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Assembly of Functional ALT-associated Promyelocytic Leukemia Bodies Requires Nijmegen Breakage Syndrome 1[1]

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ABSTRACT

Immortalized cells maintain telomere length through either a telomerase-dependent process or a telomerase-independent pathway termed alternative lengthening of telomeres (ALT). Homologous recombination is implicated in the ALT pathway in both yeast and human ALT cells. In ALT cells, two types of DNA double-strand break repair and homologous recombination factors, the Rad50/Mre11/NBS1 complex and Rad51/Rad52 along with replication factors (RPA) and telomere binding proteins (TRF1 and TRF2), are associated with the ALT-associated PML body (APB). DNA synthesis in late S-G_2 is associated with APBs, which contain telomeric DNA and, are therefore, potential sites for telomere length maintenance. Here, we show that the breast cancer susceptibility gene product, breast cancer susceptibility gene 1, and the human homologue of yeast Rap1, hRap1, are also associated with APBs specifically during late S-G_2 phase of the cell cycle. We additionally show that the localization of the double-strand break repair factors with APBs is distinct from their association with ionizing radiation-induced nuclear foci. To systematically explore the mechanism involved in the assembly of APBs, we examine the role of Nijmegen breakage syndrome 1 (NBS1) and TRF1 in this process, respectively. We demonstrated that NBS1 plays a key role in the assembly and/or recruitment of Rad50, Mre11, and breast cancer susceptibility gene 1, but not Rad51 or TRF1, to APBs. The NH_2 terminus of NBS1, specifically the BRCA1 COOH-terminal domain, is required for this activity. Although TRF1 interacts with NBS1 directly, it is dispensable for the association of either Rad50/Mre11/NBS1 or Rad51 with APBs. Perturbation of the interactions between NBS1/Mre11 and APBs correlates with reduced BrdUrd incorporation associated with APBs, consistent with decreased DNA synthesis at these sites. Taken together, these results support a model in which NBS1 has a vital role in the assembly of APBs, which function to maintain telomeres in human ALT cells.

INTRODUCTION

Telomeres, specialized structures at the ends of linear chromosomes, are comprised of GC-rich tandem repeats. The protection of telomere ends and maintenance of their length prevent chromosome degradation, end-to-end fusions, and rearrangements, events detrimental to chromosome stability and genome integrity. Yeast, germline cells, and the majority of cancer cells typically maintain telomere length by *de novo* addition of DNA by telomerase (1, 2). In addition to the standard mechanism, subsets of cells maintain telomere length through a telomerase-independent mechanism, termed the ALT[3] pathway (3).

Genetic studies in yeast have implicated a recombination-dependent mechanism in the ALT pathway. In this model, members of *RAD52* epistasis group, including the DNA recombination and DSB repair factors Rad52, Rad51, and the Rad50/Mre11/Xrs2 complex participate, along with a helicase, Sgs1, and mismatch repair factors (4–11). Two distinct ALT pathways have been proposed based on participation of either Rad50 or Rad51. Rad52, on the other hand, is required for both pathways (5, 7).

In humans, ~10–20% of tumors are telomerase negative and have been proposed to maintain telomere length by ALT pathways (3). Selection of the exact mechanism of ALT in the cells of these tumors, although not well understood, may be related to the germ layer of their origin (12). Evidence hints that homologous recombination plays a primary role in most of the mammalian ALT pathways, similar to the nonreciprocal recombination used by yeast that are telomerase defective (5, 7). Marker cassettes integrated into specific telomeres of mammalian cells can be copied to other short telomeres, presumably through a gene conversion-like recombination process (13). In mammalian ALT cells, recombination factors, including Rad51, Rad52, and the Rad50/Mre11/NBS1 complex (the human counterpart of yeast Rad50/Mre11/Xrs2), are specifically associated with a fraction of nuclear PML bodies. Also present are telomere binding factors TRF1, TRF2, replication factor RPA and telomere repetitive DNA (14–17). BrdUrd incorporation associated with the APBs in late S-G_2 phase indicates that the DNA synthesis associated with telomere lengthening may be an important function in these subnuclear compartments (16).

DNA recombination and DNA DSB repair involve a number of factors, defects in which are implicated in mammalian carcinogenesis. The product of the BRCA1 has essential roles in cellular responses to DNA damage, in checkpoint control, and in DSB repair (18–27). Consistent with these functions, BRCA1 associates with both the Rad50/Mre11/NBS1 complex and Rad51 *in vivo* (18, 20). In yeast, the Rad50/Mre11/Xrs2 complex participates in several processes, including homologous recombination and nonhomologous end-joining, S-phase replication, DNA damage response, and cell cycle checkpoint control (review in Ref. 28). Moreover, the Rad50/Mre11/Xrs2 complex is proposed to have a role in telomerase-dependent maintenance (5). Whether the Rad50/Mre11/NBS1 complex has a similar role in mammalian cells is unknown. Intriguingly, however, NBS1 physically interacts with TRF1, a telomere-specific binding protein, supporting a potential role for NBS1 complex in mammalian telomere homeostasis (16). Although the association of BRCA1 with Rad50/Mre11/NBS1 complex is critical for DNA damage response, it remains unclear whether BRCA1 plays a role in telomere length maintenance in mammalian cells. Furthermore, how the multiple repair/recombination factors with distinct function assembled in APBs during telomere elongation is also unknown.

