

Np95 is regulated by E1A during mitotic reactivation of terminally differentiated cells and is essential for S phase entry

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Terminal differentiation exerts a remarkably tight control on cell proliferation. However, the oncogenic products of DNA tumor viruses, such as adenovirus E1A, can force postmitotic cells to proliferate, thus representing a powerful tool to study progression into S phase. In this study, we identified the gene encoding Np95, a murine nuclear phosphoprotein, as an early target of E1A-induced transcriptional events. In terminally differentiated (TD) cells, the activation of Np95 was specifically induced by E1A, but not by overexpression of E2F-1 or of the cyclin E (cycE)–cyclin-dependent kinase 2 (cdk2) complex. In addition, the concomitant expression of Np95 and of cycE–cdk2 was alone sufficient to induce S phase in TD cells. In NIH-3T3

cells, the expression of Np95 was tightly regulated during the cell cycle, and its functional ablation resulted in abrogation of DNA synthesis. Thus, expression of Np95 is essential for S phase entry. Previous evidence suggested that E1A, in addition to its well characterized effects on the pRb/E2F-1 pathway, activates a parallel and complementary pathway that is also required for the reentry in S phase of TD cells (Tiainen, M., D. Spitkousky, P. Jansen-Dürr, A. Sacchi, and M. Crescenzi. 1996. *Mol. Cell. Biol.* 16:5302–5312). From our results, Np95 appears to possess all the characteristics to represent the first molecular determinant identified in this pathway.

Introduction

Terminal differentiation of a cell is marked by its functional specialization and irreversible loss of proliferation ability. Skeletal muscle cells, such as C2C12, are a well characterized model system to study this process. In vitro, myoblasts deprived of growth factors enter an irreversible postmitotic state and activate the expression of muscle-specific genes, thus becoming myocytes. Myocytes, in turn, can fuse into multinucleated, syncytial structures called myotubes (Okazaki and Holtzer, 1966). Terminally differentiated (TD)* myotubes can be induced to enter the cell cycle by growth factors. However,

they cannot progress beyond a mid-G₁ block, and hence do not enter the S phase (Tiainen et al., 1996a). Even forced expression of cell cycle regulators, such as E2Fs or the cyclin E (cycE)–cyclin-dependent kinase 2 (cdk2) complex, is unable to trigger DNA synthesis in TD muscle cells (Puri et al., 1998; Pajalunga et al., 1999; Latella et al., 2001). Conversely, early gene products of DNA tumor viruses, such as adenovirus E1A, are able to stimulate S phase entry of quiescent or TD cells (Crescenzi et al., 1995; Tiainen et al., 1996b). Thus, they constitute a powerful tool to study how postmitotic cells can be forced to proliferate, an issue of great relevance to the understanding of human cancer.

The E1A region of human adenovirus 5 produces two major mRNAs, 12S and 13S, which encode nuclear proteins of 243 and 289 amino acids, respectively. The 12S variant of E1A (hereafter referred to as E1A) is sufficient to promote cell cycle reentry and S phase in TD myotubes (Tiainen et al., 1996b) by a mechanism that is only partially elucidated. E1A displays

