SV40 T antigen interacts with Nbs1 to disrupt DNA replication control

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Nijmegen breakage syndrome (NBS) is characterized by radiation hypersensitivity, chromosomal instability, and predisposition to cancer. Nbs1, the NBS protein, forms a tight complex with Mre11 and Rad50, and these interactions contribute to proper double-strand break repair. The simian virus 40 (SV40) oncoprotein, large T antigen (T), also interacts with Nbs1, and T-containing cells experience chromosomal hyperreplication in a manner dependent on T/Nbs1 complex formation. A substantial fraction of NBS-deficient fibroblasts reinitiate DNA replication in discrete regions, and wild-type Nbs1 corrects this defect. Similarly, synthesis of an N-terminal Nbs1 fragment induced DNA rereplication and tetraploidy, in NBS-deficient but not NBS-proficient cells. Moreover, SV40 origin-containing DNA hyperreplicated in T-containing NBS-deficient cells by comparison with T-containing, Nbs1-reconstituted derivatives. Thus, Nbs1 suppresses rereplication of cellular DNA and SV40 origin-containing replicons, and T targets Nbs1, thereby enhancing the yield of new SV40 genomes during viral DNA replication.

Keywords: Nbs1; SV40 T; SV40 origin; DNA replication; endoreduplication; mammalian cells

Received December 30, 2003; revised version accepted April 12, 2004.
Nbs1 are active in this process. Nbs1 is phosphorylated by ATM at several serine residues after S-phase ionizing radiation, and these ATM-mediated phosphorylation events are essential for activating the intra-S-phase checkpoint in response to DNA damage [Gatei et al. 2000; Lim et al. 2000; Wu et al. 2000; Zhao et al. 2000]. Nbs1 forms a tight complex with two repair proteins, Mre11 and Rad50 [Carney et al. 1998]. This complex forms nuclear foci at sites of DNA damage and disappears from these sites after damage is repaired, suggesting a role for this complex in sensing DNA damage and/or in its repair [Carney et al. 1998; Nelms et al. 1998]. In this regard, experimental depletion of Mre11 leads to the accumulation of DSBs during DNA replication [Coston et al. 2001; Mizoeva and Petrini 2001]. In addition, loss of Nbs1, Mre11, or Rad50 function in mice or in chicken cells is a lethal event [Xiao and Weaver 1997; Luo et al. 1999; Yamaguchi-Iwai et al. 1999; Zhu et al. 2001]. Taken together, these findings suggest that one role of Nbs1 is to coordinate DNA replication with DNA repair.

One mechanism that slows down S-phase progression in response to DNA damage is inhibition of the firing of late replication origins [Santocanale and Diffley 1998]. In Saccharomyces cerevisiae, this inhibition depends on the checkpoint genes Mec1 and Rad53 [Santocanale and Diffley 1998; Shirahige et al. 1998]. The mechanism whereby Nbs1 participates in the S-phase checkpoint remains unknown. However, because S-phase checkpoint action requires specific modulation of DNA replication initiation, it is conceivable that one or more cellular components dedicated to the control of replication initiation is active in the checkpoint mechanism. In this regard, we have detected a heretofore unappreciated interaction between Nbs1 and T and observed that complex formation leads both to enhancement of SV40 DNA replication initiation and to endoreduplication of nonviral chromosomal DNA.

Results

Nbs1 interacts with SV40 large T antigen

We have detected an interaction between the Nbs1/Mre11/Rad50 complex and T in multiple cell lines. Cell lysates were prepared from both 293T and 293 cells, and immunoprecipitation (IP) was performed with antibodies to Nbs1 (D29) and Mre11 (D27). Immunoblotting of these precipitates, using antibody to SV40 T [pAB419], revealed that T coimmunoprecipitated with both Nbs1 and Mre11 in 293T cell extracts [Fig. 1A]. Negative results were obtained with 293 cells that lack T. Similarly, in anti-T IPs of 293T but not of 293 extracts, Nbs1, Mre11, and Rad50 all coimmunoprecipitated with T [Fig. 1B]. Similar results were obtained when U2OS cells that produce T were analyzed [data not shown].

