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c-Myc Attenuates The Ability of Doxorubicin to Reduce The Colony Formation Partially Through Regulating Nbs1 in U2OS Cells*

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Abstract c-Myc is a ubiquitous transcription factor that regulates a wide variety of genes involved in the control of cell proliferation, differentiation and apoptosis. It was demonstrated that over-expressions of c-Myc and Nbs1 attenuated the ability of doxorubicin in reduction of colony formation in U2OS cells, and it was found that this effect of c-Myc was associated with Nbs1. It can be confirmed that *Nbs1* is a target gene of c-Myc. Chromatin immunoprecipitation assays reveal that c-Myc recruited the histone acetyltransferase p300 complex to the promoter region of *Nbs1* gene, resulting in an increased level of acetylated histone H4. Furthermore, it was found that the two proximal E-box elements located in *Nbs1* promoter region were essential for the c-Myc binding. Thus, it was concluded that c-Myc attenuates the effect of doxorubicin partially through regulating *Nbs1*, implicating its roles in repair of DNA breakage induced by doxorubicin.

Key words c-Myc, Nbs1, doxorubicin, colony formation, p300
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The c-Myc oncoprotein is a ubiquitous member of a small family of highly related DNA-binding transcription factors that regulates a wide range of genes involved in the control of cell proliferation, differentiation and apoptotic cell death. c-Myc binds to the E-box DNA elements (such as CACGTG, CATGTG) as a heterodimer with Max [1–4]. c-Myc is essential for embryonic development, and both c-Myc expression and activity are tightly regulated by mitogens and other physiological stimuli in normal somatic cells. Unregulated c-Myc expression has been implicated in most types of cancer in humans [4–5]. Moreover, c-Myc deregulation promotes genomic instability[6–7].

It has been reported that c-Myc-stimulated gene transcription *in vivo* correlates with an increased acetylation levels of both histones H3 and H4 at the vicinity of E-box elements within regulatory regions of c-Myc target genes [8–10]. Evidence also shows that c-Myc can recruit co-activator TIP60 complex and GCN5/PCAF-containing complex that possess the histone acetyltransferase (HAT) activity [11–12]. The transcriptional co-activator p300 and the cAMP response element binding protein (CREB)-binding protein (CBP) are the two highly conserved large nuclear phosphoproteins that contain HAT activity. These proteins co-activate a large number of transcription factors and remodel chromatin by acetylating nucleosomal histones. A previous study indicates that c-Myc can recruit p300 to the promoter of the human telomerase reverse transcriptase gene[13].

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Nbs1, the gene product mutated in Nijmegen breakage syndrome (NBS) patients, is the p95 component of the MRN complex (Mre11-Rad50-Nbs1) that forms foci at the sites of DNA DSBs (double strand breaks)[14]. MRN complex is an essential genome caretaker that regulates crucial steps of the DSB response such as DSB detection, activity of the ataxia telangiectasia mutated kinase (ATM, a key upstream component of DSB signaling), cell cycle checkpoints [15], and induction of apoptosis [16-17]. The MRN complex participates in the resection of DNA ends, an essential step required for an error-free DSB repair by homologous recombination[18]. Furthermore, a report suggests that c-Myc is linked to the physiological function of DNA DSB repair during DNA replication to preserve the integrity of the genome due to the direct regulation of *Nbs1* gene transcription by c-Myc[19].

Doxorubicin, an inhibitor of DNA topoisomerase Ⅱ, has been widely used as a chemotherapeutic agent for cancers. This drug can introduce double-strand breaks into DNA to trigger apoptosis in proliferating cells[20]. It has also been reported that doxorubicin reduces *in vitro* colony formation by inducing either apoptosis or senescence [21-22]. A number of proteins have been found to influence the effect of doxorubicin, such as p53, BRCA1, Bax and Bcl2[23-25]. Moreover, c-Myc is required for the induction of apoptosis by doxorubicin[26]. It has been well known that DNA damage and DNA repair mechanisms play critical roles in the sensitivity and resistance of tumor cells during and after anti-cancer drug treatment and irradiation[27]. However, whether c-Myc is also involved in the process of DNA repair upon doxorubicin treatment is unclear.

