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A role for PML and the nuclear body in genomic stability

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The PML gene of acute promyelocytic leukemia (APL) encodes a cell-growth and tumor suppressor. PML localizes to discrete nuclear bodies (NBs) that are disrupted in APL cells. The Bloom syndrome gene BLM encodes a RecQ DNA helicase, whose absence from the cell results in genomic instability epitomized by high levels of sister-chromatid exchange (SCE) and cancer predisposition. We show here that BLM co-localizes with PML to the NB. In cells from persons with Bloom syndrome the localization of PML is unperturbed, whereas in APL cells carrying the PML-RARα oncoprotein, both PML and BLM are delocalized from the NB into microspeckled nuclear regions. Treatment with retinoic acid (RA) induces the relocalization of both proteins to the NB. In primary PML−/− cells, BLM fails to accumulate in the NB. Strikingly, in PML−/− cells the frequency of SCEs is increased relative to PML+/+ cells. These data demonstrate that BLM is a constituent of the NB and that PML is required for its accumulation in these nuclear domains and for the normal function of BLM. Thus, our findings suggest a role for BLM in APL pathogenesis and implicate the PML NB in the maintenance of genomic stability.

Keywords: PML; BLM; APL; Bloom syndrome; nuclear body; sister-chromatid exchange

Introduction

The cell nucleus is known to contain several highly organized domains (for review see Scheer and Weisenberger, 1994). One distinctive class of sub-nuclear domains, the PML-nuclear body (NB), appears as punctate speckles by indirect immunofluorescence microscopy and is referred to variously as Kremer Bodies, ND10, or PODs (for PML Oncogenic Domains) (for review see Hodges *et al*., 1998).

The PML gene was identified at the translocation breakpoint in APL cells that contain the chromosomal translocation t(15;17) (for reviews see Melnick and Licht, 1999; He *et al*., 1999). Gene-targeted disruption of PML demonstrates that the PML protein functions as a growth and tumor suppressor *in vivo*, and it is important for multiple apoptotic pathways (Wang *et al*., 1998a,b). Interferons, which are potent growth and tumor suppressive cytokines, increase PML expression as well as the size and the number of NBs (Lavau *et al*., 1995; Stadler *et al*., 1995; Gaboli *et al*., 1998; Hodges *et al*., 1998). However, the mechanisms by which PML exerts its tumor-growth suppressive functions are still to be determined.

PML is covalently conjugated to small ubiquitin-like modifier 1 (SUMO-1, also known as PIC1) (Sternsdorf *et al*., 1997; Müller *et al*., 1998). SUMO-1 is conjugated to various proteins that localize to different cellular compartments. In the nucleus, it concentrates in the NB where, besides PML, it covalently modifies other NB proteins such as Sp100 (Sternsdorf *et al*., 1997).

In APL cells harboring the t(15;17), the PML gene is fused to RARα leading to the generation of a PML-RARα fusion protein (Pandolfi *et al*., 1991; de The *et al*., 1991; Kakizuka *et al*., 1991; Goddard *et al*., 1991; He *et al*., 1999). The PML-RARα fusion protein is leukemogenic in transgenic mice (He *et al*., 1997, 1998; Grisolano *et al*., 1997; Brown *et al*., 1997). PML-RARα retains most of the functional domains of its parental proteins and can heterodimerize with PML (He *et al*., 1999). In APL cells, PML no longer localizes to the NB but instead forms nuclear microspeckles that are coincident with PML-RARα and RXR (Dyck *et al*., 1994; Koken *et al*., 1994; Weis *et al*., 1994). PML-RARα also disrupts the localization of other NB components such as Sp100 (Koken *et al*., 1994). Thus, it is tempting to speculate that PML-RARα interferes with their normal functions as well. Treatment of persons with APL with RA can achieve complete but temporary clinical remission of this disease, and in APL cells, it causes the degradation of PML-RARα and induces relocalization of NB components (He *et al*., 1999).

Null mutations in the BLM gene have been identified in the autosomal recessive disorder Bloom syndrome (BS), which is characterized at the cellular level by a genomic instability that includes excessive chromosome breakage and chromatid exchange (Ellis *et al*., 1995; for review see German and Ellis, 1998). The hypermutability and hyper-recombinability of BS cells very likely explain many of the clinical features of the syndrome, including the enormous cancer predisposition of persons with BS. The BLM gene encodes a DNA helicase of the RecQ family (Ellis *et al*., 1995); however, the normal function of BLM is unknown.