T is composed of multiple functional domains [Ali and DeCaprio 2001]. It interacts with p53 in its C-terminal region and binds to Rb through its LxCXE motif. The N-terminal J domain cooperates with the LxCXE motif to inactivate the function of the Rb family members, including p107 and p130, and also possesses pocket protein binding-independent transformation cooperation function [Liu and DeCaprio 2003]. The interaction of Nbs1 and SV40 T appears to proceed in the absence of the p53, the Rb binding, and the J domain. Specifically, the T mutants Δ434–TΔ444 [a p53 binding-defective mutant], K1 [a pocket protein binding-defective mutant mapping to the LxCXE], and L19PP285 and D44N [J domain mutants; Peden et al. 1990; Kierstead and Tevethia 1993; Campbell et al. 1997], all bound to Nbs1 at the same input concentrations as wild-type T [Fig. 2A].

To search for a specific Nbs1-binding domain in T, a series of C-terminal dl T mutants were coexpressed with myc-tagged Nbs1 in U2OS cells [Fig. 2B]. T fragments, containing as little sequence as the N-terminal 167 residues, readily bound to Nbs1, whereas the 1–157 mutant demonstrated decreased binding. No binding was evident to the 1–135 and 1–147 fragments. Thus, residues located between amino acids 147 and 167 appear to contribute to T/Nbs1 complex formation. In this regard, internal deletion mutants of T were also analyzed [Fig. 2B]. Full-length T lacking residues 147–201 bound Nbs1 efficiently, unlike full-length T lacking residues 147–259. Collectively, this suggests that the T/Nbs1 core binding unit is composed of redundant sequences located between residues 147 and 259.

SV40 T binds specifically to SV40 replication origins as well as nonspecifically to both single- and double-stranded DNA. Its DNA binding function is a product of its DNA-binding domain, which extends from residues 137 to 246 [Kalderon and Smith 1984; Simmons et al. 1990; Wun-Kim et al. 1993]. The Nbs1-binding unit of T, thus, overlaps part of the DNA-binding domain. Because Nbs1 also binds to DNA, we asked whether the T/Nbs1
interaction is mediated by nonspecific DNA binding. Specifically, immunoprecipitation of SV40 T or Nbs1 was performed in extracts of 293T cells in the presence of 400 µM ethidium bromide. Although DNA/protein interactions can be selectively inhibited by ethidium bromide (Lai and Herr 1992), the association of T and the Nbs1/Mre11/Rad50 complex was unaffected by this agent (data not shown). Moreover, we consistently found that the T mutant W128 (Leu156Phe; Kalderon and Smith 1984; Simmons et al. 1990), although defective in DNA binding, bound to Nbs1 like wild-type (wt) T (Fig. 2A). This implies that the T/Nbs1 interaction is not mediated by an associated DNA molecule(s).

The T-binding site on Nbs1 was also mapped and found to be located within the N-terminal region of Nbs1. Two Myc-tagged N-terminal Nbs1 fragments (amino acids 1–357 and 1–478) interacted with T when each was coexpressed with T in U2OS cells. In contrast, the Nbs1 C-terminal fragments (amino acids 474–754 and 343–754) failed to co-IP with T (Fig. 2C). The association between SV40 T and Nbs1 does not influence Nbs1 function in response to DNA damage. The Nbs1/Mre11/Rad50 complex plays an essential role in the response to DNA damage. Nbs1 is phosphorylated.
by ATM after DNA damage, and this phosphorylation is important for certain subsequent cellular responses, such as S-phase checkpoint control and cellular radiation sensitivity [Gatei et al. 2000; Lim et al. 2000; Wu et al. 2000; Zhao et al. 2000]. To test whether the presence of T impairs DNA damage-driven Nbs1 phosphorylation, we compared SDS-gel mobility of Nbs1 before and after ionizing radiation (IR) of IMR 90 primary fibroblasts and a derivative that synthesizes T [IMR90(T)]. IR-induced Nbs1 phosphorylation led to a gel mobility shift (Fig. 3A) that was unaffected by T. Similar results were obtained with 293T and 293 cells. The results of exogenous phosphatase treatment confirmed that the shift was caused by IR-induced phosphorylation [Fig. 3A]. We also immunoprecipitated SV40 T from 293T cells before and after IR. The same quantity of under- and hyperphosphorylated Nbs1 was coprecipitated with T before and after damage (Fig. 3B). This suggests that T/Nbs1 complex formation does not interfere with proper Nbs1 phosphorylation after DNA damage. Moreover, the formation of the Nbs1/Mre11/Rad50 complex was not perturbed by T [Fig. 3C], nor was IR-induced Nbs1, Mre11, and Rad50 nuclear focus formation (Fig. 3D). The intra-S-phase checkpoint response, represented by down-regulation of DNA synthesis in response to IR, was also unaffected in multiple T-containing cell species, including IMR90 (data not shown). These data imply that T does not negatively affect the above-noted Nbs1 functions.

