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# Suppression of Myc oncogenic activity by Nucleostemin haploinsufficiency

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## Abstract

Nucleostemin (NS) encodes a nucleolar GTPase which is highly expressed in stem/progenitor cells and in most cancer cells. However, little is known about the regulation of NS expression. Here we identify NS as a novel direct transcriptional target of the c-Myc oncoprotein. We show that Myc overexpression enhances NS transcription in cultured cells and in pre-neoplastic B-cells from Eμ-myc transgenic mice. Consistent with NS being downstream of Myc, NS expression parallels that of Myc in a large panel of human cancer cell lines. Using chromatin IP we show that c-Myc binds to a well-conserved E-box in the NS promoter. Critically, we show NS haploinsufficiency profoundly delays Myc-induced cancer formation in vivo. NS+/−Eμ-myc transgenics have indeed much slower rates of B cell lymphoma development, with life spans twice that of wild-type littermates. Moreover, we demonstrate that NS is essential for the proliferation of Myc-overexpressing cells both in vitro and in vivo. Impaired lymphoma development was associated with a drastic decrease of c-Myc-induced proliferation of pre-tumoural B-cells. Finally, we provide evidence that NS control cultured cell proliferation independently of p53 and that NS haploinsufficiency significantly delayed lymphomagenesis on a p53-deficient background. Together these data indicate that NS functions, downstream of Myc, as a rate limiting factor for the proliferation and transformation of cells independently from its putative role within the p53 pathway. Targeting NS is therefore expected to compromise early tumour development irrespectively of the p53 status.

### Keywords

Nucleostemin; c-Myc; p53; lymphoma; transgenic mice

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**Conflict of interest**
The authors declare no conflict of interest.

## Introduction

Deregulation of c-Myc oncoproteins is estimated to contribute to 70% of all human cancers (Dai et al 2004, Dang et al 2006, Vita and Henriksson 2006). Overexpression of Myc is sufficient to drive normal quiescent cells into cycle and to accelerate their rates of cell cycle traverse (Bouchard et al 1998). However, cells respond to this hyperproliferative response by activating apoptosis (Askew et al 1991, Evan et al 1992) through -at least in part- activation of the ARF-p53 tumour suppressor pathway (Eischen et al 1999, Zindy et al 1998) or by repressing the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL (Eischen et al 2001). Bypass of these cell cycle checkpoints and apoptotic pathways is a hallmark of Myc-driven cancers.

Myc is a transcription factor that can either activate or repress gene expression. Activation occurs through dimerization with the partner protein Max, and binding to the consensus DNA sequence CACGTG (E-box). Repression occurs through association of Myc/Max dimers with other transcription factors such as Miz-1 or NF-Y, and interference with their function (Izumi et al 2001, Mao et al 2003, Seoane et al 2002, Staller et al 2001), although it is becoming apparent that Myc may also repress transcription through E-boxes (Adhikary and Eilers 2005). A number of studies based on chromatin immunoprecipitation (ChIP) have shown that Myc associates with a large (10–20%) fraction of cellular genes in a variety of cell types (Chen and Olopade 2008, Eilers and Eisenman 2008, Fernandez et al 2003, Kidder et al 2008, Kim et al 2008, Li et al 2003, Perna et al 2011, Zeller et al 2006). Myc has therefore the potential to activate thousands of genes, which in turn coordinate a wide range of cellular processes including those essentials for cell cycle, growth and apoptosis but also ribosome biogenesis, metabolism, protein folding and self-renewal. Which specific targets contribute to Myc's diverse biological effects is therefore a real challenge and while the ability of Myc to promote cellular transformation is well established, a better understanding of the mechanisms through which Myc mediates tumourigenesis is essential for the development of therapeutic approaches to target this potent oncoprotein.

Nucleostemin (NS) is a nucleolar GTP-binding protein that was initially identified by virtue of its preferential expression in stem cell-enriched populations including the CNS, hematopoietic and embryonic stem cells (Tsai and McKay 2002) NS is also overexpressed in many cancer cell lines and in mouse and human primary tumours (Han et al 2005, Liu et al 2004, Malakootian et al 2010, Sijin et al 2004, Tsai and McKay 2002, Ye et al 2008, Zhang and Wang 2010). Accumulating evidence indicates that high NS levels may contribute to the high proliferative capacity of tumour cells and therefore to tumourigenesis. Mouse mammary tumour cells that express high levels of NS exhibit stronger *in vitro* and *in vivo* tumourigenic activities (Lin et al 2010). Moreover, NS is functionally required for the sphere-forming activity of breast cancer cells (Lin et al 2010). Accordingly, knocking-down (KD) NS expression reduces cell proliferation of *in vitro* cultured osteosarcoma cancer cells (Tsai and McKay 2002) it also decreases the tumourigenicity of HeLa cells upon injection into nude mice (Sijin et al 2004). However, it still remains to be seen whether decreasing NS function would be sufficient to inhibit spontaneous tumour development in a cancer genetic model.

Several studies have provided important insights into the molecular mechanisms of action of NS. KD of NS in cancer cells leads to p53 activation and induction of p53-dependent cell cycle arrest (Tsai and McKay 2002). It was proposed that NS growth-promoting activity in cancer cells is caused by the formation of NS-p53 complexes and direct NS-dependent inhibition of p53 function (Bernardi and Pandolfi 2003, Tsai and McKay 2002) Genetic experiments in mice have, however, highlighted p53-independent role of NS in the control cell proliferation. NS is indeed required for the proliferation of normal mouse embryonic

stem cells. NS-deficient embryos die before implantation and, importantly, this early embryonic lethality is not rescued on a p53-null background (Beekman et al 2006) Consistently, NS is required for the proliferation of normal rat bone marrow stem cells in a p53-independent manner (Jafarnejad et al 2008) NS may also contribute to cell proliferation via its role as an integrated component of ribosome biogenesis, particularly pre-rRNA processing (Romanova et al 2009). NS indeed forms a large protein complex that co-fractionates with the pre-60 S ribosomal subunit and contains proteins related to pre-rRNA processing, such as Pes1, DDX21, and EBP2, in addition to several ribosomal proteins. NS promotes the nucleolar retention of DDX21 and EBP2 and NS KD retards rRNA processing, thereby causing the accumulation of free ribosomal proteins (RPs), which are normally assembled into ribosomal subunits, and induction of ribosome stress (Romanova et al 2009). Interestingly, NS' role in ribosome biogenesis might be connected to its contribution to the regulation of the p53 pathway. RPs can indeed directly bind MDM2 and interfere with MDM2-dependent degradation of p53 (Dai and Lu 2004, Dai et al 2004, Jin et al 2004). Depletion of NS induced the interaction between some RPs (RPL5 and RPL11) and MDM2, and KD the RPs reversed, at least partly, p53-dependent arrest caused by NS depletion (Dai et al 2008). These data provide an alternative explanation for the p53-dependent role of NS in the control of cancer cell proliferation (Lo and Lu 2010). Hence, targeting NS in tumours in which the p53 pathway is intact may offer a way for eradicating growing cancer cells by cutting off the ribosome supplies for protein production, which is necessary for actively growing cancer cells and/or for activating a p53 tumour suppression response.