In this study, we demonstrate that c-Myc, as well as over-expression of Nbs1, attenuated the effect of doxorubicin. Moreover, Nbs1 was able to complement the function of c-Myc in restoration of the doxorubicin-reduced colony formation. We verify that c-Myc regulated Nbs1 expression by recruiting p300 to the Nbs1 promoter region in the vicinity of E-box elements. Based on these data, we propose that c-Myc plays a role in doxorubicin-reduced colony formation and in DNA repair in U2OS cells, partially through regulating *Nbs1* gene.

1 Materials and methods

1.1 Cells and culture

293T and U2OS cells were purchased from the Institute of Biochemistry and Cell Biology, The Chinese Academy of Sciences. Cells were maintained in IMDM, supplemented with 10% FBS, 100 mg/L penicillin and 100 mg/L streptomycin in a humidified atmosphere containing 5% CO_2 at 37℃.

1.2 The *Nbs1* promoter-reporter constructs, point mutation and luciferase assays

The *Nbs1* promoter fragment (–701 bp to +7 bp) ligated to the luciferase reporter gene in pGL3 basic (Promega), was generated by PCR from the human genomic DNA with the primers 5′ tcagagctcatg-gtgctgggcctaaac 3′, 5′ tatagatctcaggctgccttggatgag 3′ and the primers 5′ tcagagctcatggtgctgggcctaaac 3′, 5′ tatagatctcgcgctcccgggagccacgcaggctgccttggatgag 3′.

Constructs of the mutated *Nbs1* promoter for c-Myc binding study were generated by using an overlap extension PCR procedure [28]. The mutated sequences were designed as follows. c-Myc binding sites at –65, –439, and –493: –493 CAGGTG to CAGCTG; –439 CATGTG to CAGCTG; –65 CAGGTG to CAGGCA. All the constructs were sequenced to confirm the correct mutations before use.

Transient transfection of 293T cells were performed using the conventional calcium phosphate-DNA precipitation method. Transfected cells were analyzed for luciferase activity using a Promega dual-luciferase reporter assay system. Relative luciferase activity was detected by a Turner Designs TD20/20 Luminometer (Sunnyvale, CA). Firefly luciferase activity was normalized to the activity of the Renilla luciferase control. Extracts from at least 3 independent transfection experiments were assayed in triplicate. Results are shown as $\bar{x} \pm s$.

1.3 RT-PCR

Total RNA was extracted from U2OS cells according to the Promega Total RNA Isolation System manual. RNA(1 μg per sample) was reverse transcribed to cDNA in a total volume of 20 μl using a reverse transcriptase reaction kit (Promega). The resultant cDNA was diluted five fold with RNase-free water.

For detection of gene expression by PCR, the *Nbs1* primer pairs used were: sense 5′ gaaattgagttcc-gcagttgtc 3′ and antisense 5′ ggattctcatcttagccaaag 3′. The β-actin primer pairs were: sense 5′ tcgtgcgtga-cattaaggag 3′ and antisense 5′ atgccagggtacatggtggt 3′.

1.4 Western blot

U2OS cells were harvested after treatments. Cells were digested and lysed with lysis buffer for 30 min at 4℃. Total cell extracts were separated by

9% SDS-polyacrylamide gel electrophoresis (PAGE), then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-Nbs1 (Cell Signaling, 3002), anti-c-Myc (Santa Cruz, sc-764), anti-p300 (Santa Cruz, sc-565), or anti-β-actin (Sigma, A1978) antibodies, and then visualized by using the SuperSignal West Pico kit provided by Pierce Co. β-Actin was used as an internal control for normalizing the loading materials.