Here, we present evidence that BRCA1 and hRap1 are new components of APBs. The association of repair proteins with IRIF is distinct from their association with APBs. Using overexpressed dominant-negative proteins, we show that NBS1 is required for recruitment and/or assembly of a subgroup of proteins into APBs, including Mre11, Rad50, and BRCA1 but not Rad51 or TRF1. The BRCT domain of NBS1 is required for this function. Although TRF1 interacts with NBS1, it is not critical for localization of either NBS1 or Rad51 within APBs. Finally, colocalization of Mre11, Rad50, and

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[3] The abbreviations used are: ALT, alternative lengthening of telomere; DSB, double-strand break; PML, promyelocytic leukemia; APB, ALT-associated PML body; BRCA1, breast cancer susceptibility gene 1; IRIF, ionizing radiation-induced foci; NBS, Nijmegen breakage syndrome; GST, glutathione *S*-transferase; GFP, green fluorescent protein; BrdUrd, bromodeoxyuridine; BRCT, BRCA1 COOH-terminal domain; CR, conserved region.

BRCA1 at APBs is important for BrdUrd incorporation at these subnuclear sites. These results suggest that NBS1 plays a critical role in mediating the assembly of functional APBs that is important for the propagation of human ALT cells.

MATERIALS AND METHODS

Cell Lines, Cell Synchronization, and BrdUrd Labeling. ALT cell lines, including SV40 large T-antigen-immortalized human fibroblasts (GM847 and VA13) and two osteosarcoma lines (Saos-2 and U-2 OS), have previously been described (16). Cells were cultured in DMEM supplemented with 10% fetal bovine serum in 10% CO_2. Telomerase-positive cells T24 and MCF7 were cultured similarly. Cell synchronization at late S-G_2 was achieved by a double thymidine block-and-release protocol as described previously (16). For BrdUrd pulse labeling, cells were incubated 30 min with reagents in a cell proliferation kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), fixed, and stained as described below.

Immunostaining, Coimmunoprecipitation, and Western Blots. Indirect immunofluorescence staining was performed essentially as described previously (16). Briefly, cells plated on cover slips in 6-cm culture dishes were washed with PBS and fixed for 20 min in 3.7% formaldehyde in PBS with 0.1% Triton X-100. Cells were then permeated with 0.05% Saponin at room temperature for 30 min, followed by washing five times in PBS. After blocking with 10% goat serum in PBS/0.5% NP40 at room temperature for 30 min, cells were incubated with primary antibodies for 2 h at room temperature. FITC or Texas Red-conjugated secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) were used to detect primary antibodies. Cells were counter stained with 4′, 6′-diamino-2-phenylindole and mounted in Permafluor solution (Shandon Lipshaw, Pittsburgh, PA). Fluorescence microscopy was performed with a Nikon Eclipse TE300, and the captured images were processed with Metamorph software and Adobe Photoshop. Immunoprecipitation was performed in lysis 250 buffer [50 mM Tris-Cl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% NP40, and 0.5 mM phenylmethylsulfonyl fluoride] at 4°C as described previously (18). Coprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted with Rad50, Mre11, and/or NBS1 antibodies (29).

Antibodies. For TRF1 and hRap1, mouse polyclonal sera were raised against GST-TRF1 and GST-hRap1, respectively, which had been expressed and purified from bacteria. Mouse anti-Mre11(12D7), mouse anti-Rad50 (13B3), and mouse anti-Rad51 (14B4) monoclonal antibodies have been described previously (16, 18). Rabbit anti-Rad51 and mouse anti-BRCA1 monoclonal (Ab-1) antibodies were purchased from Oncogene Research Products (Boston, MA). Rabbit anti-NBS1 antibody was obtained from Novus Biologicals (Littleton, CO). Mouse anti-PML antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Sp100 antibody was purchased from MBL International Corporate (Watertown, MA).

Conditional Expression of NBS1C. To generate cell clones that express a GFP-NBS1C fusion protein, we used a tetracycline-inducible expression system (30). NBS1C (amino acids 543–754 of NBS1) was translationally fused to GFP in the pUHD10 vector. Constructs pUHD10-NBS1C and pCHTV (bearing tet regulator and hygromycin-resistant gene, respectively) were cotransfected (10:1 molar ratio) into human osteosarcoma U-2 OS cells, which were subsequently selected with hygromycin (150 μg/ml) in the presence of tetracycline (1 μg/ml). Hygromycin-resistant colonies were isolated and assayed for conditional expression of GFP-NBS1C. One of the positive clones, named UNBS1C, with tight tet-dependent regulation, was used in this study.