Little is known about the mechanisms leading to high NS expression in cancer cells. Here we identified NS as a direct transcriptional target of c-Myc and demonstrate that NS is a critical downstream regulator of Myc's-ability to provoke accelerated cell proliferation and cancer. We additionally address the relevance of NS-dependent inhibition of p53 function to the proliferation of cancer cells in an *in vivo* setting. We show that NS is haploinsufficient in the control of proliferation of cancer initiating cells and tumour development *in vivo*, in a p53-independent manner. Our data indicate that targeting NS may be therapeutically efficacious in tumours driven by c-Myc and thus irrespective of the p53 status.

## Results

### Search for novel Myc-induced transcriptional targets

Overexpression of the c-Myc oncoprotein is a feature of a many human cancers. In order to identify novel Myc-transcriptional targets that play a critical role in Myc-induced tumourigenesis, we searched for genes that are differentially expressed upon Myc overexpression using a microarray-based approach. As a cellular model, we chose the previously established human lung fibroblast-derived L1 cell line in which Myc overexpression causes a significant increase in cell proliferation and induction of anchorage-independent growth in soft agar (Brown et al 1997, Wei et al 2003). Global gene expression was determined in both parental (LF1c) and stably Myc-expressing (LF1Myc) cell lines -in duplicate- using the affymetrix Human Genome U133 array platform. Of the 44,928 probe sets analyzed, 464 showed statistically significant differences in gene expression using a cutoff of >2-fold differential expression between the mean values in LF1c and LF1Myc cells ($P<0.05$). We next compared our list of putative Myc-upregulated genes (193 out of the 464 probe sets) with the previously reported list of c-Myc-bound genomic loci as determined by a ChIP on chip experiment performed in the human acute myeloid leukemia HL60 cells (Mao et al 2003). This cross-comparison identified 4 common genes, which are likely to be direct Myc-transcriptional targets: H2BFG, OIP2, DLEU1 and E2IG3, also known as NS (Nucleostemin). Interestingly, going back to one of our previously published microarray experiments, we noticed that the gene corresponding to the hypothetical GTP-binding protein (accession number AA892598) is in fact NS. According to these data NS expression

is significantly elevated in rat fibroblast cells with endogenous c-Myc (TGR) and in c-Myc-null fibroblasts reconstituted with exogenous c-Myc expression (Myc3) compared to c-Myc null fibroblasts (HO) (O'Connell et al 2003). Hence, the ability of Myc to induce NS appears to be conserved during the evolution.

### NS is a direct Myc target

Given the above data and its previously recognized role in the control of proliferation and survival of stem/progenitor and cancer cells (Tsai and McKay 2002), we decided to confirm that NS is a bona fide Myc-target and further explore its role in Myc-induced transformation. We confirmed NS up-regulation in Myc-overexpression LF1 cells and in Myc-expressing rat fibroblasts (TGR and Myc3) using RT-qPCR and Western blot analysis (Figure 1A and 1B). We also analyzed the expression of NS in rat fibroblasts (TGR) under conditions of serum starvation, which decrease Myc mRNA and protein expression, and at various time points following serum stimulation. c-Myc is rapidly induced upon serum addition; its mRNA expression peaks at around 4 hours post-stimulation (Dang et al 2006). RT-qPCR confirmed Myc induction upon serum stimulation and, consistent with NS being regulated by Myc, a concomitant increase in NS expression was observed (Figure 1C). Similarly, NS downregulation paralleled that of Myc at later time points.

Cross-species comparison of the rat, mouse, and human NS promoter identified a canonical c-Myc binding site (E-box sequence: CACGTG) approximately 60 bp upstream of the transcription start site. To determine whether Myc can directly bind to this E-box we performed ChIP assays in both TGR rat and LF1 human fibroblasts. TGR fibroblasts were starved and serum-stimulated for 4 hours. The protein-DNA complexes were immunoprecipitated with a polyclonal c-Myc antibody ("Myc"; Figure1d) or without antibody ("NoAb") as a negative control. The immunoprecipitated DNA was analyzed using quantitative PCR (qPCR). We observed a 4.5-fold enrichment for c-Myc binding at the NS E-box upon serum stimulation (Figure 1D). A fragment containing the E-box of the known c-Myc target Nucleolin was used as a positive control. Fragments containing the E-box of glycine methyltransferase (GNMT), a gene which is not regulated by c-Myc, or completely devoid of any E-box (CHRNB4) (Frank et al 2001) were used as negative controls. Similarly, we observed a 7-fold enrichment in c-Myc binding to the NS E-box in Myc-overexpressing LF1 fibroblasts as compared to the parental LF1c cells (Figure 1E). Fragments containing the E-box of the CAD gene and PAPT, a gene which is not regulated by Myc, were used here as positive and negative controls, respectively (Brodsky et al 2005, Fernandez et al 2003). Together these data indicate that Myc is directly recruited to the E-box present in the NS promoter and therefore suggest that NS is a direct Myc-transcriptional target.

To confirm that Myc can induce NS expression in an in vivo setting we analyzed NS expression in mice overexpression Myc, the Eμ-myc transgenic mice. These mice express high levels of Myc in the B-cell lineage and develop pre-B and/or B-cell lymphoma with mean survival of about 11 weeks (Adams et al 1986). While low in wild-type bone marrow and spleen, which contain mostly early progenitors and mature B-cells respectively, NS protein levels are dramatically elevated in spleen and in tumours of the Eμ-myc tumour-bearing mice (Figure 1F). As expected, NS protein levels in the spleens of NS+/− mice are decreased by approximately 50% when compared to wild-type (NS+/+) mice (Figure 1F). NS mRNA, as measured by quantitative PCR (RT-qPCR), is significantly elevated in the spleen of the Eμ-myc tumour-free mice and perfectly parallels the level of Myc in spleen and lymphoma from the Eμ-myc tumour-bearing mice (Figure 1G). These data further confirm that that NS transcription is directly induced by Myc in vivo.

NS, just like c-Myc, is overexpressed in a variety of human cancers (Charpentier et al 2000, Tsai and McKay 2002). If NS is indeed a direct Myc-target a correlation between c-Myc and NS expression should be observed in human cancers. In order to test this possibility, we searched for genes with expression profiles most similar to c-Myc in a large panel of human cell lines (NCI60) using the National Cancer Institute's Cancer Genome Anatomy Project database. c-Myc expression varied across the available 60 cancer cell lines (red = high expression, and blue = low expression). Of the 6,817 genes in the database, NS was one of the ten genes with an expression profile most similar to c-Myc (Figure1H). These data indicate that Myc and NS are co-expressed in human cancers, an observation that is consistent with NS being downstream of Myc.