1.5 Colony forming assay

U2OS cells were plated at a density of 1×10^3 cells/well (for transiently transfected U2OS cells 1.5×10^3 cells/well were seeded) in six-well plates 2 days before treatment. Cells were treated with or without 150 nmol/L doxorubicin. Twenty four hours after treatment, cells were washed with D-Hanks and allowed to proliferate in new culture medium until control cells had grown into clearly visible colonies (2 ～3 weeks, depending on different cell lines and treatments). Plates were then stained with crystal violet, and colonies were counted and normalized to the number observed for controls (without drug). At least 2 plates of the same treatment were counted for each colony-forming assay, and the data represent experiments from at least 2 independent experiments. For accuracy, only colony-forming assays that gave greater than 50 colonies in control plates were selected for calculating an average.

1.6 Chromatin immunoprecipitation

The protocol for chromatin immunoprecipitation (ChIP) was described previously[29]. Briefly, cells were fixed and homogenized by sonication. The chromatin solution was pre-cleared with 50 μl of protein A-agarose beads (Upstate Biotechnology). The soluble fraction was collected and 5 μg of anti-acetyl-histone H3 (Upstate Biotechnology), anti-acetyl-histone H4 (Upstate Biotechnology), anti-c-Myc (Santa Cruz, sc-764) or anti-p300 (Santa Cruz, sc-565) antibodies was added. The precipitated samples were analyzed by PCR. The sequences of the primers used were: sense 5′ gtgggtggtgatgggttg 3′ antisense 5′ atgtagtttcgtgcgttt-gc 3′. The expected size of the PCR product is 304 bp, which fragment includes E-box elements located in –493 and –439.

1.7 Plasmids and transfection

c-Myc-pcDNA3.1 was constructed from the pSP271-Myc (kindly gifted from Eisenman R. N., Washington, USA). Nbs1-pcDNA3.1 was constructed from the Nbs1-pSG5 (kindly gifted from Jackson S. P., Cambridge UK). The wild type p300 (pCI-p300) were generously provided by Dr. Joan Boyes (Institute of Cancer Research, UK).

Short interference RNA (siRNA) targeting the sequence of c-Myc corresponds to the position 817 to 835 of the human *c-Myc* gene (GenBank accession NM 002467) [30]. The siRNA targeting the *Nbs1* sequence is 5′ ggcgtgtcagttgatgaaa 3′d(tt)[31]. The target sequence for p300 is 5′ tgacacaggcaggcttgac 3′[32]. The DNA fragments were synthesized and cloned into pSilencer 4.1-neo (Ambion) according to the recommended protocol. Transient transfection of U2OS cells was performed using the LipofectAMINE 2000 (Invitrogen) procedures.

2 Results

2.1 c-Myc attenuated the doxorubicin′s ability to reduce the colony formation in association with Nbs1

Doxorubicin can lead to DNA damage in cells. Colony forming assay in cells treated with doxorubicin is routinely used to assess the survival ability of cells to the DNA damage that can indirectly reflect the status for double strand DNA damage repair. To clarify the effects of c-Myc in DNA repair, we transiently transfected c-Myc siRNA and Nbs1 siRNA into U2OS cells, respectively. Colony forming assays showed that the survival ability of the cells after DNA damage was impaired when c-Myc or Nbs1 was knocked down in U2OS cells (Figure 1a). To further confirm the results, we generated the stably transfected cell lines that over-express c-Myc and Nbs1, respectively. The colony forming assays revealed that the survival ability after DNA damage was increased in the cells over-expressing c-Myc or Nbs1 (Figure 1b). These results suggest that c-Myc and Nbs1 can attenuate the effect of doxorubicin.

To address whether c-Myc affects the function of doxorubicin through Nbs1, we performed colony forming assay using both the stably and transiently transfected U2OS cells. The results of the c-Myc stably transfected cells showed that the colongenic survival of the cells was significantly decreased when endogenous Nbs1 was knocked down in the c-Myc over-expressing cells after DNA damage (Figure 1c). This indicates that the survival ability after DNA damage induced by doxorubicin treatment may be associated with Nbs1 in the c-Myc stably transfected cells. To further clarify whether Nbs1 facilitates c-Myc to enhance colongenic

survival after DNA damage, we performed transient transfection with plasmids of c-Myc siRNA, Nbs1, or c-Myc siRNA + Nbs1 in U2OS cells. The results showed that the reduction of the ability of colony formation upon c-Myc knockdown could be partially compensated by over-expression of Nbs1 (Figure 1d). Clearly, these data strongly suggest that the c-Myc function to attenuate the doxorubicin effect is associated with the participation of Nbs1.