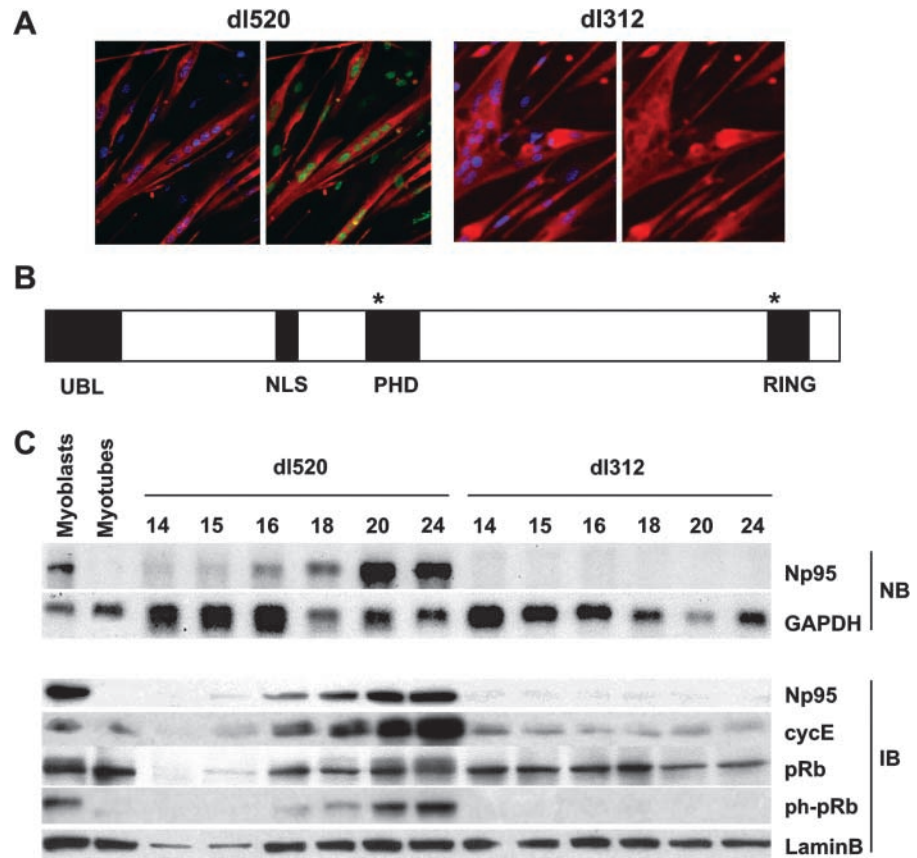
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*Abbreviations used in this paper: cdk2, cyclin-dependent kinase 2; cycE, cyclin E; MOI, multiplicity of infection; pfu, plaque-forming unit; pRb, retinoblastoma protein; TD, terminally differentiated.

Key words: Np95; cell cycle; E1A; pRb; cycE–cdk2

Figure 1. Np95 is an early E1A-induced gene.

(A) TD myotubes were infected with either *dl520* (left, MOI, 400 pfu/cell) or *dl312* as a control (right, MOI, 400 pfu/cell). 24 h later, cells were treated with BrdU. 48 h after infection, cells were fixed and stained with anti-myosin heavy chain (red) to check differentiation and anti-BrdU (green) antibodies. Nuclear counterstaining was performed with DAPI (blue). (B) Schematic of mouse Np95; the various domains of the protein are indicated (Ubl, ubiquitin-like; NLS, nuclear localization signal). Two putative pRb-binding motifs, LxCxE and IxCxE (Dahiya et al., 2000), are indicated by asterisks. (C) TD myotubes were infected with either *dl520* (MOI, 400 pfu/cell) or *dl312* (MOI, 400 pfu/cell), and mRNA or proteins were harvested at the indicated time points. RNAs (2 μ g Poly(A)⁺, top) were analyzed in Northern blot (NB) with the indicated probes. Proteins (40 μ g of total cellular lysates, bottom) were immunoblotted (IB) with the indicated antibodies. pRb and ph-pRb are a pan-anti-pRb and a specific anti-phosphoRb, respectively (see Materials and methods). The levels of Np95 before (myoblasts) and after (myotubes) differentiation are also shown.



multiple interactions with key components of cellular growth-regulatory pathways, including retinoblastoma protein (pRb)-family proteins (the so-called pocket proteins) and the transcriptional coactivators p300 and CBP (Arany et al., 1994, 1995). Although these interactions are required for the ability of E1A to immortalize and transform cells (Eckner, 1996), its capacity to reactivate the cell cycle segregates with binding to pocket proteins, whereas binding to p300 is not required (Stein et al., 1990; Tiainen et al., 1996b; Puri et al., 1997).