## NS haploinsufficiency protects mice from Myc-induced tumourigenesis

We next asked whether high levels of NS is required downstream of Myc for facilitating Myc-induced tumourigenesis. Targeted deletion of NS in mice leads to early embryonic lethality (Beekman et al 2006). However, NS+/− mice appear completely normal, despite expressing half of NS protein. We therefore tested whether loss of one NS allele would affect Myc-induced lymphoma development. NS+/− mice were bred onto a C57/Bl6 background for more than 10 generations and were then crossed to C57/Bl6 Eμ-Myc transgenics. NS+/+; Eμ-Myc and NS+/−; Eμ-Myc animals were then followed for their course of disease. As expected, most NS+/+; Eμ-Myc succumbed to lethal lymphoma between 90 and 200 days (Figure 2). Strikingly, lymphoma development was profoundly delayed in NS+/−; Eμ-Myc mice compared to NS wild-type Eμ-Myc littermates (Figure 2; $p<0.0001$, log-rank test). The mean survival of Eμ-Myc NS heterozygous mice was twice that of the mean survival of the NS wild-type Eμ-Myc littermates (Figure 2). All NS+/+ mice died within 40 weeks (n=24), whereas 50% (13 out of 26) of the NS+/− animals were still alive at that time. The lymphomas that developed in NS+/−; Eμ-Myc animals were typical pre-B/B-cell lymphomas that arise in Eμ-Myc mice (data not shown). Therefore, NS haploinsufficiency markedly compromises Myc-mediated tumourigenesis.

## NS is a critical regulator of Myc-induced cell proliferation

Having established the relevance of NS in Myc-induced tumourigenesis, we next investigated the underlying mechanism by dissecting the role of NS in Myc functions. To this end, we first knock down NS by siRNA in an established embryonic rat fibroblast Rat1A cell line expressing an inducible form of Myc, the c-Myc/estrogen receptor (ER) fusion protein (Mai et al 1996). In agreement with the above data, NS expression increased as a result of c-Myc induction (c siRNA + OHT); transfection of NS siRNAs completely prevented this up-regulation (Figure 3A). In agreement with its oncogenic nature, increased levels of Myc drive cellular proliferation. NS knock down had only a marginal effect on cell proliferation in the absence of Myc induction in this experimental setting; however, preventing Myc-induced NS up-regulation by siRNA profoundly decreased cell proliferation upon OHT-mediated Myc induction (Figure 3B). As expected, in addition to stimulate cell proliferation Myc-induction promoted an increase in spontaneous apoptosis (Meyer et al 2006). However, we did not observe any effect of NS KD on the extent of Myc-induced apoptosis in this experimental setting (data not shown). Induction of c-Myc in Rat1A Myc/ER cells causes anchorage-independent growth in soft agar (Qi et al 2004). Accordingly, cells transfected with control siRNA grew large colonies upon Myc induction. Strikingly, cells transfected with NS siRNAs grew very few and smaller colonies (Figure 3C); there was a 3.6-fold reduction in the ability of these cells to form colonies as a result of NS knockdown. Together these results indicate that NS is dispensable for Myc-induced apoptosis but functions as a critical mediator of c-Myc induced proliferation.

To further confirm this view in an in vivo setting we assessed cell proliferation in response to Myc overexpresion in the Eμ-Myc transgenic mice using BrdU incorporation assays and FACS-analyses (Figure 3D). As expected, we observed an increase in cell proliferation both in bone marrow and splenic pre-tumoural B cells from the Eμ-Myc mice. Importantly, a decrease in cell proliferation was observed in normal pre-B cells of NS+/− mice compared to NS+/+ mice, and this difference was more pronounced in the pre-tumoural B cells that expressed Myc. From these observations we concluded that NS is a rate-limiting factor in the proliferation of pre-B cells and that NS haploinsufficiency limits the ability of Myc to efficiently drive proliferation and expansion of these cells *in vivo.*

Myc overexpression induces p53-dependent apoptosis in part by inducing expression of the Mdm2-antagonist Arf (Dominguez-Sola et al 2007) and in part by promoting a DNA-damage response (DDR) (Ray et al 2006, Vafa et al 2002, Wang et al 2008). Accordingly, we measured a significant increase in apoptosis in pre-tumoural B cells isolated both from bone marrow and spleen of the Eμ-Myc mice as compared to wild-type counterparts; apoptosis was assessed using caspase3/7 glow assays (Figure 3E) and FACS analysis (data not shown). As expected, we observed stabilization of p53 and induction Ser-18 phosphorylation of p53, an event which occurs specifically in response to DDR (Chao et al 2000) in pre-tumoural and tumoural B cells of the Eμ-Myc mice. However, the extent of p53 stabilization and phosphorylation and the percentage of spontaneous apoptotic cells were comparable in NS+/−; Eμ-Myc and NS+/+; Eμ-Myc animals (Figure 3F). Hence, NS+/− mice do not exhibit a defect in DDR signaling and p53-dependent apoptotic response on a sensitized Eμ-Myc background; an observation which is consistent with the in vitro data presented above.

## NS is required for cell proliferation and tumourigenesis independently of p53

Previous transfection studies indicated that NS, through a direct physical interaction with p53, dampens p53-growth suppressive activities (Tsai and McKay 2002). We therefore tested whether the critical role for NS in the regulation of cell proliferation and tumourigenesis is linked to its ability to regulate p53 function. We first established stable NS KD lines in the human colon cancer cells lines HCT116 and isogenic HCT116 p53KO (kind gift from Prof B. Vogelstein). The cells were infected with an empty lentiviral vector (EV) as control (Figure 5A). Notably, Myc and NS are co-expressed at high levels in both parental HCT116 and HCT116 p53KO cell lines (data not shown). A robust NS KD was observed in both of these cell lines upon infection with two different lentiviral NS-shRNA constructs. Strikingly NS KD decreased the ability of these cells to grow in vitro irrespective of p53 status as assessed using growth curve assays (Figure 4B), FACS analysis (Figure 4C) and soft agar assays (Figure 4D). The FACS analysis indicated that NS KD resulted in a decreased percentage of cells in S-phase and an increased proportion of cells in the G2/M phase of the cell cycle. The cell cycle block was accompanied with an increase in the sub-G1 fraction, which is likely the result of increased cell death. Importantly, these growth inhibitory effects were observed in both p53 competent and p53KO cells.

In order to assess a putative p53-dependent role of NS in tumour formation, NS heterozygous mice were crossed with p53-null mice (Jacks et al 1994). T cell development proceeds normally in p53-deficient mice although they eventually succumb to thymic lymphomas between 15 and 25 weeks of age (Donehower et al 1992) and only few of them survive past week 30 (Hursting et al 1994). Strikingly, we observed that partial reduction of NS expression was sufficient to significantly delay tumour-formation on p53-null background and extended the mean survival of p53-deficient mice twofold (log rank test: $P<0.001$; Fig 5). In our study, 4 out of 19 NS+/−; p53-null mice survived past 40 weeks while all NS+/+ controls died within 30 weeks (n=6). This finding indicates that NS

haploinsufficiency delays lymphomagenesis in a manner that is independent of its role in the regulation of p53 function.