Recombinant Adenoviruses. Constructs directing the expression of NBS1C, GFP, or GFP-TRF1Δmyb were prepared according to a simplified adenovirus amplification system with minor modifications (31). To facilitate virus amplification and evaluation of the titer, TRF1Δmyb and NBS1C were both tagged with GFP. Adenoviruses were produced and amplified in E1-immortalized human embryonic kidney cells 293. ALT cells were infected and harvested at 36 h after infection. To perform double staining (such as Mre11 with PML), cells were treated with 1% H_2O_2 after fixation until the GFP signal was fully quenched and then stained as described above.

RESULTS AND DISCUSSION

Association of BRCA1 with PML Bodies Is Specific to ALT Cell. BRCA1 plays an essential role in the maintenance of genome stability, which is partially attributed to its interaction with the DNA damage response and repair complex NBS1/Rad50/Mre11 (18). In ALT cells, the NBS1/Rad50/Mre11 complex is localized with APBs during late S-G_2 phase, suggesting its involvement in telomere length maintenance (16, 17). We first asked if BRCA1 is involved in the ALT pathway. Indirect immunofluorescence studies showed that BRCA1 colocalized with APB-associated proteins, TRF1, Sp100, and NBS1 in several ALT cells, including GM847 (Fig. 1*A*). Although BRCA1 forms speckles in S-phase cells, they are not APB associated (32). However, when ALT cells are enriched at late S-G_2 phase, after release from a double-thymidine block, BRCA1 associates with APB foci in ~24% of cells, compared with 5–7% in unsynchronized culture (Fig. 1*B*). These data suggest that the association of BRCA1 with APBs is specific for late S-G_2 stage, similar to that of NBS1 and Rad51 (Fig. 1*B* and Ref. 16). In contrast, colocalization was not observed in telomerase-positive cells such as human bladder carcinoma cells, T24, and human breast cancer cells, MCF7 (Fig. 1*C*), suggesting that the association of BRCA1 with APBs is specific to late S-G_2 in ALT cells. These data implicating BRCA1 in the ALT pathway are consistent with its roles in homology-directed DNA repair and microhomology-mediated DNA end-joining (27, 33).

Colocalization of Multiple DSB Repair Factors and hRap1 at APBs. BRCA1 is known to colocalize with NBS1/Mre11/Rad50 complex and Rad51 in response to treatment of cells with DNA damaging agents (18, 20). The NBS1/Mre11/Rad50 complex and Rad51 participate in distinct DNA repair events (34, 35). However, both colocalize with APBs in ALT cells (14–16). To demonstrate these functionally distinct recombination factors and BRCA1 are localized in APBs, we performed pair-wise coimmunostainings in either randomly growing or synchronized ALT cells at late S-G_2 (Fig. 1*D*). We found that Rad51 colocalized with BRCA1 (Fig. 1*D*, *a–d*), NBS1 (*e–h*), and Mre11 (*i–l*) reciprocally, suggesting that these factors may work in concert during a recombination-mediated telomere lengthening that potentially occurs in APBs.

In addition to recombination proteins, it is known that many factors are found at APBs, including telomere repeat binding proteins TRF1 and TRF2 (14, 16). The human homologue of yeast Rap1, hRap1, was recently identified as a telomere-associated protein, which contains a BRCT and is critical for telomere length regulation (36). Using an antibody specific for hRap1, we detected immunoreactivity that colocalized with APBs (Fig. 1*E*). Similar to BRCA1 and the NBS1/Mre11/Rad50 complex, the association of hRap1 with APBs is also most evident when cells are arrested at the late S-G_2 phase (data not shown). Combined, the results of these localization experiments suggest that a wide spectrum of both telomere and DSB repair-associated factors may be operational in human ALT pathway.

Colocalization of Double-Strand Break Repair Factors with IRIF Is Distinct from APB Association. NBS1/Mre11/Rad50 complex and Rad51 are known to play an important role in the repair of ionizing radiation-induced DSBs. Although NBS1/Mre11/Rad50 and Rad51 form nuclear foci upon ionizing radiation, Rad51 cannot be found to be colocalized with NBS1 or Mre11 in a given cell (Ref. 37 and our unpublished observations). This observation is in sharp contrast to what was observed in Fig. 1*D* that both groups of proteins are colocalized at APBs in ALT cells. Therefore, it is likely that recombination-dependent telomere length maintenance is mechanistically related but not identical to DSB repair-associated homologous recombination.