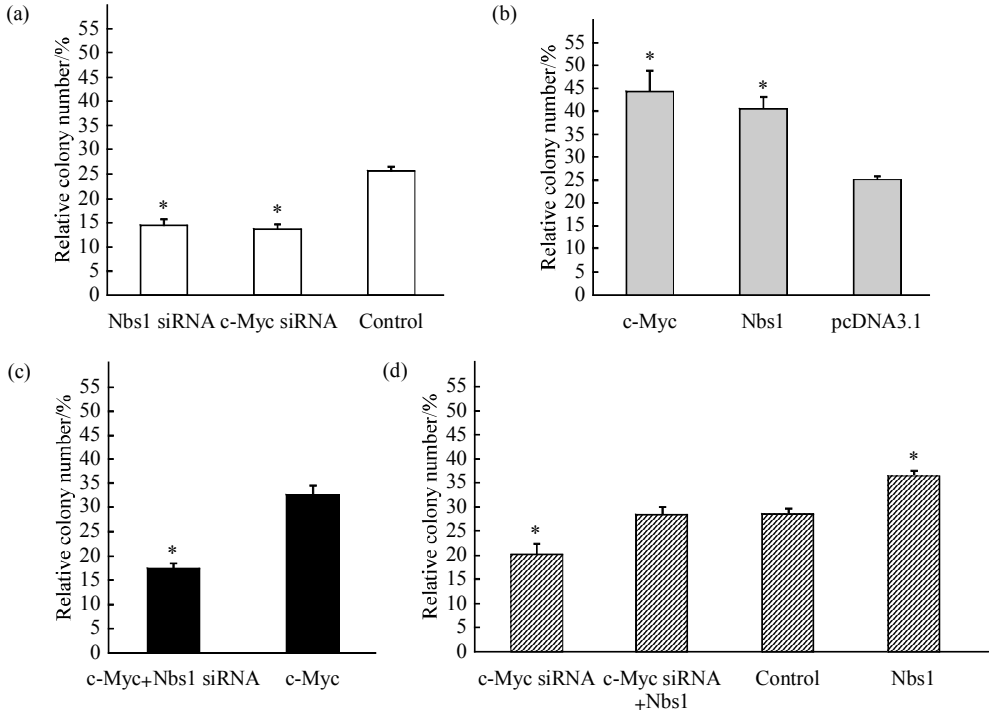

Fig. 1 c-Myc and Nbs1 attenuated the effect of doxorubicin

(a) Colony forming ability of U2OS cells in which either c-Myc or Nbs1 was knocked down. The pSilencer-4.1 empty plasmid was used as a negative control. Cells were treated with doxorubicin (150 nmol/L) for 24 h after transfection for 48 h. *$P < 0.01$ (n=3). (b) Colony forming assays for U2OS cells stably transfected with c-Myc or Nbs1, and pcDNA3.1 cell line was used as a control. Cells were treated with doxorubicin (150 nmol/L) for 24 h. *$P < 0.01$ (n=3). (c) The ability of colony formation was impaired when Nbs1 was knocked down with siRNA in cells over-expressing c-Myc. Cells were treated with doxorubicin at 150 nmol/L for 24 h after transfection for 48 h. *$P < 0.01$ (n=3). (d) Nbs1 partially compensated the ability of colony formation impaired by DNA damage after doxorubicin treatment. pcDNA 3.1 empty plasmid was used as negative control. *$P < 0.01$ (n=3).