Here we show that BLM and PML co-localize in the NB and BLM is delocalized in APL and PML−/− cells. Furthermore, we provide the first evidence that PML and the NB are implicated in the control of genomic stability and in the regulation of normal BLM function.

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Results

The BLM helicase localizes to the NB

Using rabbit polyclonal anti-BLM antibodies, we observed that the BLM protein in normal cells is present in characteristic nuclear dots (Figure 1a). Purified antibodies reacted specifically with BLM as determined by Western blot and indirect immunofluorescence analyses of normal cells and of BS cells that contain null mutations in BLM (Figure 1a,b and see Ellis *et al*., 1999; Neff *et al*., 1999).

The discrete nuclear distribution of BLM strongly suggested that protein–protein interactions are important for its localization. We therefore undertook a yeast two-hybrid screen to identify BLM-interacting

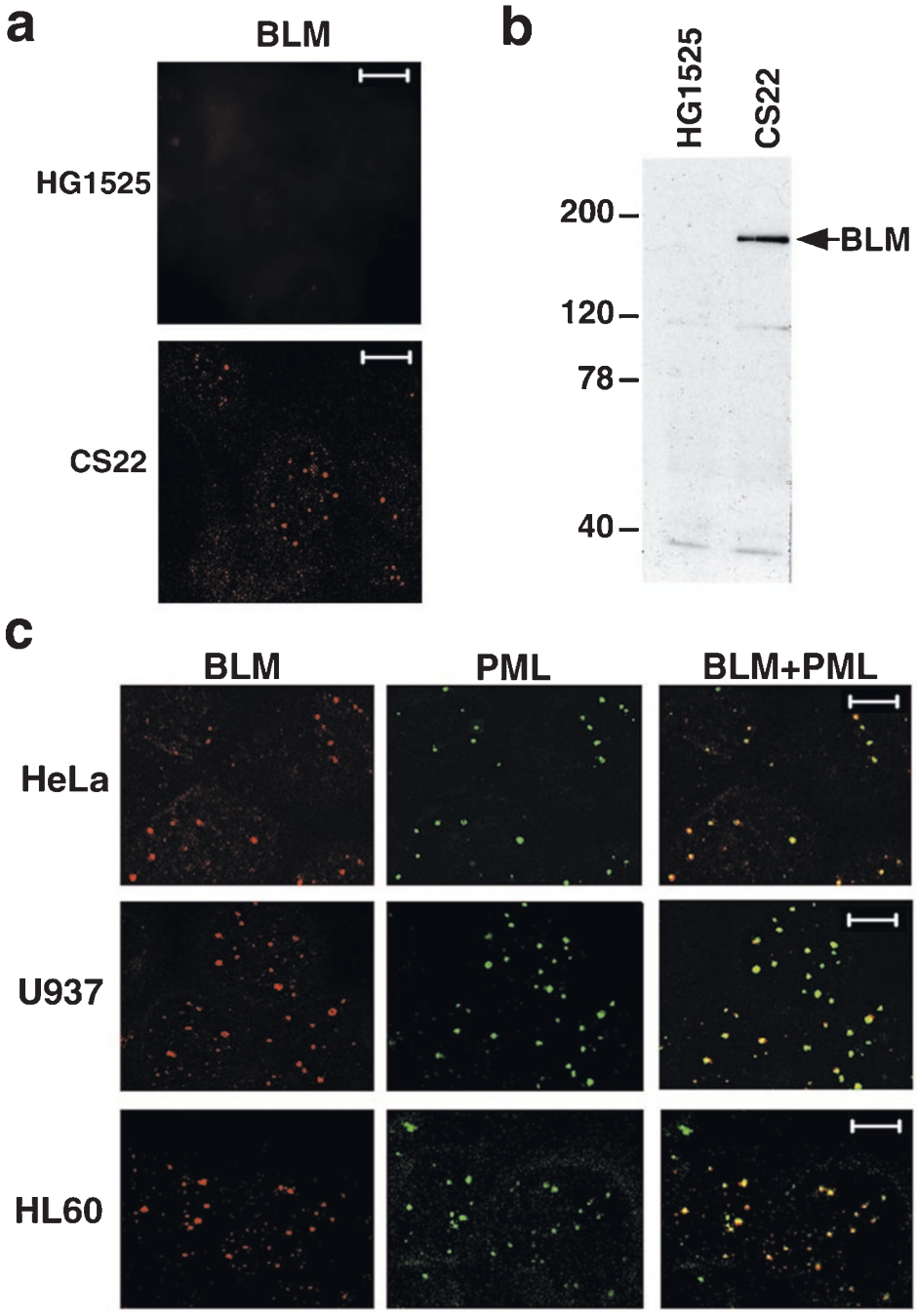

Figure 1 Localization of BLM to the NB. Rabbit polyclonal anti-BLM antibodies were raised to a histidine-tagged, N-terminal 431 amino-acid segment of BLM produced in *E. coli* and purified by nickel chelation chromatography (Beresten *et al*., 1999). (**a**) Indirect immunofluorescence analysis of BLM expression in lymphoblastoid cell lines derived from a normal (CS22) and a BS person (HG1525). BLM is shown by red fluorescent signal. The bar represents 5 μM. (**b**) Western blot analysis of BLM expression in HG1525 and CS22. Equal amounts of protein were separated by electrophoresis on a 6% SDS–PAGE. Western blot analysis was carried out with the rabbit polyclonal anti-BLM antibody. The arrow points to the BLM protein which has an apparent molecular weight of approximately 180 kD. (**c**) Co-localization of BLM and PML in HeLa, U937 and HL60 cells. PML and BLM are shown by green and red fluorescent signal, respectively, and co-localization of the two is revealed by the yellow color in the superimposed panels (right panels). The NB varies in size from 0.2 to 1 micron (Weis *et al*., 1994); in our analysis, we have counted those dots that are greater than 0.2 micron in size. The bar represents 5 μM