Binding of E1A to pocket proteins releases transcription factors of the E2F family (Nevins, 1990) that are master regulators of the G₁/S transition and are able to induce DNA synthesis in a variety of quiescent, non-TD cells (Johnson et al., 1993; Lukas et al., 1996). E2Fs act in part by transcriptionally up-regulating the kinase activity of the cycE-cdk2 complex (Müller and Helin, 2000). However, neither the overexpression of several E2Fs nor that of cycE-cdk2 could force the re-entry into S phase of TD myotubes (Pajalunga et al., 1999; Latella et al., 2001). Thus, E1A must be activating complementary pathways that contribute to the G₁/S transition of TD myotubes. An investigation of such pathways is warranted by both their central role in the control of cell proliferation and their possible alterations in neoplastic cells. The present studies were undertaken in an attempt to elucidate this issue.

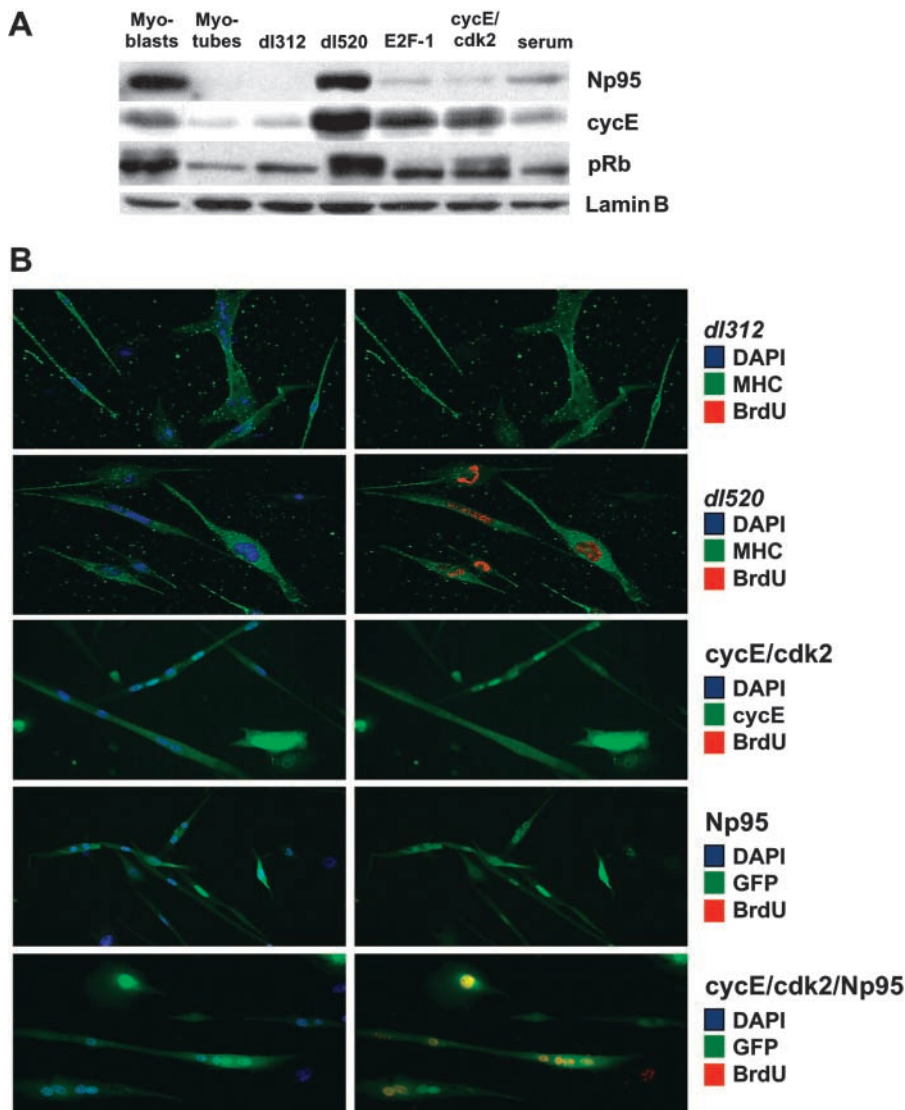
Results and discussion

Np95 is induced early in the reentry of TD myotubes in the cell cycle

To gain insight into the molecular mechanisms leading to reactivation of TD cells, we used a cDNA subtraction ap-