## Discussion

NS is highly expressed in various human cancer cell lines and primary tumours (Han et al 2005, Lin et al 2010, Liu et al 2004, Malakootian et al 2010, Tsai and McKay 2002, Ye et al 2008, Zhang and Wang 2010). However, the mechanisms leading to this up-regulation is poorly understood. Previous reports had highlighted a correlation between NS expression and the presence of the active Myc oncogene (Dave et al 2006, Rosenwald et al 2002, Shaffer et al 2006). In this manuscript we further extend such a correlation to a large panel of human cancer cell lines (NC60) and show dramatic accumulation of NS in Myc-driven B-cell lymphomas isolated from Eu-Myc mice. Importantly, we now provide direct mechanistic insights underlying the correlation between Myc and NS by identifying NS as a novel direct Myc-transcriptional target. Although we cannot exclude that additional mechanisms account for high NS expression in human tumours, our findings also provides an explanation for the up-regulation of NS in human cancers as Myc is activated in a high percentage of human malignancies.

Critically, our data indicate that NS is not just one additional component of the complex transcriptional networks induced by Myc but it is a critical regulator of tumour development and a key mediator of Myc-induced tumourigenesis in vivo. Decreasing NS levels by 50% (haploinsufficiency) is sufficient to drastically limit Myc-induced lymphoma development *in vivo*. Our data indicate that this delay is not a consequence of an impaired DDR or p53-dependent apoptotic response but rather of an altered ability of Myc to accelerate cell cycle traverse of the tumour initiating cells. The exact biochemical mechanism underlining the role of Myc-induced NS in cell proliferation is unresolved but could be linked to its previously recognized role in ribosome biogenesis. Depletion of NS indeed retards rRNA processing, thereby causing the accumulation of free ribosomal proteins (RPs), accompanied by a substantial decrease in overall protein synthesis (Romanova et al 2009). Interestingly, Myc is a critical regulator of ribosome biogenesis and translation via its ability to up-regulate the transcription of rRNA and ribosomal proteins and auxiliary factors that are required for rRNA processing, ribosome assembly and export of mature ribosomal subunits from the nucleus to the cytoplasm (van Riggelen et al 2010). The ability of Myc to regulate the expression of yet another regulator of this process, NS, is therefore entirely consistent with this view. Importantly, an increasing number of observations indicate that Myc's contribution to tumourigenesis is at least partly connected to its ability to modulate ribosome biogenesis. In Eu-Myc mice, the same model system utilized in the study herein, loss of one allele of Rpl24 or Rpl38 decreased the incidence of lymphoma by 20% and delayed tumour onset by over 100 days (Barna et al 2008). Notably, as shown herein for NS, decreased Rlp24 gene dosage impeded the ability of Myc to stimulate cell cycle progression independently from the cell cycle program established at the transcriptional level by Myc hyperactivation. By analogy it is therefore tempting to speculate that Myc-induced cell proliferation and tumourigenesis are dependent on Myc's ability to stimulate ribosome biogenesis and protein synthesis, at least in part via induction of NS expression.

Finally, we show that the function of NS in tumourigenesis is independent of its role in the regulation of the p53 tumour suppressor pathway. Indeed, NS haploinsufficiency was sufficient to significantly delay lymphomagenesis on a p53-null background. Our data therefore indicate that in contrast to strategies targeting MDM2/MDMX, the efficacy of which rely on the presence of an intact p53 pathway (Marine 2010, Toledo and Wahl 2007) targeting NS could form an alternative valuable therapeutic approach in management of cancers with a disabled p53 pathway. The presence of two putative GTP binding sites within

NS makes it a potential target for molecules that mimic or compete for GTP binding. It has recently been reported that AVN-944, a potent specific IMP dehydrogenase inhibitor that is currently in Phase I trials for the treatment of hematologic malignancies and in Phase II trials for solid tumours, leads to the rapid degradation of the NS protein in tumour cell lines (Huang et al 2009). The effect is a consequence of targeting of the GTP-uncoupled NS for proteasomal degradation by MDM2 and appears to be specific as it was not observed with the two other nucleolar proteins nucleophosmin and nucleolin. Modulation of NS levels using AVN-944 could therefore be particularly effective in tumours overexpressing MDM2.

## Material and Methods

### Cell lines and culture conditions

Human lung fibroblast cells (LF1) (Brown et al 1997) expressing the catalytic subunit of telomerase (hTERT), simian virus 40 (SV40) large T antigen (LT), and SV40 small T antigen (ST), and c-Myc (Wei et al 2003) were cultured in Ham's F10 nutrient mixture supplemented with 15% fetal bovine serum (FBS). The pair of $p53^{+/+}$ and $p53^{-/-}$ human colon cancer cells HCT116 was obtained from Bert Vogelstein (Johns Hopkins University) and293T packaging cells were obtained from W. Hahn (Dana Farber Cancer Institute). The cells were cultured in DMEM supplemented with 10% FBS. Immortalized embryonic Rat-1 fibroblast lines including TGR-1 (TGR) (Prouty et al 1993), TGR-1 bearing homozygous *c-myc* deletion HO15.19 (HO) (Mateyak et al 1997) HO cells reconstituted with c-Myc expression (Myc3) (Mateyak et al 1999) and Rat1A expressing the c-Myc/estrogen receptor (Myc/ER) fusion protein (obtained from S. McMahon, Kimmel Cancer Center) were cultured in DMEM supplemented with 10% calf serum (Hyclone). For the experiments requiring quiescent cultures, cells were grown to confluence and serum starved in media containing 0.1% calf serum for 48 hours. The cells were stimulated to enter the cell cycle upon addition of media with 10% calf serum. All cells were incubated at 37o C in an atmosphere of 5% $CO_2$.

### Lentiviral and retroviral vectors

The lentiviral vector pLKO.1-puro expressing NS shRNA1 (targeting all variants of human NS at GCTAAACTGTTCTCTGTATAA) or NS shRNA2 (targeting all variants of human NS at CAGCAAGTATTGAAGTAGTAA) was obtained from the RNAi Consortium at the Broad Institute. Lentivirus production was achieved according to the protocol available through the RNAi Consortium, using 293T packaging cells and FuGENE 6 transfection reagent (Roche). HCT116-infected cells were selected in 1 μg/ml puromycin.

### RNA interference (RNAi)

siRNAs were transiently transfected using nucleofection (Amaxa Biosystems). $1\times10^6$ cells per each sample were combined with 100 μl Nucleofector solution and 1.5 μg siRNA. Following nucleofection, cells were transferred directly to the culture dish. For HCT116 cells, Kit V and the D-32 setting was used for nucleofection. For TGR and Rat1A Myc/ER cells Kit R and the T-16 setting was used for nucleofection. The following siRNAs were used:

NS: Rat NS siRNA sense/antisense:

*GACAUUGUGUUAGAAGUUUtt/AAACUUCUAACACAAUGUCtg* (Silencer pre-designed siRNA, Ambion);

Human NS siRNA1 sense/antisense:

*GGUGAUUGAAGCCUCCGAUdTdT/AUCGGAGGCUUCAAUCACCdTdT* (Dharmacon);

Human NS siRNA2 sense/antisense:

*GGCUUACAAGGAGCAUGCAAGUUGU/*
*ACAACUUGCAUGCUCCUUGUAAGCC* (Stealth RNAi, Invitrogen).