To distinguish the relationship between these two types of nuclear

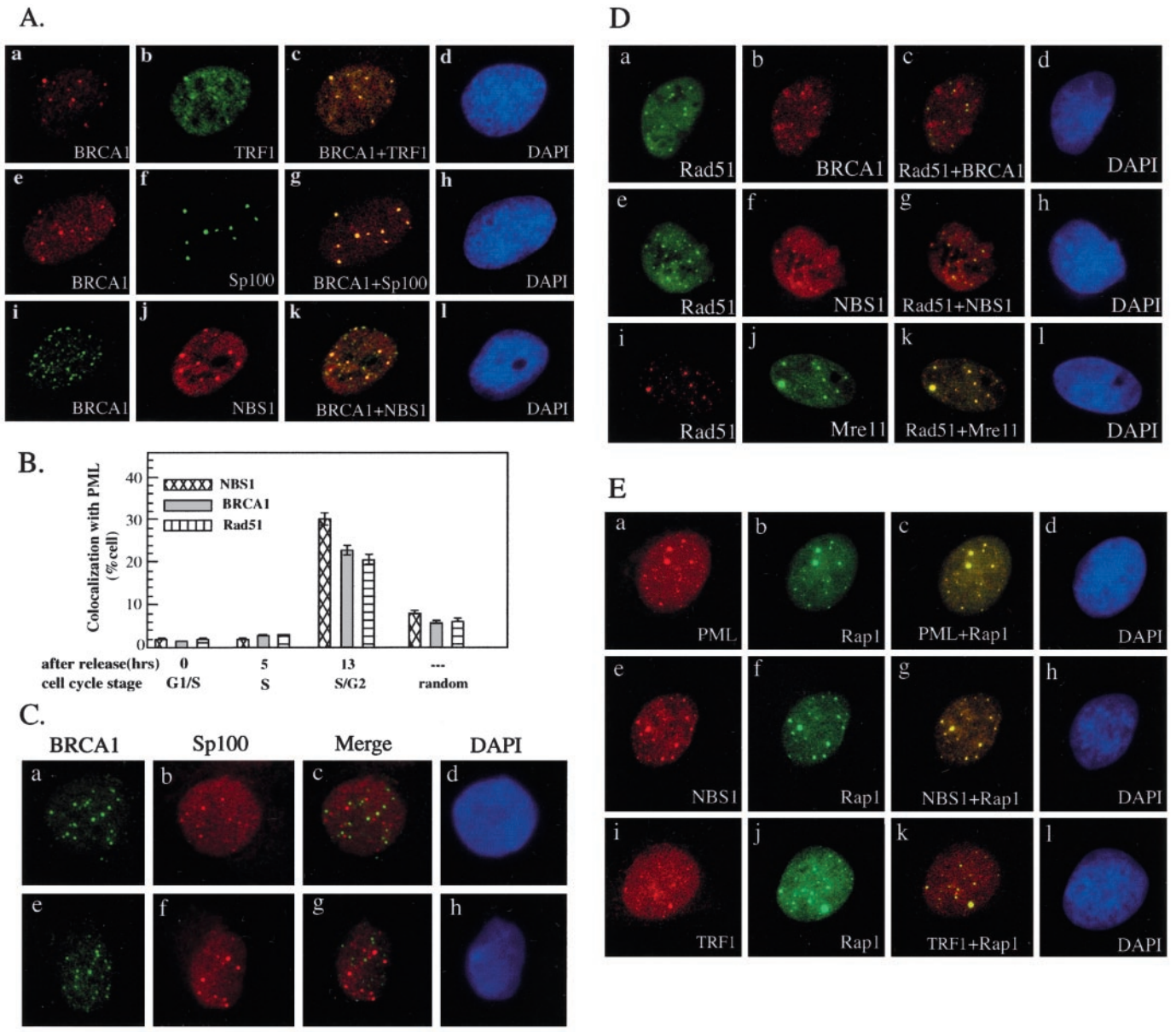


Fig. 1. BRCA1 is a component of the APB in the ALT line GM847. *A*, BRCA1 colocalizes with APB in ALT cells, GM847, as demonstrated by immunofluorescent colocalization with TRF1 (*a–d*), Sp100 (a PML body marker; *e–h*), and NBS1 (*i–l*). Images in *red* represent the detection by a Texas-red-conjugated secondary antibody, whereas *green* represents FITC. Nuclei were visualized by 4′, 6′-diamino-2-phenylindole staining. *B*, association of BRCA1, NBS1, and Rad51, with APBs is most evident in cells enriched at late S-G_2 phase. Cells were either actively growing or synchronized by a double thymidine block and harvested at different time points upon release. For each point, at least 200 cells were counted, and the results were summarized from three independent experiments. *C*, BRCA1 does not associate with PML bodies in telomerase-positive lines such as human bladder carcinoma cells, T24 (*a–d*) and human breast cancer cells, MCF7 (*e–h*). Panels *a* and *e* were immunostained for BRCA1, *b* and *f* for Sp100, whereas *c* and *g* show merged images. *D*, Rad51 colocalizes with BRCA1 (*a–d*), NBS1 (*e–h*), and Mre11 (*i–l*) using antibodies specific for each antigen and procedures described above. *E*, immunostaining with a mouse anti-hRap1 antibody demonstrated its colocalization with APB marker proteins PML (*a–d*), NBS1 (*e–h*), and TRF1 (*i–l*) using antibodies specific for each antigen and procedures described above.

foci, we test whether IRIF and the localization to APBs are dependent on each other. After treatment with γ-irradiation, increased percentage of cells exhibited granular nuclear speckles of BRCA1, Rad51, and NBS1, characteristic of DNA repair foci. These DNA-damaged induced foci did not colocalize with PML or TRF1 in ALT cells such as human osteosarcoma U-2 OS (Fig. 2) and Saos-2 cells (data not shown). However, it was noted that a small fraction of cells demonstrated APB-associated foci positive for the BRCA1, NBS1, and Rad51 antigens in irradiated and mock-irradiated cells. These same patterns of localization were also observed at earlier times (1.5 and 4 h) after exposure to ionizing radiation (data not shown). Combined, these findings suggest that DNA damage-induced foci and APB-associated foci form independently and are morphologically different.