Targeting Nbs1 by SV40 large T antigen is essential for SV40 T-mediated endoreduplication

Expression of T in permissive cells, such as CV-1 induces reinitiation of DNA synthesis within a single cell cycle, resulting in the production of cells with >8N DNA content [Friedrich et al. 1992, 1994; Perry and Lehman 1998]. We have found that, in semipermissive human cells, such as IMR90 primary human fibroblasts, this T-dependent cellular DNA endoreplicative response is more apparent when cells become arrested at G2/M after ionizing radiation [IR, Fig. 4A]. To test whether T/Nbs1 complex formation contributes to T-induced endoreduplication, we infected IMR 90 cells with retroviruses encoding a NeoR marker and either wild-type T or T (Δ147–259 amino acids). Cells were selected for G418 resistance. Flow cytometry was used to measure DNA content before and 48 or 72 h after IR. A significant percentage of T-producing cells (>12% at 72 h after IR) underwent a second round of DNA replication without mi-
nosis, whereas only ~2% of the T- (Δ147–259) or mock-infected cells revealed an 8N DNA content (Fig. 4B). Synthesis of a T mutant defective in Rb binding (K1), origin DNA binding [W128] or p53 binding [434–444] also led to the accumulation of cells with an 8N DNA content (Fig. 4B,C). Similar results were obtained when another primary fibroblast cell line, BJ, was similarly analyzed (data not shown). These results are compatible with the hypothesis that T/Nbs1 complex formation allows endoreduplication of cellular DNA.

To test the T/Nbs1-endoreduplication hypothesis more stringently, we analyzed the effect of T synthesis in NBS-deficient cells before and after reconstitution with Nbs1. Indeed, T did lead to endoreduplication in NBS-deficient cells and, wild-type Nbs1 coexpression led to dramatic suppression of this effect (Fig. 5A). This result strongly suggests that Nbs1 can antagonize SV40 T to suppress the development of multiple rounds of DNA replication within one cell cycle. On the other hand, as shown earlier, there is also evidence suggesting that an excess of SV40 T can bind to Nbs1 to inactivate its endoreduplication suppression function.

Expression of an N-terminal Nbs1 fragment can induce endoreduplication in NBS-deficient cells in the absence of SV40 T expression

Our finding that Nbs1 can suppress SV40 T-mediated endoreduplication suggests that Nbs1 participates in restricting cellular DNA to one round of replication in each cell cycle. In this regard, NBS-deficient GM07166 cells revealed low, endogenous levels of endoreduplication (Fig. 5A). Surprisingly, when the [1–478 amino acids] Nbs1 fragment was expressed in these cells, endoreduplication was markedly enhanced [Fig. 5A, Nbs1[1–478]/vector]. The enhancement was similar to that observed when these same NBS-deficient cells were engineered to express SV40 T (Fig. 5A, mock/SV40 T). Synthesis of a C-terminal [343–754] Nbs1 fragment did not result in this effect (Fig. 5A). This suggests that the Nbs1 [1–478 amino acids] fragment can, with relative specificity, disrupt cellular DNA replication control. Conceivably, it disrupts the control[s] that prevent[s] hyperreplication by interacting with and sequestering/perturbing the function of critical proteins that participate in the relevant replication control pathway[s]. For example, the Nbs1 N-terminal fragment interacts with certain E2F family members known to participate in the regulation of DNA replication [Maser et al. 2001].