2.2 c-Myc and p300 up-regulated *Nbs1* expression *in vivo*

Next, we intended to validate whether c-Myc truly regulates the expression of *Nbs1*. We first demonstrate that both the Nbs1 protein and mRNA levels were increased remarkably in the cells stably transfected with c-Myc plasmid, compared with the pcDNA3.1 control cells (Figure 2a, b), implicating the association of Nbs1 expression with c-Myc function. We then transiently transfected p300, p300 + c-Myc, and pcDNA3.1 (control) plasmids into U2OS cells, respectively, and found that p300 up-regulated Nbs1; and co-transfection of both p300 and c-Myc up-regulated Nbs1 expression at a much higher level than p300 alone (Figure 2c, d). To further test whether *Nbs1* is regulated by endogenous c-Myc and p300, we used RNA interference (RNAi) to knockdown endogenous c-Myc or p300 in U2OS cells, and we found that both Nbs1 protein and mRNA expression levels were decreased significantly upon c-Myc siRNA or p300 siRNA transfection (Figure 2e, f). These gene silencing experiments provide further evidence that *Nbs1* expression was regulated by endogenous c-Myc and p300.

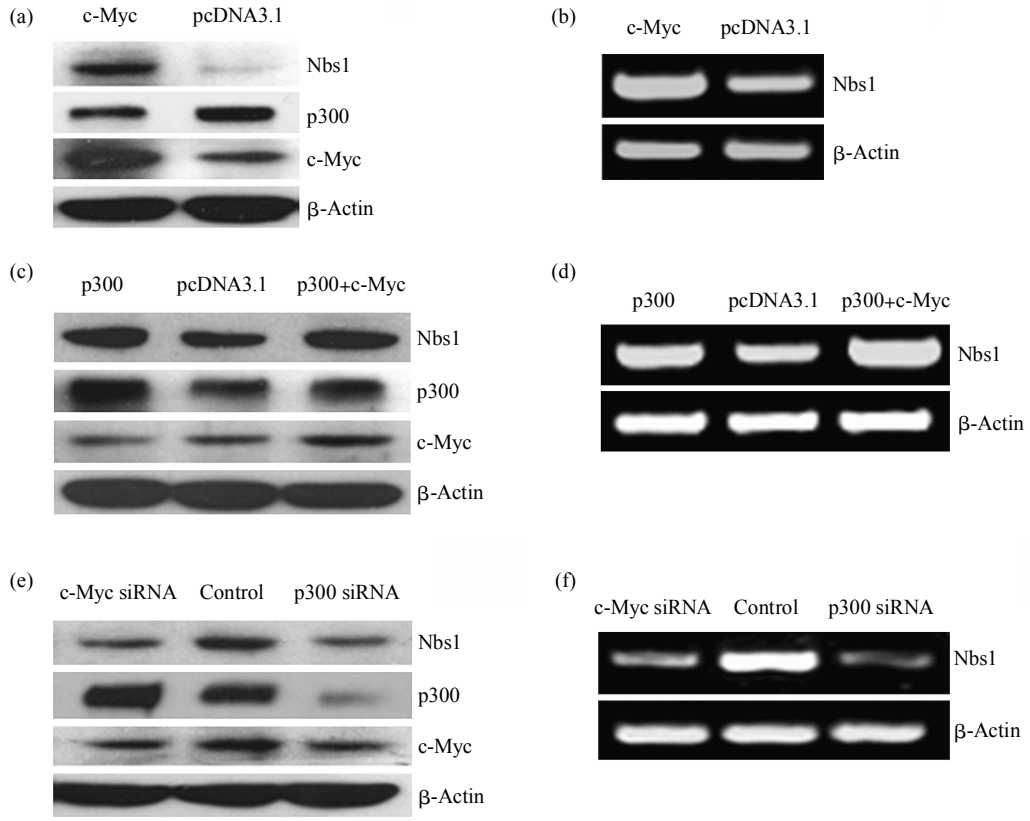

Fig. 2　c-Myc regulated Nbs1 protein and RNA expression in U2OS cells

(a) Nbs1 protein level in the stable c-Myc-transfected cells. Western blots: The stable pcDNA3.1 transfection cells was used as the control. (b) *Nbs1* mRNA level in stable c-Myc-transfected cells. RT-PCR: the stable pcDNA3.1-transfected cells was used as the control. (c) Nbs1 protein level in cells over-expressing p300 or p300+c-Myc. Western blots: Cells were transiently transfected with plasmids p300, p300+c-Myc, or pcDNA3.1 (control). (d) *Nbs1* mRNA level in cells transiently transfected as described in (c). (e) Nbs1 protein level in cells in which p300 or c-Myc was knocked down. The empty pSilencer-4.1 plasmid was used as the control. (f) *Nbs1* mRNA level in cells in which p300 or c-Myc was knocked down.