Interrelationships of DSB Repair and Telomere-associated Factors. The association of the above factors with telomeres or APBs in ALT or telomerase-positive cells, their colocalization with BrdUrd under various conditions, and their inclusion in IRIFs are summarized in Table 1. The results were obtained from experiments using either GM847 (ALT line) or T24 (telomerase positive) cells in this study or from other studies using various cell lines (14–16, 19, 37–40). Cumulatively, these data indicate that the distribution of both DSB repair and telomere-associated factors in the nucleus appears to be dynamic and multifaceted. In both telomerase-positive and ALT cells, the NBS1 complex is associated with telomeres and APBs, within which DNA synthesis takes place during late S-G_2 of the cell cycle. The NBS1 complex also associates with replication forks throughout

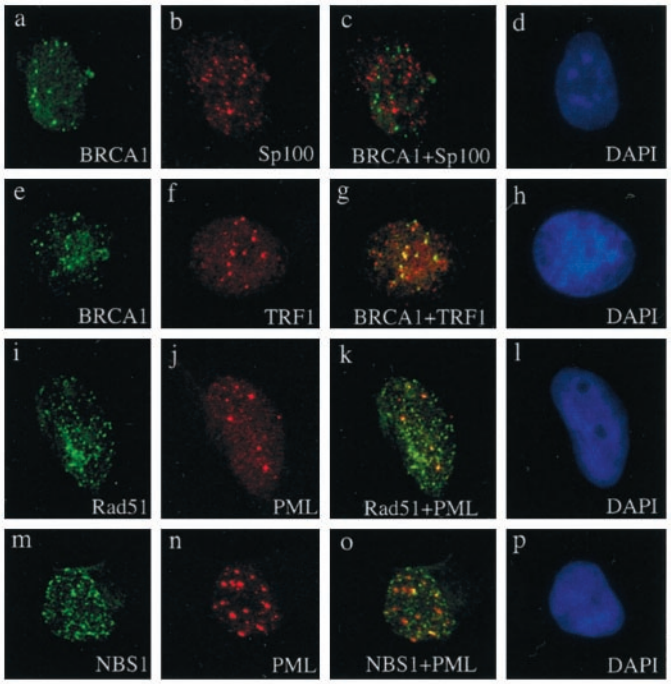


Fig. 2. Association of DSB repair factors with IRIF is distinct from their association with APBs. U-2 OS cells (an ALT line) were harvested and fixed 8 h after radiation (12 gray) and subsequently stained with indicated antibodies. DNA damage-induced foci positive for BRCA1 (usually outnumber PML bodies and are smaller and more homogeneous) do not colocalized with Sp100 (*a–d*) or TRF1 (*e–h*). On the other hand, in a fraction of cells, BRCA1 foci characteristic of APB bodies (larger and brighter but fewer in number) are still present along with newly appearing repair foci that colocalize with TRF1 (*e–h*) after irradiation, suggesting that BRCA1 foci induced by γ-irradiation and colocalization with APBs are independent events. Similar observations were obtained for Rad51 (*i–l*) and NBS1 (*m–p*).

Table 1 *Interrelationships of DSB repair and telomere-associated factors*

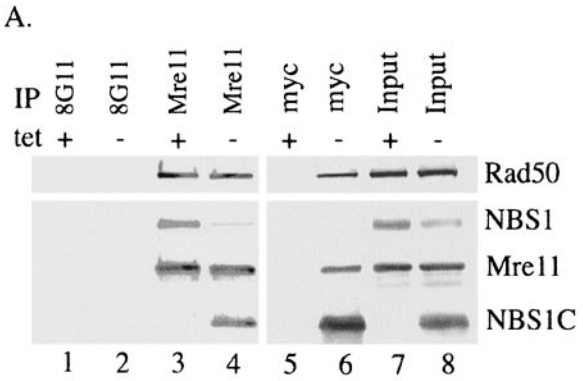
	Association w/telomeres or APBs		Colocalize w/BrdUrd			
Factors	ALT cells	TERT(+) cells[a]	S phase	DDI	@APBs	IRIF
Mre11	+	+	+	+	+	+
NBS1	+	+	+	+	+	+
BRCA1	+	−	−	+	+	+
Rad51	+	−	−	+	+	+
Rad52	+	−	?	?	+	+
hRap1	+	+	−	−	+	−

[a] TERT(+), telomerase positive; DDI, DNA damage induced.

S-phase progression and is associated with DNA damage-induced foci when cells are irradiated. On the other hand, BRCA1 and Rad51 are associated with APBs and are also found in replication foci in the presence of DNA damage, regardless of the cell type.

