR confirmed increased DKK3 expression 48 hours after MYCN siRNA transfection with DKK3 mRNA levels 3 fold higher in IMR32 cells compared with SCR treated controls and 2.5 fold higher in SKNBE(2c) cells than SCR controls. Conversely, DKK3 expression in MYCN+ Tet21N cells was ~2 fold less than MYCN- Tet21N cells (Fig. 4A).

TP53INP1. In IMR-32 cells, TP53INP1 mRNA expression increased 1.5 fold at 16 hours and ~7 fold 48 hours after MYCN siRNA transfection. Levels of TP53INP1 were lower in p53 mutant SKNBE(2c) cells, with ~2 fold increase in TP53INP1 observed in SKNBE(2c) cells by qRT-PCR. In contrast, TP53INP1 levels were ~2 fold higher in MYCN+ Tet21N cells compared with MYCN- Tet21N cells (Fig. 4B).

$p57^{cip/kip2}$. Levels of $p57^{cip/kip2}$ mRNA increased ~6 fold in IMR-32 cells 48 hours after siRNA transfection, however there was no change in p53 mutant SKNBE(2c) cells confirming the microarray observations, or in Tet21N MYCN+ and MYCN- cells (Fig. 4C).

$p21^{WAF1}$ protein levels after irradiation in MYCN siRNA treated IMR-32 cells. The p53 response to DNA damage was examined after MYCN knockdown in IMR-32 cells (Fig. 4D). Levels of MDM2 induction at 6 hours were 4 fold higher in MYCN compared to SCR siRNA treated IMR-32 cells and levels of p53, $p21^{WAF1}$ and hypo RB were similar in MYCN and SCR siRNA treated cells after irradiation.

DISCUSSION

Investigation of possible downstream targets of MYCN using MYCN knockdown to alter gene expression gives an insight into genes affected by high level MYCN expression that may not be identified by investigating ectopic MYCN expression in a non-*MNA* cell line alone. MYCC binds some of its downstream targets with a high affinity, and another group of genes with low affinity. MYCC binding at low affinity sites sharply increased with increasing levels of MYCC.[22] The low affinity binding sites will probably be most affected by high levels of MYCN expression resulting from *MNA*. The current study has identified a number of genes involved in regulation of the G_1 checkpoint that are differentially expressed after MYCN knockdown.

MYCN effects on $p21^{WAF1}$ during normal growth conditions. We previously reported that *MNA* neuroblastoma cells treated with MYCN siRNA accumulated in the G_1 phase of the cell cycle 48 hours after transfection.[1] In p53 wt cell lines this was accompanied by an increase in $p21^{WAF1}$ protein expression and hypo RB, with no change in p53 levels. In p53 mutant SKNBE(2c) cells MYCN knockdown increased the G_1 population, without an increase in $p21^{WAF1}$ or hypo RB.[1] Data from the present study, suggests that increases in $p21^{WAF1}$ levels in p53 wt IMR-32 cells after MYCN knockdown may be due to decreased SKP2 and increased TP53INP1 levels (Table 1, Figs. 3A and 4B). SKP2 is part of the SCF^{skp2} complex (composed of: Rbx, Cul1, SKP1 and SKP2). Ubiquitination of