2.3　c-Myc and p300 were present at *Nbs1* promoter, and activation of *Nbs1* by c-Myc required the E-boxes in *Nbs1* promoter

To ascertain whether c-Myc up-regulates *Nbs1* expression at the transcriptional level, we first examined whether the *Nbs1* promoter region contains any c-Myc binding sites. We cloned and analyzed the *Nbs1* promoter (–701 bp to +7 bp), and detected three E-boxes (–439: CATGTG[3-4]; –65 and –493: CAGGTG[33]) as putative c-Myc binding sites (Figure 3a). We then transiently transfected the plasmids of c-Myc, p300 and c-Myc+p300, respectively, together with the *Nbs1* promoter-reporter, into 293T cells for luciferase reporter assay. The results showed that p300 and c-Myc were able to up-regulate the activity of *Nbs1* promoter, and c-Myc could co-operate with p300 to promote the transcription activity of the *Nbs1* promoter (Figure 3b). To determine whether the putative c-Myc binding sites are truly responsible for c-Myc-mediated activation, point mutations were introduced into the E-boxes at –493 (CAGGTG to CAGCTG), –439 (CATGTG to CAGCTG) and –65 (CAGGTG to CAGGCA), respectively. The wild type promoter construct and the various mutant constructs were transiently transfected into 293T cells, and the luciferase activity was measured after the transfection. The point mutation experiments demonstrated that the mutation at –493 remarkably decreased the *Nbs1* promoter activity; while the –439 mutant also exhibited certain extents of decrease in *Nbs1* promoter activity in cells transfected with c-Myc or p300 (Figure 3c). Meanwhile, the –65 mutant did not affect the *Nbs1*

promoter activity (Figure 3c). Thus, these data indicate that the E-box elements located around –493 and –439 are the c-Myc binding sites for c-Myc to activate *Nbs1* transcription. Moreover, the results also imply that the E-box elements may be the sites where c-Myc recruits p300 to the *Nbs1* promoter.