## Microarray analysis

Total RNA was extracted from exponentially growing cells using Trizol reagent (Invitrogen). The experiment was conducted in two independent repetitions. Synthesis of double stranded cDNA, followed by conversion to target cRNA, and hybridization to the Affymetrix Human Genome U133 array set was carried out by the manufacturer's instructions (Affymetrix). The probe arrays were washed and stained using the GeneChip Fluidics Station 400, and scanned using the Agilent GeneArray scanner. Data was normalized to a target intensity of 1500, and analysis was carried out using Microarray Suite 5.0, MicroDB 3.0, and Data Mining Tool 3.0 software (Affymetrix).

## Quantitative real-time PCR (qPCR)

Total RNA was extracted from cultured exponentially growing cells or murine spleens and tumours, using Trizol reagent (Invitrogen). RNA samples were treated with DNase I, and two micrograms of total RNA from each sample were reverse transcribed with a high-capacity cDNA archive kit (Applied Biosystems). Quantitative reverse transcriptase PCR (RT-qPCR) assays were performed using Fast SYBR Green 2x Master Mix, following the manufacturer's instructions (Applied Biosystems). Primer pairs used: Rat NS: *AGGATGGTGATGATCAAGAACATG* and *GATGGTTTACTTGCTGTTGATTGC*; Rat GAPDH: *ACCACCAACTGCTTAGCCCC* and *TTCTGAGTGGCAGTGATGGC*; Rat c-Myc: *CCAGCAGCGACTCTGAAGAAG* and *GATGACCCTGACTCGGACCTC*; Human NS (amplifies all isoforms): *TCGGGTTGGAGTAATTGGTTTC* and *TGTAAGCCCCATGGATACACC*; Human actin: *AAGGATTCCTATGTGGGCGA* and *TCCATGTCGTCCCAGTTGGT*; c-Myc*: AGTGCTGCATGAGGAGACAC* and *GGTTTGCCTCTTCTCCACAG;* Mouse NS: *TGCAGCAGTCATGAAGAAGG* and *CCGAAGACCGAAAAAGGATT;* Mouse Actin-beta: *GCTTCTAGGCGGACTGTTACTGA* and *GCCATGCCAATGTTGTCTCTTAT;* Mouse TATA box binding protein (Tbp): *TCTACCGTGAATCTTGGCTGTAAA* and *TTCTCATGATGACTGCAGCAAA;* Mouse Rlp13a: *CCTGCTGCTCTCAAGGTTGTT* and *TGGTTGTCACTGCCTGGTACTT.* The primers were designed across exon junctions with the Primer Express software, version 3.0 (Applied Biosystems) or using Primer 3, version 0.4.0. Gene expression levels and errors on the gene expression levels were calculated using qBasePLUS 1.0 analysis software (Hellemans et al 2007), which uses delta–quantification cycle (delta–Cq) model normalization to multiple reference genes (Vandesompele et al 2002) qPCR assays were conducted in triplicates.

## Chromatin immunoprecipitation

The ChIP protocol was carried out using the ChIP Assay kit (Upstate). Each ChIP assay was performed using $5 \times 10^6$ cells. Cross-linking was performed by incubating cells for 10 minutes at room temperature in 1% formaldehyde in culture media. Cells were lysed in the SDS lysis buffer supplied with the kit. DNA was sonicated and chcromatin was immunoprecipitated with the 4 mg c-Myc polyclonal antibody (Upstate) or a no antibody control at 4°C overnight. Antibody-bound chromatin was collected using protein A agarose, followed by multiple washes, elution of DNA and decrosslinking at 65°C overnight. DNA was recovered by phenol/chloroform extraction/ethanol precipitation and qPCR reactions were carried out using: Rat ChIP primers: NS E-box at position −67: *ACTTTCCGGCAGCTTCTTGA* and *GTCTGGAGCAGAAGAGGTCTCAG*; control primers targeting E-box for nucleolin at position 574: *CGCGTCCGAGGCAGTG* and

*TCCATCTACCGTCACGGTCAG*; an irrelevant E-box not bound by c-Myc, glycine methyltransferase (GNMT): *CAAGCGCCGTCAGGATG* and *CAGTACGGCTGCGGGTG*; and a promoter without an E-box, neuronal acetylcholine receptor beta 4 (CHRNB4): *TGCCTCGGGTGAACTAAGATG* and *GCCTCATTCGTCTTGGGAACT*; as reported in (Frank et al 2001) Human ChIP primers: NS E-box at position −55: *CTCGTCAGTGGCTTCAGTTCAC* and *GACCCCACTTACTAGGCCTTTTC*; The following control primers targeting E-box for carbamoyl-phosphate synthetase 2 (CAD) at position −454: *ACGTGGTTCCAGTGGAGTTTG* and *CGACCCGTCCTCCAACACTA*; and a gene not expressed in LF1 cells, testis-specific poly(A) polymerase (PAPT): *CGTTGCACCAAGTCATGTGG* and *CCTCCTCGACAGCCCTTAAA*; as reported in (Brodsky et al 2005, Fernandez et al 2003).

### Immunoblot analysis

Protein lysates from exponentially growing rat fibroblasts were prepared using Laemmli buffer. Proteins were separated by 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Antibodies used: anti-Nucleostemin (R&D Systems); anti-GAPDH (Ambion); Secondary antibodies were horseradish peroxidase (HRP)-conjugated (Jackson Immunoresearch).

Lysis of mouse organs and immunoblotting were performed as described before (De Clercq et al 2010). Organs were harvested from moribund animals and from age-matched non-Myc-expressing control mice of indicated NS status. The primary antibodies used were: anti-vinculin (clone hVIN-1, Sigma); anti-CD45R (B220) (clone RA3-6B2, Santa Cruz); anti-Nucleostemin (Chemicon); anti-myc (clone 9E10, Abcam), anti-p53 (clone 1C12, 1:1000, Cell Signaling); anti-caspase-3-cleaved (Cell Signaling); anti-γH2AX (Cell Signaling). The secondary antibodies used were HRP-conjugated (Amersham).

### Flow cytometry

As previously reported (Schorl and Sedivy 2003), flow cytometric analysis was performed using a BD Biosciences FACS-Caliber instrument and CellQuest and Modfit software. Exponentially growing cells were fixed in ethanol and stained with propidium iodide (Sigma) to a concentration of 50 μg/ml or sulforhodamine 101 (Molecular Probes) to a concentration of 3 μg/ml. Propidium iodide or sulforhodamine 101 emissions were recorded in the FL-2 channel. Cell size analysis of live, unfixed cells was performed by measurements of forward scatter.