The endoreduplication effect mediated by the 1–478 Nbs1 fragment was detected only when cells were Nbs1-deficient. When this same fragment was expressed in endogenous wild-type Nbs1-producing cells, such as IMR90, no increment over the low, basal level of endoreduplication was observed (Fig. 5B). This was the case, even though the expression level of the 1–478 Nbs1 fragment was similar in IMR90 and NBS-deficient GM07166 cells. Notably, when the fragment was expressed in wt-Nbs1-reconstituted GM07166 cells, its endoreduplica-
tion-inducing effect was largely suppressed (Fig. 5C). This suggests that the Nbs1 fragment can disrupt cellular DNA replication control only when endogenous, wild-type Nbs1 function is deficient. This implies that NBS deficiency is a prerequisite for the Nbs1 N-terminal fragment to induce endoreduplication and suggests that intact Nbs1 may establish a safeguard mechanism that prevents DNA hyperreplication, even when certain replication control pathways are disturbed.

NBS deficiency leads to reinitiation of DNA replication at regions close to putative replication initiation sites

To more directly test the Nbs1 replication control hypothesis, we performed Fluorescence In Situ Hybridization (FISH) analysis on interphase cells using a DIG-labeled probe for sequences at a specific site near the /H9252-globin locus where DNA replication is initiated (Kitsberg et al. 1993; Aladjem et al. 1995; Avni et al. 2003). FITC-conjugated secondary antibody against DIG was used to detect replication initiation foci at this site (Fig. 6A; Table 1A). Diploid cells in which the specific segment to be probed is unreplicated should reveal two FISH dots, three dots if one of the two relevant replication origins has fired, or four dots if both have fired. If there are more than four dots overlying two chromosomes, at least one segment of the probed region has rereplicated in the relevant cell cycle.

In a culture of unperturbed IMR90, most cells contained two, three, or four dots, and only ~2% of cells contained more than four dots (Table 1A). In contrast, >20% of the aforementioned NBS-deficient cell line contained more than four dots—even in the unperturbed state (Table 1A). Examples of NBS-deficient cells with two, three, four, or more than four replication dots are illustrated in Figure 6A. Expression of wild-type Nbs1 in NBS-deficient cells reduced the percentage of cells with more than four dots by more than fourfold (Table 1A). Probes that recognize DNA regions containing known sites of DNA synthesis initiation near the HSP70 and Lamin B2 genes (Taira et al. 1994; Abdurashidova et al. 2000) yielded similar results (data not shown). Almost all NBS-deficient cells (GM07166) were characterized by normal chromosomal numbers.

We also performed FISH analysis on metaphase chromosomes. In ~97% of IMR90 cells [wtNbs1], we detected two pairs of dots at the β-globin replication initiation region per cell, one on each chromatid, implying that, in G2-arrested cells, the adjacent DNA has replicated once (Table 1B). In contrast, ~26% of NBS-deficient cells (GM07166) contained two or more β-globin replication dots on one chromatid, and 10% contained two or more
occasionally, we observed that certain GM07166 cells carry an additional Chromosome 11 (three copies of Chromosome 11), where the /H9252-globin gene is located (<1%; Fig. 6B). These findings strongly suggest that NBS deficiency permits more than one round of DNA replication at certain loci close to replication origins. The additional round of DNA replication appears regional and does not cover the entire chromosome, in keeping with the prior observation that only low/background levels of gross chromosomal endoreduplication and hyperploidy were detected in NBS-deficient cells (see Fig. 5A).

Nbs1 suppresses SV40 T-mediated viral DNA replication

Our study suggests that SV40 T binds to Nbs1 and, thereby, perturbs the normal control of cellular DNA replication initiation. One outcome appears to be a state of cellular endoreduplication. This being the case, it seemed reasonable to ask whether this process is relevant to the phenomenon of autonomous replication of the viral genome, an event that depends on repetitive firing of the viral origin in a single S phase.