proteins. One candidate that emerged by screening with an N-terminal BLM segment was SUMO-1. (A full report of the yeast two-hybrid screen and molecular analysis of specific candidates identified in the screen will be published elsewhere.) Because it was known that nuclear SUMO-1 accumulates in the NB (Boddy *et al*., 1996; Müller *et al*., 1998) and that BLM also concentrates in nuclear dots, we investigated using indirect immunofluorescence whether BLM co-localizes with PML in the same subnuclear structures. Anti-BLM staining of unsynchronized cultures of various human cell lines had identified two predominant nuclear localization patterns of BLM (Figure 1c, left panels): (i) discrete, bright dots and (ii) diffuse microspeckles. As with anti-BLM, staining cells with anti-PML exposed discrete bodies and diffuse microspeckles (Figure 1c, central panels, and see Hodges *et al*., 1998). Superimposition of the stainings with anti-BLM and anti-PML revealed co-localization of BLM and PML to the same discrete, bright dots (Figure 1c, right panels). In microscopic analysis of 174 HeLa cells, coincidence of BLM and PML in the dots was detected in 85% of the cells; the levels of BLM were too low to detect in 12% of the cells; and BLM and PML did not co-localize in 3% of the cells. In 15 randomly selected cells analysed by confocal microscopy, the coincidence of BLM and PML in the dots was greater than 90%: 159 dots co-localized, 17 dots were BLM only, and ten dots were PML only.

BLM is delocalized from the NB in the presence of PML-RARα

As stated above, PML is delocalized from the NB in APL cells that contain the PML-RARα fusion protein due to t(15,17) translocation. This fusion protein is present in the APL cell line NB4 (He *et al*., 1999). Staining NB4 cells with anti-PML exposed PML protein predominantly in nuclear microspeckles (Figure 2) that, as earlier studies have shown, co-localize with the RXR transcription factor (Dyck *et al*., 1994; Koken *et al*., 1994; Weis *et al*., 1994). Staining NB4 cells with anti-BLM also revealed BLM in diffuse microspeckles (Figure 2), of which the majority did not appear to co-localize with PML (Figure 2), suggesting that BLM is not localized to the same microspeckles as PML-RARα and RXR.

Treatment of NB4 cells with 1 μM RA for 24 h caused a dramatic re-localization of both PML and BLM to the NB (Figure 2). These observations demonstrate that the normal localization pattern of BLM requires intact NBs because its localization can be reconstituted by RA treatment, as has been shown for PML and Sp100 (Dyck *et al*., 1994; Koken *et al*., 1994; Weis *et al*., 1994; Hodges *et al*., 1998). We conclude therefore that BLM localizes to the NB.