For flow-cytometric analysis of *in vivo* 5-bromodeoxyuridine (BrdU) incorporation in mouse B220+ cells, bone marrow and spleens were isolated from 6–7 weeks-old mice of indicated genotypes. BrdU injection of mice, magnetic sorting-based isolation of B220+ from organs (MACS, Miltenyi Biotech), fixation, and indirect immunofluorescent BrdU labeling, were performed as described previously (De Clercq et al., 2010). FACS analysis was performed using FACSCanto (Becton & Dickinson).

### Luminescence Cell Apoptosis Assay

B220+ cells were isolated from bone marrow and spleens of 6–7 weeks-old mice using magnetic sorting (MACS, Miltenyi Biotech), as described previously (De Clercq et al 2010). Directly after the isolation, the cells were counted and apoptosis was measured using Caspase-3/7 Glo luminescence assays (Promega) according to manufacturers protocol.

### Soft agar assay

The soft agar assay was performed as described (Zhang et al 2005). Briefly, 2 ml of 0.6% agarose (SeaPlaque Agarose, Cambrex) in media (DMEM/10% FBS) heated to 42°C was layered on to each well of a six-well plate. Once solidified, 10,000 cells were added to 2 ml of 0.3% agarose in media at 42°C and overlaid on to each well. For experiments with the Rat1A Myc/ER cells, 2 ml media supplemented with 200 nM 4-hydroxytamoxifen (OHT) was added over the solid agarose the following day. Colony growth was assessed after 2 weeks.

### Mouse strains

NS +/−, Eμ-Myc, and p53 −/− mouse strains and their PCR genotyping were described earlier (Adams et al 1986, Beekman et al 2006, Jacks et al 1994). All mice were backcrossed to C57Bl/6 strain for a minimum of 10 generations. Animals displaying signs of reduced vitality or with visible tumours were euthanized.

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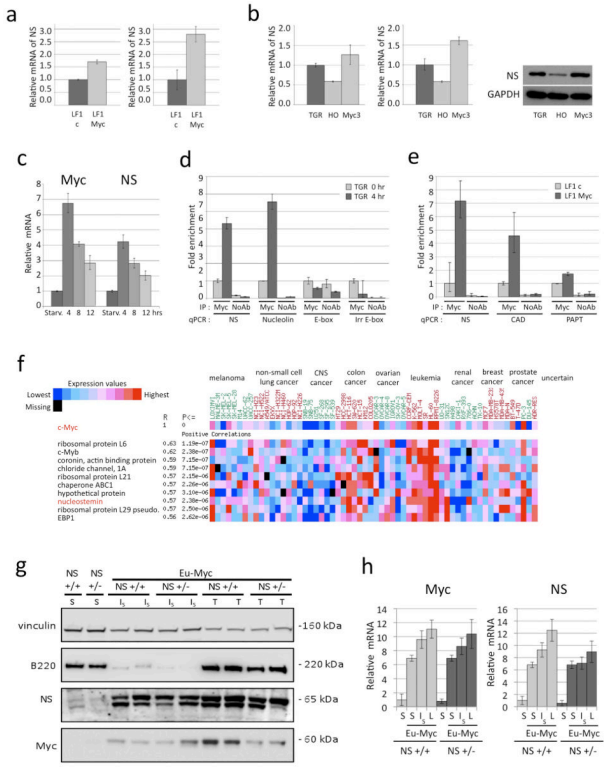



**Figure 1. NS is a direct transcriptional target of c-Myc**

(a) Expression of NS in human LF1 lung fibroblasts (LF1 c) and LF1 cells with c-Myc overexpression (LF1 Myc) relative mRNA of NS in these cells assessed by RT-qPCR. (b) Expression of NS in rat fibroblasts wild-type for c-Myc (TGR), c-Myc −/− (HO), and in c-Myc−/− reconstituted with ectopic c-Myc overexpression (Myc3). NS mRNA expression, confirmed by qPCR (middle panel), corresponds to the expression of NS protein (right panel). (c) NS and c-Myc show similar patterns of mRNA expression under conditions of serum starvation and stimulation in TGR cells. (d) c-Myc binds directly to the NS promoter in rat cells. ChIP was performed with a polyclonal c-Myc antibody (Upstate), or with no antibody (negative control), in TGR cells that were serum starved to prevent c-Myc expression (0 h), or serum stimulated for four hours to induce c-Myc expression (4 h). qPCR was performed using primers spanning a NS E-box, a Nucleolin E-box (positive control), a promoter with an E-box that has been previously shown not be bound by c-Myc (GNMT, negative control), and a promoter with no E-box (CHRNB4, negative control). (e) c-Myc binds directly to the NS promoter in human cells. ChIP was performed in LF1 control cells (LF1 c) and in LF1 cells with c-Myc overexpression (LF1 Myc), using c-Myc antibody (Upstate), or no antibody (negative control). qPCR primers were spanning: NS E-box, CAD E-box (positive control), and a gene not expressed in LF1 cells, testis specific poly(A) polymerase beta (PAPT) (negative control). (f) NS is highly overexpressed in all c-Myc-driven lymphoma-related tissues but not in healthy spleens. Western blot analysis on spleens (S) and tumours (T) derived from NS+/+; Eμ-Myc and NS+/−; Eμ-Myc. Lysates from healthy spleens from NS +/− and WT C57BL/6 mice were used as controls. Vinculin served as loading control. Two splicing variants of NS are detected with anti-NS antibody (Malakootian et al 2010). S – healthy spleen, $I_S$ – lymphoma-infiltrated spleen, T – tumour. (g) NS mRNA expression strongly correlates with c-Myc expression. As expected, NS mRNA levels in spleens (S) from non-c-Myc overexpressing N+/− animals are about half of the ones detected in the wild type (NS +/+) mice. In spleens from Eμ-Myc animals (Eμ-Myc) the difference becomes less pronounced due to strong c-Myc driven transactivation of

NS expression. .S – healthy spleen, $I_S$ – lymphoma-infiltrated spleen, T – tumour. Relative expression levels are presented as averages from three mice per each genotype. (h) Expression of NS correlates with c-Myc expression in the NC60 cancer cell lines. Analysis of a dataset available from the National Cancer Institute's Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/) reporting expression profiles for 6,817 genes across 60 cancer cell lines. Red = high expression; Blue = low expression. NS was included as one of the ten genes with expression profiles most similar to c-Myc.