In an effort to address this question, we introduced an integrated SV40 replication origin into IMR90 cells by infecting with a retroviral vector. The vector encodes the murine ecotropic retroviral receptor (ER) and contains an SV40 replication origin. Subsequently, these cells were infected with another retrovirus encoding wild-type T or with an empty vector (pBaba-Neo-oric−) that carries a neo-resistance gene but lacks an SV40 replication origin. After several passages, genomic DNA from these cultures was purified and digested with SalI and HindIII, a step that results in the generation of an intact and unique SV40 origin-containing fragment detectable by Southern blotting (Fig. 7A). The integrated SV40 origin-containing fragment became amplified when T was expressed (Fig. 7B). In contrast, cells infected with empty vector or T(Δ147−259), which encodes a protein defective in replication origin binding, did not amplify this fragment (Fig. 7B).

We also asked whether wild-type Nbs1 affects this process. Forced expression of an ectopic, wild-type Nbs1 allele [amino acids 1–754] in the NBS-deficient cells suppressed T-mediated viral DNA replication in these cells (Fig. 7C, cf. lanes 2 and 4). In contrast, the Nbs1(1–478) fragment led to enhanced T-mediated viral DNA repli-
A BAC clone containing a large DNA segment [clone RP11-645I8] of the β-globin locus, which includes a DNA replication initiation site [Kitsberg et al. 1993; Aladjem et al. 1995], was used as a FISH probe.

bInterphase nuclei from primary IMR90 cells, NBS-deficient GM07166 cells [NBS], and wild-type [wt] Nbs1-reconstituted GM07166 cells [NBS+/− [Nbs1wt]] were analyzed by FISH using this probe. The number of cells containing two, three, four, or more than four dots at the β-globin locus is indicated.

bMetaphase nuclei of IMR90 and GM07166 cells were analyzed by FISH, using the above-noted probe. The number of IMR90 and GM07166 cells with two pairs of β-globin dots, one on each sister chromatid [sc one on each sc], with multiple dots on one sc [two or more on one sc], and with multiple dots on more than one sc [two or more on more than one sc], are shown. The percentage of cells in each category is shown in parentheses.

### Table 1. β-Globin locus FISH analysis of NBS-deficient cells (GM07166)

| Cell lines | Total number | 2 dots | 3 or 4 dots | >4 dots |
|------------|--------------|--------|-------------|--------|
| IMR90      | 807          | 423 (52.4%) | 365 (45.2%) | 19 (2.4%) |
| NBS−/−     | 219          | 67 (30.6%)  | 102 (46.6%) | 50 (22.8%) |
| NBS+/−     | 432          | 232 (53.7%) | 177 (41.0%) | 23 (5.3%)  |

| Cell lines | Total number | 1 on each sc | 2 or more on one sc | >2 or more on more than one sc |
|------------|--------------|--------------|---------------------|-------------------------------|
| IMR90      | 129          | 125 (96.9%)  | 4 (3.1%)            | 0 (0.0%)                      |
| NBS−/−     | 110          | 70 (63.6%)   | 29 (26.4%)          | 11 (10.0%)                    |

A large fraction of NBS-deficient cells spontaneously dereplicated their DNA in regions close to replication initiation sites, FACS analysis did not reveal significant numbers of cells with DNA contents of >8N. However, when the N-terminal fragment [amino acids 1–478] of Nbs1 was expressed in NBS-deficient cells, profound endoreduplication was observed independent of SV40 T function. This phenomenon was observed only in NBS-deficient cells, and not in NBS-proficient cells such as IMR90. Taken together, these findings, in part, suggest that Nbs1 may contribute to the activation of a checkpoint that prevents additional refiring of replication origins that have already fired.
florid, T-mediated endoreduplication likely requires at least one as-yet-undefined function of this viral protein. Whatever this step(s) may be, like T, the 1–478 fragment of Nbs1 also induced marked endoreduplication. Notably, it was only detected in NBS-deficient cells. Conceivably, unopposed by wild-type Nbs1, this mutant polytetramer interacts in an abnormal manner with certain cell protein(s) (which might include one or more polytetramers with which intact Nbs1 normally interacts), an outcome of which is a gross override of rereplication control. It will eventually be interesting to determine whether T and this particular Nbs1 mutant operate by perturbing the same pathway(s).