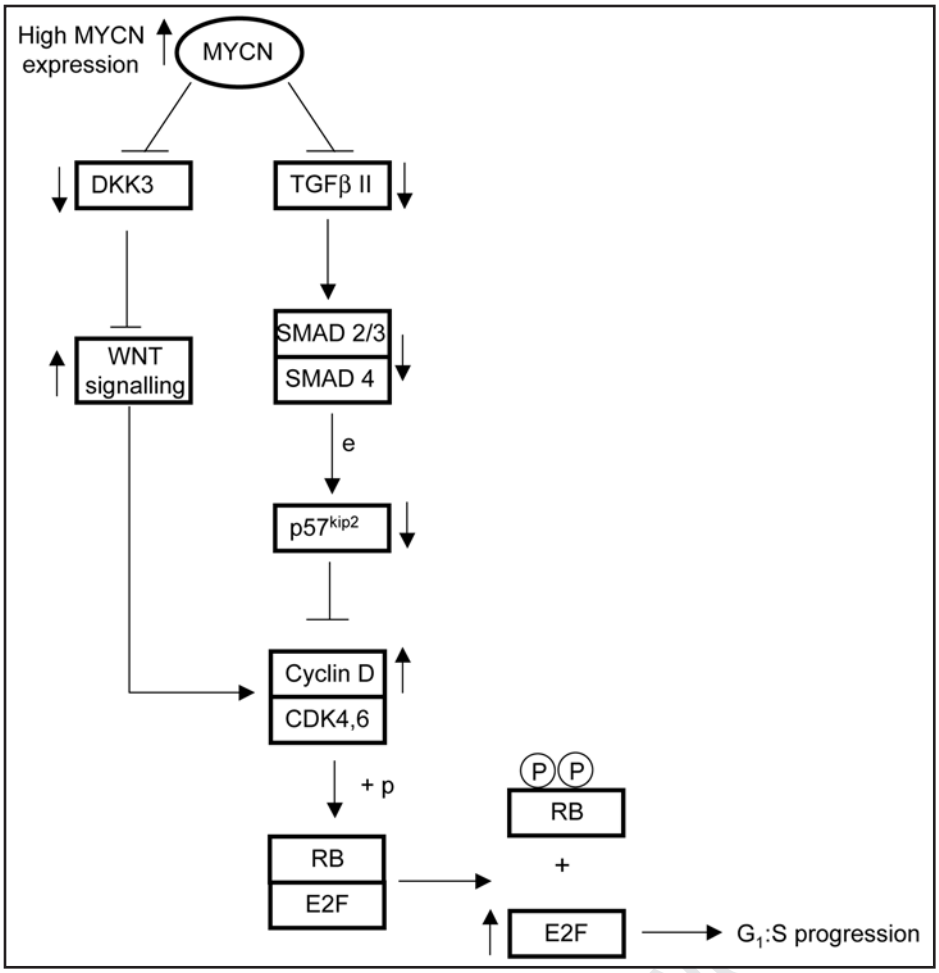

Figure 5. Proposed mechanism of how MYCN might act via DKK3 and $p57^{cip/kip2}$ to affect the G_1 checkpoint in *MNA* neuroblastoma cells. Phosphorylation (p+) and increased expression (e).

$p21^{WAF1}$ by SCF^{skp2} requires the functional interaction of $p21^{WAF1}$ with cyclin E-CDK2 complex.[23] Reduction of SKP2 after MYCN knockdown may decrease SCF^{skp2} mediated degradation of $p21^{WAF1}$, allowing $p21^{WAF1}$ to accumulate and induce a G_1 arrest in p53 wt cells.

TP53INP1 has been reported to cause a G_1 arrest and apoptosis.[24] TP53INP1 regulates p53 and p73 transcriptional activity, and in particular has been found to increase p53-dependent $p21^{WAF1}$ transcription.[25] The increase in $p21^{WAF1}$ and MDM2 after irradiation in IMR-32 cells after MYCN knockdown may also be due to increases in TP53INP1 levels (Fig. 4D). It has previously been demonstrated that MDM2 is a downstream transcriptional target of MYCN,[26,27] but in this study we did not observe any change in MDM2 levels after MYCN knockdown at the RNA or protein level.

SKP2 levels were reduced in p53 mutant SKNBE(2c) cells after MYCN knockdown (Fig. 3A), however no increase in $p21^{WAF1}$ levels were observed,[1] *$p21^{WAF1}$/CDKN1C* is a p53 induced gene.[28] Levels of TP53INP1 did not increase in p53 mutant SKNBE(2c) cells which is possibly because TP53INP1 is induced after cellular stress by p53 mediated transcription.[25] However, loss of p53 activity does not explain the result for the p53 wt $MYCN^+$ Tet21N cells, where high MYCN expression increases levels of TP53INP1.