proach to identify genes induced by E1A in TD myotubes. TD C2C12 mouse myotubes were infected with either the adenovirus *dl520*, which expresses only the 12S E1A mRNA, or the control adenovirus *dl312*, in which the E1A gene is deleted (Jones and Shenk, 1979). The efficiency of S phase re-entry, measured 48 h after *dl520* infection, was ~70–80% (Fig. 1 A). To select for genes induced by E1A at early stages, mRNAs from infected cells were harvested at 14, 15, 16, 18, 20, and 24 h after infection. These time points precede (by at least 6 h) the onset of DNA replication (occurring ~30 h after infection). Pooled mRNAs were used for the cDNA subtraction procedures (see Materials and methods).

Among the genes induced by E1A, we isolated Np95, which encodes a previously described murine nuclear phosphoprotein, and whose expression is cell cycle regulated (Fujimori et al., 1998; Uemura et al., 2000; Miura et al., 2001; Fig. 1 B). Fig. 1 C shows the mRNA and protein expression of Np95, before and after terminal differentiation of C2C12 cells, and after their reentry in the cell cycle induced by infection with *dl520*. The expression of Np95 was virtually abolished by terminal differentiation, both at the mRNA and protein levels (Fig. 1 C, compare Myoblasts with Myotubes), and reinduced by infection with *dl520*, but not with *dl312*. Np95 mRNA and protein started to be detectable ~15–16 h after infection, and continued to increase until the last time points were checked (20–24 h after infection; Fig. 1 C). Because the expression of the E1A mRNA was first detectable at 15 h after infection (unpublished data), as also previously reported (Tiainen et al., 1996a), we concluded that Np95 is an early E1A-induced gene.



Np95 is specifically induced by E1A and complements cycE-cdk2 in the induction of reentry in the cell cycle of TD myotubes

To establish a role for Np95 in the sequence of events activated by E1A, we performed a series of experiments. First, we investigated the induction of Np95 by different stimuli. As shown in Fig. 2 A, overexpression of E1A (*dl520* lane) potentially induced the expression of Np95. Stimulation with serum, or overexpression of either E2F-1 or of cycE-cdk2, only exerted minute effects (Fig. 2 A), possibly attributable to a small percentage of myoblasts contaminating the myotube cultures. As a control, we checked the levels of cycE and the phosphorylation status of pRb. As expected, both E1A and E2F-1 induced the expression of cycE (Tiainen et al., 1996b; Pajalunga et al., 1999), whereas both E1A and cycE-cdk2, but not E2F-1, induced hyperphosphorylation of pRb (Tiainen et al., 1996b; Pajalunga et al., 1999; Mal et al., 2000; Latella et al., 2001; Fig. 2 A). Thus, in TD myotubes Np95 expression depends essentially on the activation by E1A of pathways besides those relying on the stimulation of the activity of the cycE-cdk2 complex or the presence of high levels of free E2F-1.

Figure 2. Role of Np95 in the reentry in the cell cycle of TD myotubes. (A) TD myotubes were infected with *dl520* (MOI, 400 pfu/cell), *dl312* (MOI, 400 pfu/cell), or adenoviral vectors encoding E2F-1 (MOI, 400 pfu/cell), cycE (MOI, 100 pfu/cell)/cdk2 (MOI, 400 pfu/cell), or stimulated with 5% FCS, as indicated on top. 48 h after infection, total cellular lysates were extracted and immunoblotted (40 μ g) with the indicated antibodies. Lanes myoblasts and myotubes are as in Fig. 1 C. (B) TD myotubes were infected with adenoviral vectors, as indicated on the right. MOIs were as follows: cycE, 100 pfu/cell; cdk2, 400 pfu/cell; and Np95, 60 pfu/cell. 24 h later, cells were treated with BrdU. 48 h after infection, cells were fixed and stained with anti-BrdU (red), and either anti-myosin heavy chain (green, top two rows, MHC), or anti-cycE (green, middle row) antibodies, or detected by epifluorescence (green, bottom two rows, to detect GFP-Np95). Nuclear counterstaining was performed with DAPI (blue). For each condition (horizontally paired panels), two pictures of the same microscopic field are shown. All left panels display the blue (DAPI) and green (either MHC, or cycE, or GFP) channels. All right panels display the green (either MHC, or cycE, or GFP) and red (BrdU) channels. Stainings are also indicated by a color code (squares).