Marked amplification of the viral genome is essential for SV40 propagation, but the factors that optimize this process have, in the past, been incompletely defined. In this regard, we observed that a chromosomally integrated SV40 origin-containing segment was more extensively amplified in NBS-deficient than in wild-type Nbs1-reconstituted GM07166. This suggests that, by targeting Nbs1, SV40 T creates an environment that contributes to maximal amplification of its own genome.

Results suggesting that Nbs1 regulates DNA replication control—both cellular and viral—are consistent with the observation that Nbs1 interacts with E2F1, a protein known to associate with certain replication initiation sites, and locates near these regions in S-phase cells [Maser et al. 1997]. Because T can also perturb E2F1 function by displacing it from the Rb protein, one wonders whether it can also displace it and its partner Nbs1 from certain replication origins. This is, however, not likely the case, because the association of Nbs1 with the β-globin and B2 lamin replication initiation sites was not affected by T, as revealed by the results of chromatin immunoprecipitation (ChIP) assays [X. Wu and D. Livingston, unpubl.].

Nbs1 also operates in S-phase checkpoint control. In response to IR delivered during S phase, the protein becomes phosphorylated by ATM at several sites, and these phosphorylation events are important for Nbs1 function in the ensuing intrareplication checkpoint [Lim et al. 2000]. Although it remains unclear how Nbs1 contributes to the slowdown of DNA replication following DNA damage, it is conceivable that, when S-phase DNA damage is sensed or after replicons have fired during a normal cell cycle, Nbs1 communicates with the DNA replication machinery to ensure that no further initiation events occur.

However, it should also be noted that the signals that activate Nbs1 checkpoint function and its hyperreplica-
tion suppression function might be different. For example, T–Nbs1 complex formation did not interfere with DNA damage-induced Nbs1 phosphorylation [Gatei et al. 2000; Lim et al. 2000, Wu et al. 2000, Zhao et al. 2000], and one or more of these phosphorylation events partakes in the activation of the intrareplication checkpoint [Lim et al. 2000]. Consistently, this checkpoint operates normally in the presence of T. In contrast, the interaction of T and Nbs1 did interfere with the function of Nbs1 in preventing hyperreplication. How signals are generated and transduced to Nbs1 when the duplication of a given replicon has been initiated and/or is complete remains unclear.

Nbs1 forms a tight complex with Mre11 and Rad50 [Carney et al. 1998]. Both of these proteins are essential for DSB repair [Johzuka and Ogawa 1995; Moore and Haber 1996]. In mammalian cells, damage-induced foci containing Mre11 are detected during S phase, and focus formation depends on Nbs1 function [Maser et al. 1997; Carney et al. 1998; Dong et al. 1999]. Depletion of Mre11 from Xenopus extracts leads to dramatic accumulation of DSBs during DNA replication [Costanzo et al. 2001]. The Nbs1 S-phase checkpoint function suppresses DNA replication initiation once damage is sensed, and Nbs1 activates Mre11 and Rad50 through a direct association, leading to repair of the relevant DNA damage. The studies described in this report reveal a new function for Nbs1 in preventing DNA hyperreplication during the cell cycle. This finding reinforces the notion that Nbs1 is a multifunctional protein that contributes to proper DNA replication and the maintenance of genome stability.

Materials and methods

Cell culture

GM07166 was obtained from the Coriell Human Mutant Cell Repository. IMR90, 293T, 293, and U2OS cells were from ATCC. Murine ecotropic retroviral receptor [ER, Albritton et al. 1989] was first introduced into IMR90 and GM07166 cells by retroviral infection. These viruses were generated using the Phoenix amphotropic packaging cell line from ATCC. Subsequently wild-type Nbs1, Nbs1 mutants, wild-type T, and T mutants were introduced into these ER+ cells with suitable recombinant murine ecotropic retroviruses generated in Phoenix amphotropic packaging cells (ATCC). GM07166 cells were immortalized by infection with a retrovirus [pBabe/hygro] encoding hTERT [Meyerson et al. 1997]. U2OS cells that stably express SV40 T [U2OS(T)] were generated by transfecting U2OS cells with CMV T neo [Campbell et al. 1997] and selecting for G418-resistant cells.