NBS1 Is Essential for the Recruitment and/or Assembly of the NBS1/Mre11/Rad50 Complex into APBs. Elucidating the assembly of functional APBs is central to understanding the ALT pathway. Given the complex spectrum of APB-associated factors, we sought to identify a few key molecules that are critical for their assembly. Because TRF1 is known to interact with both telomeric DNA (41) and NBS1 (16), we hypothesized that both proteins are critical for recruitment and/or assembly of at least BRCA1, Mre11, and/or Rad50 into APBs. To test this model biochemically and cell biologically, we established a subclone of U-2 OS cells, named as UNBS1C, that harbors a stably integrated tet-off system that controls the expression of a GFP-tagged truncated form of NBS1, specifically, a COOH-terminal region encompassed by amino acids 543–754. The rationale for this design is that, although the COOH-terminal region of NBS1 is sufficient for nuclear localization and binding Mre11, the mutated complex does not associate with IRIF (42, 43), an activity that apparently resides within the NH_2-terminal part of the NBS1. If NBS1 is required for APB association, we expected that conditional expression of GFP-NBS1C would result in formation of Rad50/Mre11/NBS1C complexes incapable of APB association. In Fig. 3*A*, immunoblot data demonstrate that removal of tetracycline from the media resulted in a significant induction of GFP-NBS1C expression (compare *Lanes 7* and *8*). Coimmunoprecipitation with α-Mre11 and α-myc and immunoblot detection showed that Rad50 and Mre11 are present in a complex with GFP-NBS1C (Fig. 3*A*, *Lanes 3–6*).

The effects of the truncated NBS1C/Mre11/Rad50 complex on the association of Mre11 and BRCA1 with APBs and DNA synthesis are summarized in Fig. 3*B*. There was a significant reduction in the localization of BRCA1 at APBs upon removal of tetracycline ($P = 0.0153$). The most dramatic effect was on Mre11-associated APBs ($P < 0.0001$), which are normally detected in ~30% of the cells arrested at late S-G_2 stage (16). In contrast, Mre11-associated APBs were completely abolished in late S-G_2 cells upon induction of GFP-NBS1C expression. Consistent with these localization data were the fraction of APBs that displayed BrdUrd incorporation, which was also effectively reduced upon the expression of GFP-NBS1C ($P = 0.0157$). These data suggest that DNA synthesis was reduced in

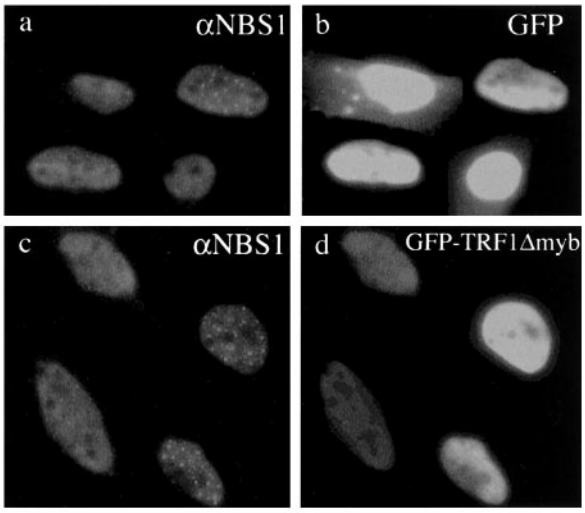


B.

tet	+	-
Mre11	6.4±0.8%	<0.4%
BRCA1	5.3±1.2%	2.1±0.8%
Rad51	5.9±1.4%	4.9±0.9%
TRF1	7.0±1.2%	6.1±1.2%
BrdUrd	3.2±0.6%	1.1±0.6%

Fig. 3. Association of Mre11/Rad50 or BRCA1 with APBs depends on NBS1. *A*, Myc-tagged GFP-NBS1C (amino acid 543–754) competed with the endogenous NBS1 in the formation of NBS1/Mre11/Rad50 complex. Cell lysates prepared from UNBS1C (U-2 OS subclonal line expressing GFP-NBS1C under a tet-off system) were immunoprecipitated with anti-Mre11 antibodies and immunoblotted using antibodies against Rad50, NBS1, and/or Mre11. In the presence of tetracycline, GFP-NBS1C was not expressed and Mre11 coimmunoprecipitated with endogenous NBS1 and Rad50 (*Lane 3*). Upon removal of tetracycline to induce the expression of GFP-NBS1C (induced for 40 h), immunoprecipitation with anti-Mre11 antibodies predominantly brought down GFP-NBS1C instead of the endogenous NBS1 (*Lane 4*). Similarly, Mre11 and Rad50 were specifically immunoprecipitated with GFP-NBS1C by anti-myc antibodies (*Lane 6*) but not control antibodies, mouse anti-GST, 8G11 (*Lanes 1* and *2*). Whole cell lysates from UNBS1C either with (*Lane 7*) or without tetracycline (*Lane 8*) were used to perform straight Western blots probed with each of the specific antibodies as indicated. *B*, effects of the expression of GFP-NBS1C on the colocalization of Mre11, BRCA1, Rad51, TRF1 with APBs. Actively growing UNBS1C cells were plated on the coverslips in the presence or absence of tetracycline in the culture medium. These cells were harvested and immunostained with antibodies against Mre11, BRCA1, Rad51, or TRF1 along with PML (or Sp100). BrdUrd labeling was also performed to evaluate DNA synthesis in APBs (16). Cells that showed positive colocalization of a factor with PML were counted and their percentage relative to the total cell number is presented. A total of at least 140 cells were assessed for each experiment, and the results were summarized from three independent experiments.