E1A reactivates proliferation of TD muscle cells by a mechanism that includes release of E2Fs and activation of the cycE-cdk2 complex, but also requires other pathway(s) that are presently not molecularly defined (Tiainen et al., 1996b; Latella et al., 2001). From these results, Np95 appears to possess the requisites to be part of such a pathway. If so, its overexpression might complement that of cycE-cdk2 toward induction of the S phase in TD myotubes. Thus, we coexpressed Np95, cycE and cdk2 in C2C12 myotubes, through adenoviral vectors. When the three proteins were coexpressed, \sim 20% of the myotubes entered S phase within 60 h after infection (Fig. 2 B and Table I). Conversely, no reentry in the cell cycle was induced by expression of Np95 alone, by the cycE-cdk2 combination, or several other negative controls (Fig. 2 B and Table I).

Np95 is essential for entry into S phase

Taken together, the previous results suggest that Np95 could be a critical effector in the progression of a cell through the cycle. The fact that Np95 cooperates with cycE-cdk2 to overcome the G₁ block in TD cells further suggests that its action might be exerted physiologically at the G₁/S transition. To address this question, we switched to NIH-3T3 cells in which functional

Table 1. Np95 cooperates with *cycE-cdk2* to induce S phase entry of TD myotubes

Infection ^a	BrdU positive cells ^b
	%
dl520	78
dl312	<0.3
<i>cycE-cdk2</i>	<0.3
<i>cycE-cdk2/TRACK</i>	<0.3
Np95	<0.3
<i>cycE-cdk2/Np95</i>	19.4

^aAdenoviral vectors encoding *cycE*, *cdk2*, and Np95 were used at an MOI of 100, 400, and 60, respectively. Optimal MOIs were established in preliminary experiments (unpublished data). TRACK is a control empty adenovirus used at an MOI of 120. 24 h after infection, cells were treated with BrdU and fixed 24 h later. TD myotubes were identified by staining with anti-myosin heavy chain.

^b350 myotubes per plate were counted and scored for BrdU positivity. The experiment shown is typical and representative of three experiments.

ablation of the expression of a protein could be more easily achieved. In these cells, the expression of Np95 is known to be cell cycle regulated (Uemura et al., 2000; Miura et al., 2001). Indeed, in serum-starved NIH-3T3 cells, Np95 was undetectable (Fig. 3 A). Its expression started to be clearly detectable after 9 h of restimulation with serum, i.e., 6 h before the onset of S phase (Fig. 3 B). These results mirrored those obtained in TD myotubes stimulated with E1A, and suggested the reactivation of Np95 occurs by similar mechanisms in cells that display either a reversible or an irreversible cell cycle arrest.

Next, we attempted functional ablation of Np95, by an antisense approach. Serum-starved NIH-3T3 cells were microinjected with a morpholino-modified antisense oligonucleotide of 25 bp that matched the sequence of Np95 and overlapped its ATG. As a control, we microinjected a similar oligonucleotide bearing four mismatched bases, including one in the start codon (see Materials and methods). Serum and BrdU were then added to the cells that were fixed 24 h later. As shown in Fig. 4 A, the expression of Np95 and BrdU incorporation was dramatically reduced in cells microinjected with the antisense oligonucleotide, but not with the mismatched control. Around 80% of the cells microinjected with the antisense oligonucleotide were inhibited in their progression into S phase (Fig. 4 B), thus demonstrating that Np95 is essential for the progression through the cell cycle.

Np95: an oncogene candidate?