Antibodies

Monoclonal antibodies against SV40 T (pAB419 and pAB416) and against Nbs1 [EE15 and D29] have been described previously [Harlow et al. 1981; Wu et al. 2000]. Polyclonal Ab (D27) against Mre11 was raised against the C-terminal fragment of human Mre11 [Mre11566–700]. Monoclonal antibody against Rad50 was purchased from Novus Biologicals.

Plasmids

Full-length Nbs1 and Nbs1 fragments were subcloned into a mammalian expression vector, pCDNA3β, that carries the sequence encoding the myc epitope [Chen et al. 1998]. Full-length T and its derivative mutants were subcloned into either pCDNA3β [Scully et al. 1997] or pSG5 [Stratagene]. Alleles encoding full-length Nbs1 with or without a myc-tag and myc-tagged Nbs1 fragments [1–478, 343–754] were inserted into a retroviral vector, pBabe-puro, that was modified by adding an IRES-EGFP sequence from pRES-EGFP (Clontech). The retroviral vector, pBabe/neo/oric−, was generated by replacing the SV40 promoter of pBabe/neo with the comparable DNA region of pBaba/puro/oric−, a gift from Kathy Rundell [Northwestern University, Chicago, IL]. The retroviral vector, pBabe-Blasticidine, encoding ER, was constructed by replacing the Hygromycin marker of pBabe/neo/eric− with a Blasticidine marker, a gift from Peiqing Sun [The Scripps Research Institute, LaJolla, CA]. Detailed information on plasmid construction will be provided upon request.

Immunostains

Cells were lysed in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl at pH 8.0, 0.5% NP-40). Primary antibodies were incubated with cell lysates at 4°C for 3 h, followed by adding protein A- or G-Sepharose beads for another hour's incubation. Beads were washed four times with NETN before SDS-gel sample buffer was added. Primary antibodies were routinely rocked with blots overnight at 4°C. HRP-conjugated secondary antibodies were used at 1:3000 dilution (Amersham).

For immunostaining, cells were fixed in 70% methanol and 30% acetone for 15 min at −20°C. After 1 h of drying at room temperature and three subsequent PBS washes, cells were stained with antibody recognizing Nbs1 [rabbit polyclonal, Oncogene] and/or T [monoclonal pAB419] for 4 h at room temperature. Subsequently, cells were stained with FITC-conjugated anti-mouse and Rhodamine-conjugated anti-rabbit antibodies [Jackson Immuno-Research] for 1 h at room temperature.

Analysis of DNA content by flow cytometry

At each time point, cells were trypsinized, washed once with PBS, and fixed with prechilled 70% ethanol. After incubating for several hours or overnight at 4°C, cells were washed with PBS and resuspended in a buffer containing 38 mM Na Citrate, 70 µM propidium iodide [PI], and 20 µg/mL RNAse A, incubated for 30 min at 37°C in the dark, and subjected to flow cytometry analysis.

Fluorescence In Situ Hybridization (FISH) analysis

FISH was performed as described [Heng et al. 1992; Heng and Tsui 1993; Boggs and Chinnault 1997]. Human BAC clones that were used as probes for DNA sequences at the B2-Lamin, HSP70, and β-globin loci were obtained through the Children's Hospital Oakland Research Institute (CHORI). The BAC clone numbers are: B2-Lamin, RP11-21113; HSP70, RP11-425A7; and β-globin, RP11-645B8.

Acknowledgments

We thank James DeCaprio and Ole Gjoerup for sharing SV40 T DNA-encoding plasmids and for many helpful discussions. We also thank William Hahn and Robert Weinberg for providing pBabe-hygro-htERT, Peiqing Sun for pBabe-hygro-ER, and...
Kathy Rundell for pBabe-puro-oric. William Hahn graciously helped us in the immortalization of GM07166 with hTERT.

The publication costs of this article were defrayed in part by advertisement in accordance with 18 USC section 1734 solely to indicate this fact.

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*Genes Dev.* 2004, 18:
Access the most recent version at doi:10.1101/gad.1182804

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