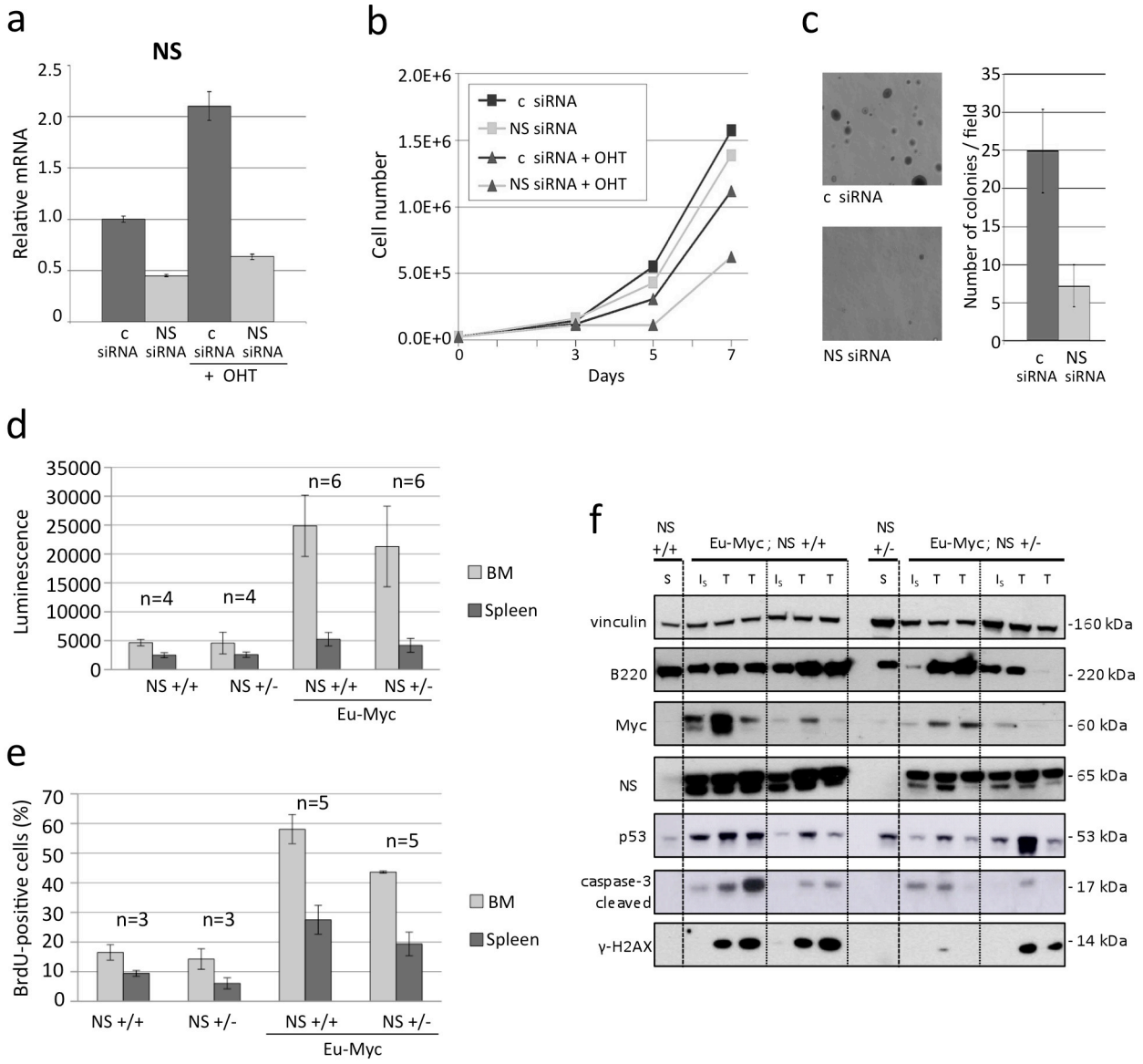



**Figure 2. NS haploinsufficiency delays c-Myc-induced B cell lymphoma**

(a) Kaplan-Meier curves showing the disease-free survival of Eμ-Myc mice of the indicated NS status. “n” designates the number of mice in each group (P<0.001, log rank test). (b) The graph represents the average life spans of NS+/+; Eμ-Myc and NS+/−; Eμ-Myc mice. (***; P<0.001).

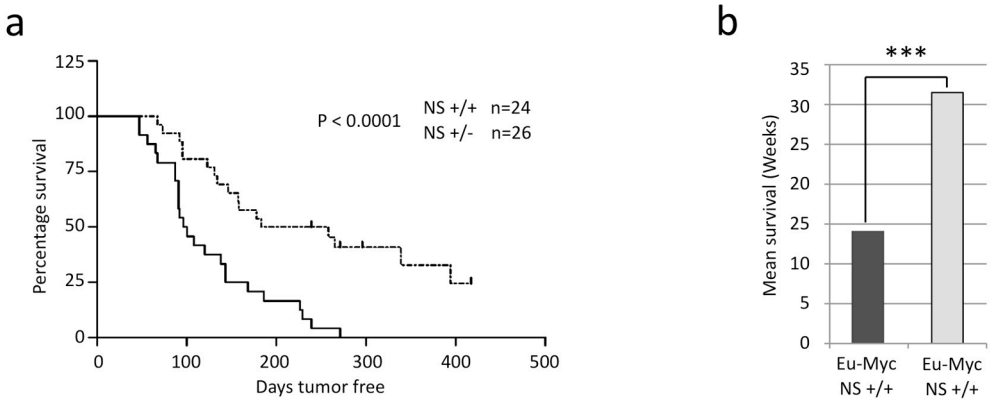
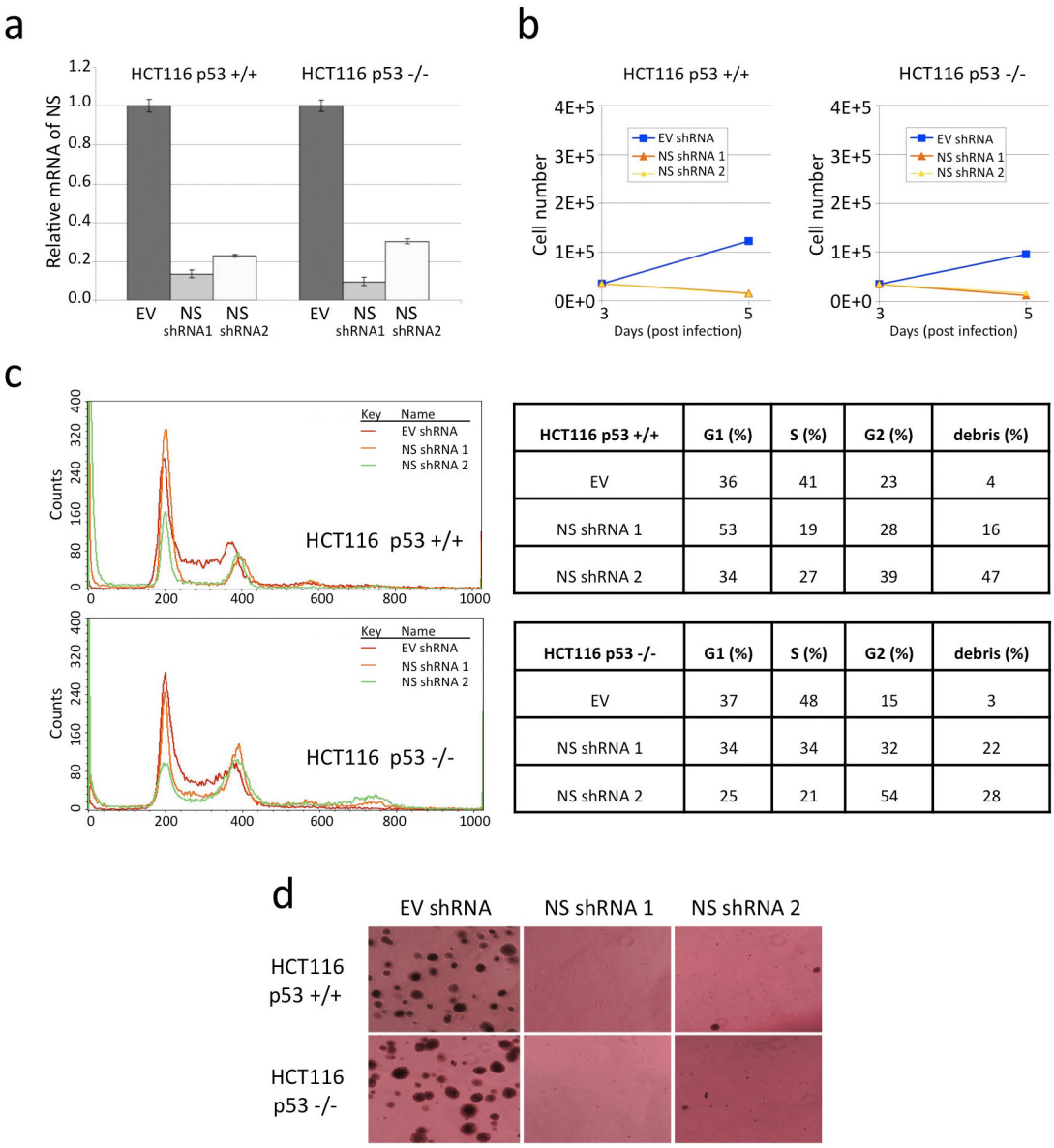