Terminal differentiation exerts a remarkably tight control on cell proliferation. Because TD cells constitute the majority in an adult mammal, the understanding of how they can be forced to reenter the cell cycle might be very relevant to human cancer. TD myotubes are extremely refractory to the mitogenic action of several oncogenes and growth-promoting inducers (Tiainen et al., 1996b; Latella et al., 2001), but sensitive to the action of E1A, and therefore represents an invaluable tool to dissect critical mechanisms in cell cycle progression. Previous evidence suggests that E1A, in addition to its well characterized effects on the pRb/E2F-1 pathway, activates a parallel and complementary pathway that is also required for the reentry in S phase (Tiainen et al., 1996b; Alevizopoulos et al., 1998, 2000; Latella et al., 2001). In this study, we show that Np95 has all the characteristics to be the first molecular determinant identified in this pathway. Np95 appears to constitute a critical

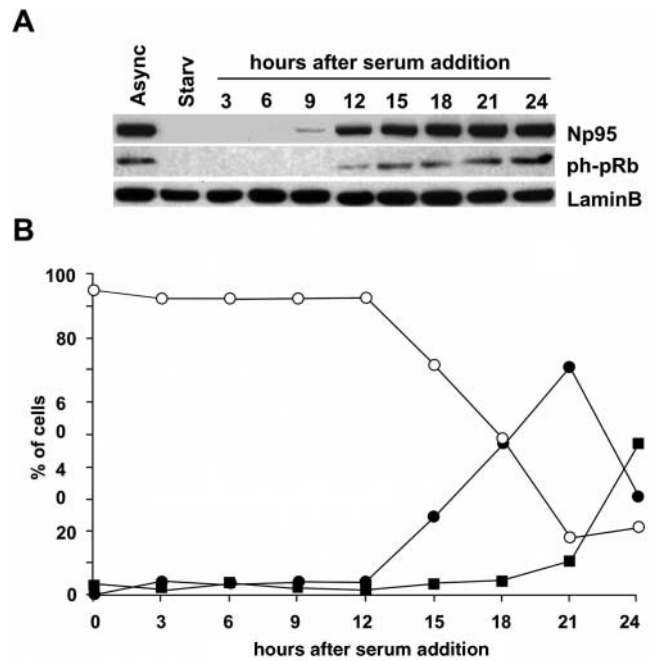


Figure 3. Cell cycle-dependent regulation of Np95 in NIH-3T3 cells. (A) NIH-3T3 cells were serum-starved for 48 h, and then stimulated with 10% FCS for the indicated time points. (A) Immunoblots (40 μ g of total proteins) with the indicated antibodies. The levels of Np95 in serum-starved cells (Starv) or in an asynchronously growing population (Async) are also shown. (B) FACS[®] profiles: open circles, G0/G1; closed circles, S; squares, G2/M.

effector of this pathway, because it can complement the overexpression of *cycE-cdk2* toward stimulation of S phase in TD myotubes. In addition, the function of Np95 is physiologically required for progression through the cell cycle, as witnessed by its tight regulation and by the dramatic effects of its functional ablation in NIH-3T3 cells.

Many important questions remain to be addressed. How is the transcription of Np95 regulated under physiological conditions? What is the physiological function of Np95? How does it signal downstream? With regard to the first question, we note that overexpression of *cycD-cdk4* was recently shown to induce S phase entry of TD myotubes (Latella et al., 2001). Thus, it is possible that Np95 is a transcriptional target of a pathway that includes the kinase activity of this complex, a possibility that we are currently testing.

As far as the function of Np95 is concerned, a human gene (*ICBP90*) that displays remarkable similarity to mouse Np95 (74% identity and 84% overall similarity at the amino acid level), was recently isolated in a one-hybrid system as an inverted CCAAT box-interacting protein of the topoisomerase II α promoter (Hopfner et al., 2000), and shown to be involved in the transcriptional control of this gene. Although it remains to be established whether *ICBP90* and Np95 are orthologues, our results suggest the possibility that Np95 acts in transcriptional control. In addition, the presence in Np95 of many structural and functional domains, including a ubiquitin-like domain, a PHD finger, pRb-binding motifs, and a RING domain, suggests that Np95 might control several protein-protein interactions and enzymatic activities required for S phase entry. The absolute requirement for Np95 in this critical phase of the cell