A.



B.

Adenovirus	*(a). GFP*	*(b). GFP-TRF1Δmyb*
NBS1	7.3±1.1%	11.4±1.0%
Mre11	6.2±1.0%	9.1±1.0%

Fig. 4. NBS1 and Mre11 associate with APBs in the presence of overexpressed GFP-TRF1Δmyb. *A*, actively growing GM847 cells were infected with high-titer adenovirus bearing a GFP or GFP-TRF1Δmyb translational fusion, which act as a dominant negative. Cells were harvested at 36 h after infection and stained against NBS1 (*a* and *c*). *b* and *d* show the fluorescent signal of GFP alone (the control) or GFP-TRF1Δmyb in the same field of cells as in *a* and *c*, respectively. Note that some cells overexpressing GFP or GFP-TRF1Δmyb form APB-characteristic NBS1 foci. *B*, fractions of GM847 cells containing APB-characteristic NBS1 or Mre11 foci in cells infected with indicated adenoviruses as described in (*A*). Cells containing more than five APB-characteristic foci for NBS1 or Me11 were counted, and the percentage (*versus* the total population) is presented. For each experiment, at least 220 cells in total were evaluated, and the results are summarized from two independent experiments.

the APBs. Although not investigated here, the data imply an impairment of telomere length maintenance because telomere DNA is the only type known to be present at APBs (14). In contrast to the elimination of Mre11 and reduction of BRCA1 in APBs, the association of TRF1 and Rad51 proteins was only slightly reduced (Fig. 3*B*), although GFP-NBS1C also binds to TRF1 (16). Thus, the mechanism involved in the recruitment of Rad51 to APBs remains unknown.

To eliminate the possibility that the above results were attributable to clonal variations, we designed a system for the expression of GFP-NBS1C using adenovirus, with which highly efficient infection and expression could be achieved. Several ALT lines, including GM847 and VA13, were infected and assessed for the cellular effects of GFP-NBS1C overexpression. Results similar to those with the inducible U-2 OS subline UNBS1C were obtained (data not shown).

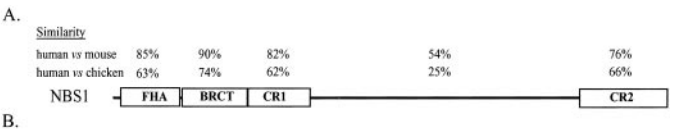
These findings suggest that NBS1 is essential for recruitment and/or association of the Mre11/Rad50 complex and BRCA1 with APBs and for APB-specific DNA replication during late S-G_2 phase. It was noted that short-term expression of GFP-NBS1C (within 4 days) did not correlate with significant cell cycle arrest, as quantified by fluorescence-activated cell sorting analysis and immunostaining of the cell cycle marker phosphohistone H3 (data not shown). On the other hand, persistent expression of GFP-NBS1C for >2 weeks led to cell death (data not shown), suggesting that the association of intact NBS1 complex with APBs may be required for growth and proliferation of human ALT cells.

The Association of the NBS1/Mre11/Rad50 Complex with APBs Is Independent of TRF1. We next tested whether TRF1 is required for the association of NBS1, Mre11, and Rad51 with APBs. We used a recombinant adenovirus capable of directing expression of GFP-TRF1Δmyb that forms a nonauthentic TRF1Δmyb/TRF1 dimer, which acts as a dominant negative by depleting endogenous TRF1 from telomere DNA (41, 44). We reasoned that its overexpression would reduce or inhibit the association of NBS1 with telomeres and/or APBs. Unexpectedly however, NBS1 antigen is detected with APBs in a fraction (Fig. 4*B*) of GM847 cells in the presence of overexpressed GFP or GFP-TRF1Δmyb (Fig. 4*A*). Similar results were obtained in two other ALT lines, U-2 OS and VA13 (data not shown). Interestingly, instead of a reduction, a slight increase of the association was observed in cells overexpressing GFP-TRF1Δmyb, compared with the control cells expressing GFP alone (Fig. 4*B*). Taken together, these results suggest that the association of NBS1/Mre11/Rad50 with APBs is independent of TRF1. It is possible that the association could be mediated by another APB component, by other telomeric factors, or by telomere DNA because all of the members of NBS1/Mre11/Rad50 complex have intrinsic DNA binding activity (34, 35).