Other potential effects of MYCN on the cell cycle. We have found that MYCN acts on two pathways that converge on cyclin D/CDK4/6 complexes at the G_1 checkpoint, providing a potential mechanism by which MYCN acts independently of $p21^{WAF1}$ (Fig. 5). Components of the WNT signaling and transforming growth factorβ (TGFβ) pathways are affected by MYCN knockdown (Fig. 2 and Table 1). DKK3, a member of the Dickkopf family of secreted WNT antagonists, was upregulated 48 hours after MYCN knockdown in both cell lines. DKK3 has been shown to modulate the activity and localization of β-catenin, a mediator of WNT signaling, targeting it to the cell membrane, reducing the amount of β-catenin in the nucleus so preventing its transcriptional activity.[17] Stabilized β-catenin, the product of positive regulation of WNT signaling, transcriptionally upregulates cyclin D,[29] MYCC,[30] c-jun and fra-1,[31] all of which affect the cell cycle. GSK-3β which is downstream of β-catenin in WNT signaling, also negatively regulates cyclin D via phosphorylation of Thr 286, targeting cyclin D for degradation.[32] The increase in DKK3 levels observed after MYCN knockdown may cause a G_1 arrest through the negative regulation of β-catenin and cyclin D. This p53 independent mechanism may be responsible for the accumulation of cells in G_1 after MYCN knockdown in p53 mutant SKNBE(2c) cells.[1]

Transforming growth factor β (TGFβ) is a potent inhibitor of cell proliferation and induces differentiation and growth arrest in certain cell types. TGFβ signaling pathway targets include bone morphogenic proteins, Smad transcription factors and activins.[33] Levels of TGFβ2 and $p57^{cip2}$ expression increased 48 hours after MYCN knockdown in p53wt IMR-32 cells. $p57^{cip2}$, a cyclin dependent kinase (CDK) inhibitor that targets cyclin D-CDK4/6 complexes is the downstream transcriptional target of TGFβ signaling which causes a G_1 arrest.[34] Therefore, deregulated MYCN expression in *MNA* cell lines may repress TGFβ signaling in order to prevent cyclin D inhibition by $p57^{cip2}$. An increase in $p57^{cip2}$ expression was not observed in the SKNBE(2c) cells. *$p57^{cip2}$/CDKN1C* is a paternally imprinted/gene,[35] it is possible that the other allele may be methylated or lost in SKNBE(2c) and Tet21N cells, in which $p57^{cip2}$ levels do not change after MYCN knockdown.

MYCN effects on $p21^{WAF1}$ induction after DNA damage. Cyclin A1 (CCNA1), the only cyclin to be induced by γ-irradiation[36] had increased expression sixteen hours after MYCN knockdown in IMR-32 cells (Fig. 2A). Cyclin A1 expression did not change in SKNBE(2c) cells, in line with cyclin A1 being a p53 inducible gene.[37] Cyclin A1 binds to CDK2 and plays a role in meiosis in male germ cells. It also phosphorylates Ku70, a DNA repair protein responsible for repairing double strand breaks (DSB). Cyclin A1 is involved in non-homologous end joining after DNA damage. Cyclin $A1^{-/-}$ cells are DSB repair deficient and have increased radiosensitivity.[36] MYCN downregulation of cyclin A1 might partly explain our previous observations of *MNA* neuroblastoma cell lines undergoing apoptosis after irradiation in preference to a G_1 arrest in which DNA damage would be repaired.[1]

This study sought an explanation for the effects of MYCN knockdown on the G_1 checkpoint in proliferating cells. Our data suggest a possible mechanism by which MYCN might repress $p21^{WAF1}$ expression by increasing SKP2 and decreasing TP53INP1 expression. In addition MYCN may also act independently of $p21^{WAF1}$ by decreasing levels of the WNT antagonist DKK3. This study has identified new pathways potentially regulated by MYCN which warrant further investigation and may provide novel therapeutic targets for *MNA* neuroblastoma.

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