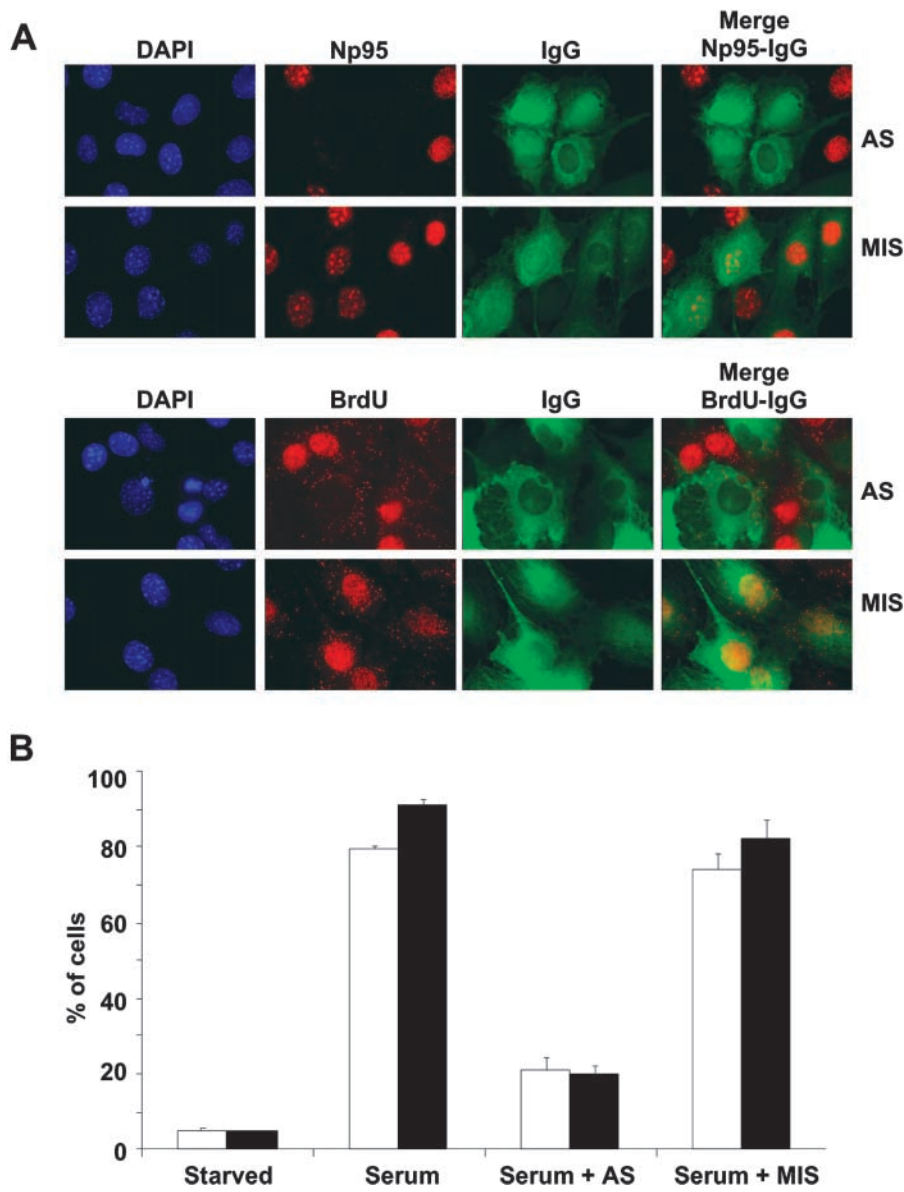


Figure 4. Functional ablation of Np95 in NIH-3T3 cells. (A) NIH-3T3 were serum starved for 48 h, and then microinjected with the Np95 antisense (AS) oligonucleotide or its mismatched (MIS) control. IgGs were also microinjected to identify microinjected cells. BrdU and 10% FCS were then added, and cells were fixed 24 h later and stained as indicated. Nuclear counterstaining was performed with DAPI. (B) A quantitative assessment was performed on three independent experiments, in which at least 150 microinjected cells per experiment were counted. Results are shown as a percentage of microinjected cells (\pm SD) that were Np95-positive (solid bars) or BrdU-positive (empty bars).

cycle warrants further investigations on its possible role in malignant transformation.