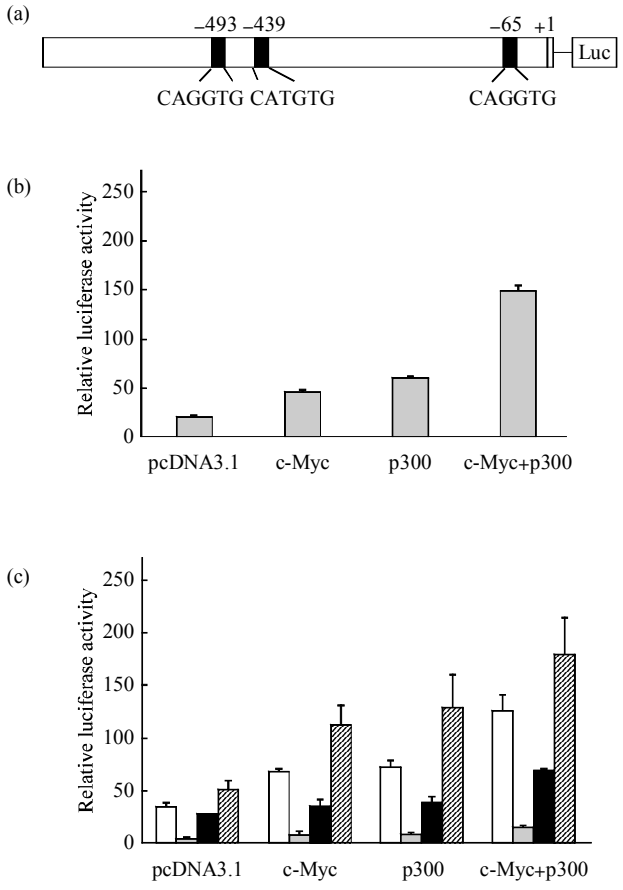

Fig. 3 Determination of c-Myc binding sites at *Nbs1* promoter and the transcriptional activation of *Nbs1* promoter by c-Myc and p300

(a) Schematic diagram of human *Nbs1* promoter reporter and the putative binding sites of c-Myc. (b) Activation of *Nbs1* promoter by c-Myc, p300 and c-Myc + p300. (c) Point mutations of the putative E-boxes affected the transcriptional activation of *Nbs1* promoter. □: wt; ▨: –493mut; ■: –439mut; ▨: –65mut.

2.4 c-Myc and p300 were present at the *Nbs1* promoter region resulting in alteration of the histone acetylation status of the promoter *in vivo*

To establish whether c-Myc actually binds to the E-box elements in *Nbs1* promoter, and whether c-Myc can recruit p300 to the vicinity of E-box elements *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays with anti-c-Myc, anti-p300, anti-Ac-H3 or anti-Ac-H4 antibodies. Because the E-box situated around –493 is very close to the one around –439 at the *Nbs1* promoter, the primers used in our ChIP experiments will amplify a 304 bp fragment containing both E-box elements. The ChIP assays showed that over-expression of c-Myc increased the yield of the 304 bp promoter fragments immunoprecipitated by anti-c-Myc antibody (Figure 4a), indicating that these two putative E-box elements were bound by c-Myc *in vivo*. Meanwhile, the amount of fragment precipitated by anti-p300 antibody was also increased upon c-Myc over-expression (Figure 4b), implicating that p300 was recruited to the vicinity region of the E-box elements by c-Myc. To investigate whether p300 remodels chromatin by acetylating nucleosomal histones in *Nbs1* gene, we assessed the acetylation status of the nucleosomal histones H3 and H4 at the *Nbs1* promoter region. The results showed that over-expression of c-Myc remarkably enhanced the H4 acetylation level, whereas the acetylation status of H3 remained unaffected (Figure 4c, d). Collectively, these data indicate that c-Myc may recruit p300 to the *Nbs1* promoter at the E-box elements to remodel the chromatin by acetylating histone H4.

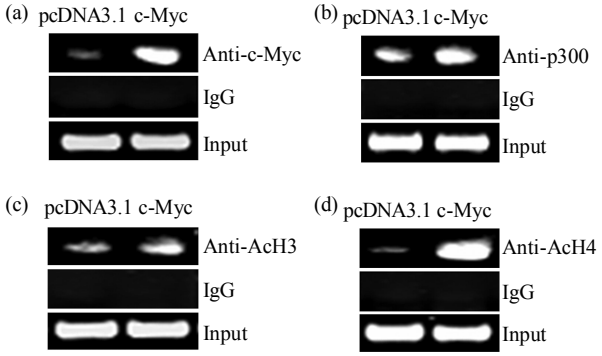

Fig. 4 c-Myc recruited p300 to *Nbs1* promoter to enhance H4 acetylation

(a) The binding of c-Myc to the E-box elements at *Nbs1* promoter. (b) p300 was recruited to the E-box elements by c-Myc. (c) The acetylation of H3 was not altered. (d) The H4 acetylation level was enhanced significantly. ChIP assays were performed in cells stably transfected with c-Myc or pcDNA3.1. The amounts of precipitated endogenous *Nbs1* promoter DNA fragments were determined by PCR and visualized with gel electrophoresis.