Structure Requirements for Association of NBS1 with DNA Damage-induced Foci Is Distinct from those Required for APBs. On the basis of the results described above, we concluded that NBS1 plays a central role in the assembly of the functional APBs, which depend on the recruitment of Mre11, Rad50, and BRCA1. Fig. 5 summarizes several sets of experiments designed to elucidate the structural requirement of NBS1 for association with the Mre11/Rad50 complex and localization with APBs. The FHA and BRCT domains are two known conserved modules residing at the NH_2-terminal part of NBS1 (Fig. 5*A* and Ref. 45). In addition, two regions that are also relatively conserved from human to chicken were found and designated as CR1 (amino acids 197–280) and CR2 (amino acids 643–754), respectively (Fig. 5*A*). CR1 is juxtaposed to several SQ-containing phosphorylation motifs that are important for DNA damage response (46–49). CR2 is the interaction domain for several proteins including Mre11 and TRF1 (16, 42, 43).

Using this structural knowledge, we generated a panel of NBS1 mutants and determined their ability to associate with Mre11 complex and APBs by coimmunoprecipitation and/or immunostaining (Fig. 5*B*). Mutation of a conserved residue (His45Ile, Fig. 5*B*) in the FHA domain reduced the association of NBS1 with APBs. Similar reduc-

A.



B.

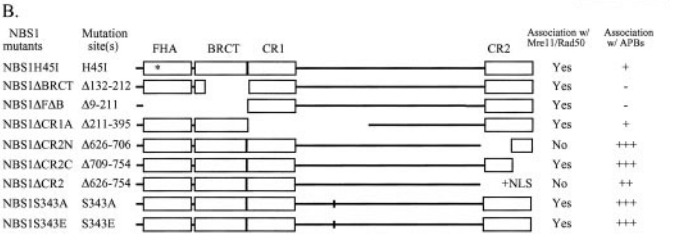


Fig. 5. Structural requirements for the association of NBS1 with Mre11/Rad50 or APBs. *A*, diagram showing the signatory molecular modules in the NBS1 protein. Besides the known NH_2-terminal FHA and BRCT domains, NBS1 contains two relatively CRs (CR1, a.a. 197–280 and CR2, a.a. 643–754). CR1 is juxtaposed to BRCT. CR2 resides at the COOH terminus. The similarities of these signatory domains among human, mouse, and chicken are shown. *B*, a panel of NBS1 deletion mutants COOH-terminally tagged with GFP was constructed and expressed in U-2 OS cells. Colocalization of the expressed NBS1 deletion mutants with Mre11/Rad50 or APBs (represented by TRF1 and PML) were determined by immunostaining as described in "Materials and Methods." Note that NBS1 lacking the BRCT domain no longer associates with APBs.

tions were also observed with several other mutants such as NBS1ΔCR1A (CR1 completely deleted) and NBS1ΔCR2. Strikingly, deletion of the BRCT domain (or both FHA and BRCT domains) completely eliminated the association of NBS1 with APBs. In contrast, deletion of Mre11 interaction domain (NBS1ΔCR2N) did not affect this association, suggesting that the association of NBS1 with APBs is Mre11 independent. Furthermore, expression of NBS1ΔBRCT in U-2 OS cells reduced the colocalization of Mre11/Rad50 and BRCA1, consistent with our observations derived from UNBS1C (data not shown). Serine 343 of NBS1 has been identified as a critical phosphorylation site during DNA damage responses (46–49). However, changing serine 343 to alanine (unphosphorylatable) or glutamate (mimicking phosphorylation) did not affect the association of NBS1 with APBs, suggesting that DNA damage-induced modifications did not play a role in relocalization of NBS1. This result is consistent with our observation that association with DNA damage-induced foci by DSB factors is distinct from their ability to localize with APBs. Taken together, these results strongly suggest that the BRCT domain of NBS1 may mediate an interaction with a PML body component, and this interaction is sufficient to bring in NBS1 and its associated factors critical for telomere elongation to APBs in ALT cells.

In summary, our results implicated the BRCA1 and NBS1 in the human ALT pathway. We established that NBS1 is essential for the assembly of functional APBs, which are probably critical for telomere elongation in human ALT cells. Association of Mre11/Rad50 and BRCA1 with APBs depends on the integrity of NBS1, especially the BRCT module. The recruitment of Rad51 at APBs is NBS1 independent, consistent with the notion that Rad50 complex and Rad51 are required for the generation of different types of ALT cells in yeast (5). Although TRF1 is an integral part of the telomeres, it is dispensable for the recruitment of NBS1 and Rad51 to APBs. Therefore, TRF1 may not be essential for telomere length maintenance in the ALT pathway, but its role at telomeres could be more structurally related. Recombination-associated events responsible for telomere elongation in ALT cells would provide a unique stage for functional cross-talk between BRCA1, Rad51, and the NBS1/Mre11/Rad50 complex, which, in parallel to their roles in DNA recombination and/or DSB repair, contributes to the maintenance of genome stability. Determining whether the BRCA1/NBS1/Mre11/Rad50 complex functions mechanistically in the actual telomere lengthening process or serves as a signal transducer to recruit other recombination and/or DNA synthesis factors awaits additional studies.

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Assembly of Functional ALT-associated Promyelocytic Leukemia Bodies Requires Nijmegen Breakage Syndrome 1

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