Materials and methods

Cells and microinjection procedures

C2C12 myoblasts (Yaffe and Saxel, 1977) were cultured in collagen-coated dishes in DME supplemented with 10% FBS. Differentiation was induced by serum deprivation for 72 h (Tiainen et al., 1996b). Unless otherwise stated, 50 mM 1- β -D-arabinofuranosylcytosine (Ara-C) was added during the first 48 h to eliminate undifferentiated cells. Ara-C-purified myotubes contained >90% of the nuclei in the culture.

For microinjections, morpholino-modified oligonucleotides (GeneTools Inc.) were prepared at a concentration of 0.5 μ M. The following oligos were used in Fig. 4: antisense, 5'CATGATGCCGATGACTCTCTCACG3' (the antisense codon corresponding to ATG is underlined); and control (bearing four mismatches), 5'CAAGATCCCGATGACTGTCTGACGCAAGATGCCGATGACTGTCTGACG3'. Microinjection was performed with a microinjector (model Axiovert 100; ZEISS).

Adenoviruses

The *dl520* and *dl312* adenoviruses have been described previously (Haley et al., 1984; Zerler et al., 1987; Wang et al., 1993). The Ad-cdk2 and Ad-cycE recombinant adenoviruses have also been described previously (Latella

et al., 2001). Ad-E2F-1 was a gift of J. Nevins (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC; DeGregori et al., 1997). The Ad-Np95 adenovirus was generated according to He et al. (1998) by placing the cDNA for Np95 (Fujimori et al., 1998) under the control of the cytomegalovirus immediate-early promoter enhancer. This virus also expressed the green fluorescent protein under the control of a second copy of the cytomegalovirus promoter. All adenoviruses were grown and titrated in the permissive 293 cell line (Harrison et al., 1977). The multiplicity of infection (MOI) for all biological experiments is expressed as plaque-forming unit (pfu), as established by titration on 293 cells per infected cells.

RNA extraction, Northern blotting, and cDNA subtraction

Total RNAs were prepared by guanidine-cesium chloride gradient according to published methods (Sambrook et al., 1989). Poly(A)⁺ RNA was prepared with a commercial kit (Amersham Pharmacia Biotech), based on the oligo-dT method, according to the manufacturer's instructions. The cDNA subtraction was performed with the PCR select cDNA subtraction kit (CLONTECH Laboratories, Inc.) according to the manufacturer's instructions (Diatchenko et al., 1996).

Protein studies

Preparation of cellular lysates, immunoprecipitation, and immunoblotting were performed as described previously (Fazioli et al., 1993). Immunofluorescence procedures were also as described previously (Tiainen et al., 1996b; Latella et al., 2001). Antibodies used were as follows: rabbit anti-

rum to cyC (Santa Cruz Biotechnology, Inc.), mAb clone G3-245 to pRb (BD PharMingen; pRb in all figures), rabbit antiserum to pRb phosphorylated on serine 807/811 (Cell Signaling; ph-pRb in all figures), mAb against E2F-1 (a gift of K. Helin, European Institute of Oncology, Milan Italy), goat antiserum against lamin B (Santa Cruz Biotechnology, Inc.), rat Th-10a mAb to Np95 (Muto et al., 1995), mouse mAb to BrdU (Becton Dickinson), and rabbit antiserum to muscle-specific myosin heavy chain (a gift of G. Cossu, Stem Cell Research Institute, Rome, Italy). In immunofluorescence experiments, mAbs were detected either with donkey anti-mouse Cy3 (red) or donkey anti-mouse FITC (green) secondary antibodies. The Np95 mAb was detected with a goat anti-rat Cy3 (red). pAbs were detected with donkey anti-rabbit FITC (green). Pictures were acquired with a color-chilled camera (model 3CCD-C5810; Hamamatsu Corporation). Blots were digitalized with an AGFA scan system. All images were managed with Adobe Photoshop®.

M. Crescenzi and P.P. Di Fiore dedicate this paper to the memory of Franco Tatò.

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