**Figure 3. NS is essential for c-Myc-induced proliferation**

(a) Knockdown of NS mRNA by RNA interference in Rat1A Myc/ER cells. Cells were transfected with either non-targeting control oligos (c siRNA) or siRNA oligos targeting NS mRNA (NS siRNA). Approximately 4 hours after the cells were seeded, c-Myc overexpression was induced by addition of 200 nM 4-hydroxytamoxifen (OHT). (b) Growth curves of the Rat1A Myc/ER cells after NS knockdown. Cells were transfected with either non-targeting control oligos (c siRNA) or siRNA oligos targeting NS mRNA (NS siRNA). 2 days after plating, c-Myc was induced in some cells were with addition of OHT (+OHT). (c) Soft agar growth after NS knockdown. Cells were transfected with either non-targeting control oligos (c siRNA) or siRNA oligos targeting NS mRNA (NS siRNA). (d) Flow-cytometric analysis of in vivo 5-bromodeoxyuridine (BrdU) incorporation in $B220^+$ cells from bone marrow (BM, gray bars) and spleen (black bars) isolated of 6–7 weeks-old mice of indicated genotypes. Data represent the mean (±SD) of biological replicates; “n” designates the number of animals in each group. (e) Apoptosis measured using Caspase-3/7 Glo luminescence assays (Promega). Pre-tumoural $B220^+$ cells were isolated from bone marrow (BM) and spleens of 6–7 weeks old mice of indicated genotypes. The graph shows average luminescence values from independent biological replicates (±SD). “n” designates the number of animals in each group. (f) Western blot of protein lysates from tumours and healthy or lymphoma-infiltrated spleens. S – healthy spleen, $I_S$ –lymphoma-infiltrated spleen, T – tumour.




| HCT116 p53 +/+ | G1 (%) | S (%) | G2 (%) | debris (%) |
|---|---|---|---|---|
| EV | 36 | 41 | 23 | 4 |
| NS shRNA 1 | 53 | 19 | 28 | 16 |
| NS shRNA 2 | 34 | 27 | 39 | 47 |

| HCT116 p53 -/- | G1 (%) | S (%) | G2 (%) | debris (%) |
|---|---|---|---|---|
| EV | 37 | 48 | 15 | 3 |
| NS shRNA 1 | 34 | 34 | 32 | 22 |
| NS shRNA 2 | 25 | 21 | 54 | 28 |

**Figure 4. NS depletion impedes cell cycle progression independently of p53**

(a) Knockdown of NS mRNA in HCT116 cells (p53+/+ or p53−/−), using lentiviral vectors expressing either NS shRNA1, NS shRNA2, or EV control. (b) Growth curves of HCT116 p53+/+ and p53−/− cells following NS knockdown. (c) Cell cycle analysis of HCT116 p53+/+ and p53−/− cells following NS knockdown. Left panel shows cell cycle profiles of HCT116 cells expressing either EV, NS shRNA1, or NS shRNA2. The right panel represents the distribution of the cells throughout the cell cycle as determined by the analysis of the cell cycle profiles. The upper section shows HCT116 p53+/+ cells, whereas the lower one the HCT116 p53−/− cells. (d) Soft agar growth assay of the HCT116 of indicated genotypes, expressing the indicated RNAs.

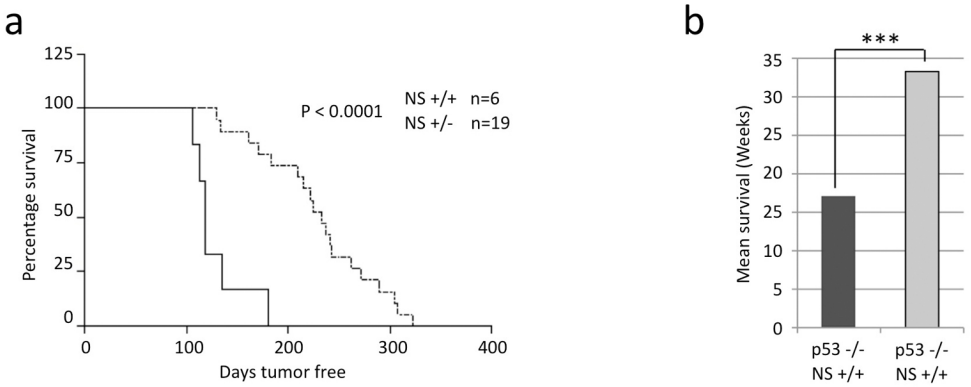



**Figure 5. NS haploinsufficiency delays tumourigenesis in absence of p53**

(a) Kaplan-Meier curve of p53-deficient transgenic mice (C57Bl/6 genetic background), WT or heterozygous for NS. “n” designates the number of mice in each group ($P<0.001$, log rank test). (b) The mean survival of animals of the indicated genotypes. (***; $P<0.001$).