3 Discussion

Doxorubicin reduces colony formation by inducing apoptosis and senescence [21-22]. Doxorubicin induced double-strand breaks in DNA, which initiate the DNA damage checkpoints. One of the key proteins in these pathways is the tumor suppressor p53, which triggers cell cycle arrest and induces DNA damage repair, and such responses are needed for cell survival, or alternatively, for apoptosis [21]. Our results in this report show that over-expression of c-Myc attenuated the effect of doxorubicin in reducing the colony formation in U2OS cells (Figure 1a, b). This indicates that the apoptosis or senescence induced by DNA damage is probably decreased by the introduction of c-Myc. Thus, the process in which c-Myc attenuates doxorubicin's effect on colony formation may be linked to DNA damage repair.

Moreover, we found that Nbs1 was also able to attenuate the effect of doxorubicin to reduce the colony formation (Figure 1a, b). Nbs1 is the p95 component of the MRN complex, which is an essential genome caretaker that regulates crucial steps of the DSB response such as DSB detection, activity of ATM, and cell cycle checkpoints [15]. The MRN complex participates in the resection of DNA ends, an essential step required for an error-free DSB repair by homologous recombination [18]. Since c-Myc is a transcription factor that regulates a wide range of genes, it is possible that c-Myc participates in DNA damage repair through regulating the DNA repair-related genes. Some authors reported that certain DNA repair-related genes were constitutively up-regulated in c-Myc transgenic mouse livers compared with wild-type controls [34]. Chiang *et al.* [19] reported that c-Myc regulated *Nbs1* expression and they identified a c-Myc binding site (E-box) in the intron 1 of *Nbs1* gene. Here we found that c-Myc could target *Nbs1* promoter to regulate its transcription (Figure 2～4), and attenuate the effect of doxorubicin on colony formation partially through Nbs1(Figure 1c, d). These data further confirm that c-Myc may facilitate DNA repair.

It has been known that c-Myc-stimulated gene transcription *in vivo* correlates with an increased acetylation of both histones H3 and H4 in the vicinity of E-box elements within regulatory regions of Myc target genes [8-10]. The transcriptional co-activators p300/CBP co-activate a large number of transcription factors and remodel chromatin by acetylating nucleosomal histones. A previous study revealed that p300 may function as a co-activator that was recruited by c-Myc to the promoter of the human telomerase reverse transcriptase gene [13]. Here, our results demonstrate that p300 regulated *Nbs1* transcription (Figure 2c, d, e, f), and c-Myc recruited p300 to the vicinity region of the E-box elements to remodel the chromatin by acetylating H4 at the vicinity region of the E-box elements. (Figure 4a, b, d).

Together, data presented in this report establish that c-Myc can attenuate the potential of doxorubicin in reduction of colony formation in U2OS cells partially through regulating the expression of *Nbs1*, implicating the roles of c-Myc in control of DNA damage repair through Nbs1.

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c-Myc 部分通过调控 Nbs1 减弱阿霉素降低 U2OS 细胞集落形成的能力 *

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摘要　c-Myc 是一种泛在性的转录因子，它调控着许多涉及细胞增殖、分化、凋亡等生命活动的基因．实验结果表明在骨肉瘤细胞 U2OS 中过表达 c-Myc 和 Nbs1 都能减弱阿霉素降低集落形成的能力．进一步的实验证实 c-Myc 的这种作用与 Nbs1 有关，*Nbs1* 是 c-Myc 的靶基因．染色质免疫沉淀实验显示，c-Myc 招募组蛋白乙酰化酶 p300 复合物到 *Nbs1* 启动子区，引起了组蛋白 H4 的乙酰化，定位在 *Nbs1* 启动子区的相邻的两个 E-box 对 c-Myc 的结合是必要的．上述结果说明 c-Myc 减弱阿霉素的作用部分是通过调控 Nbs1 来实现的．这也提示了 c-Myc 在阿霉素诱导的 DNA 损伤修复中起作用．

关键词　c-Myc，Nbs1，阿霉素，集落形成，p300

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