BLM is delocalized in PML−/− cells

The dispersed distribution of BLM in NB4 cells and its reconstitution after RA treatment suggested that BLM's presence in the NB is either directly or indirectly dependent on normal PML protein. We tested this hypothesis by transfecting GFP-BLM into PML−/− primary keratinocytes from mice in which the PML gene had been ablated by homologous recombination/ES cell technology (Wang *et al*., 1998b). In PML−/− cells, GFP-BLM failed to localize in the NBs but acquired a diffuse nuclear localization pattern (Figure 3a), whereas in PML+/+ cells GFP-BLM co-localized with the endogenous PML proteins. Conversely, absence of BLM, the situation in BS cells, had no effect on the localization of PML to the NB (Figure 3b), indicating that PML's localization to the NB is independent of BLM. PML is therefore essential for the accumulation of BLM in the NB even though direct physical interaction between PML and BLM has not been detected (our unpublished observations).

PML−/− cells exhibit higher numbers of SCEs

To investigate whether the delocalization of BLM affects its biological function, we analysed the number

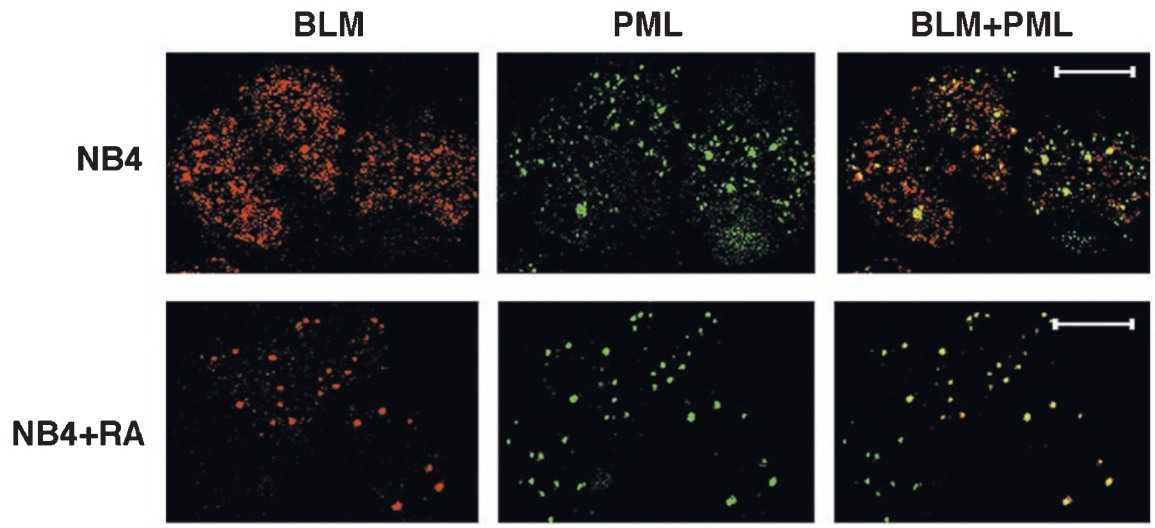

Figure 2 Localization of BLM in the presence of the PML-RARα fusion protein. Distribution of BLM in NB4 cells, with and without treatment of 1 μM RA for 24 h. BLM and PML were presented in microspeckles, some of which apparently overlapped in untreated NB4 cells; upon RA treatment, the two proteins re-localized to the NB. Superimposed figures are shown in the right panels. To allow visualization of the microspeckles, laser intensity for image captures was maximized. The bar represents 10 μM

of SCEs per metaphase in primary PML+/+ and PML−/− murine embryonic fibroblasts (MEF). The average chromosome number in the PML−/− cells was 40, the same as that in wild-type cells. In 35 PML+/+ MEFs at metaphase, the mean number of SCEs was seven, whereas in 44 PML−/− MEFs the mean number of SCEs was 14 (Figure 4). The twofold increase of SCEs in the absence of PML is statistically significant ($P<0.005$), suggesting that the localization of BLM to the NB is important for its normal function.

Discussion

BLM represents the first DNA helicase found to localize in the NB. DNA helicases operate in processes requiring access to the DNA, that is, replication, repair, recombination, and transcription. BLM's localization to the NB implicates the NB in one or more of these processes. In this respect, it is intriguing that PML has been recently found to regulate transcription (Doucas *et al*., 1999; Zhong *et al*., 1999). Here we provide evidence for a role of PML and the NB in maintaining genomic stability. We have demonstrated that the presence of PML is required for BLM's localization to the NB. The increased levels of SCEs in the absence of PML strongly suggest that normal localization of BLM to the NB is necessary for its proper function. Thus, either BLM is exerting some of its functions in the NB, or important biosynthetic/regulatory events occur there that in turn modulate BLM function.

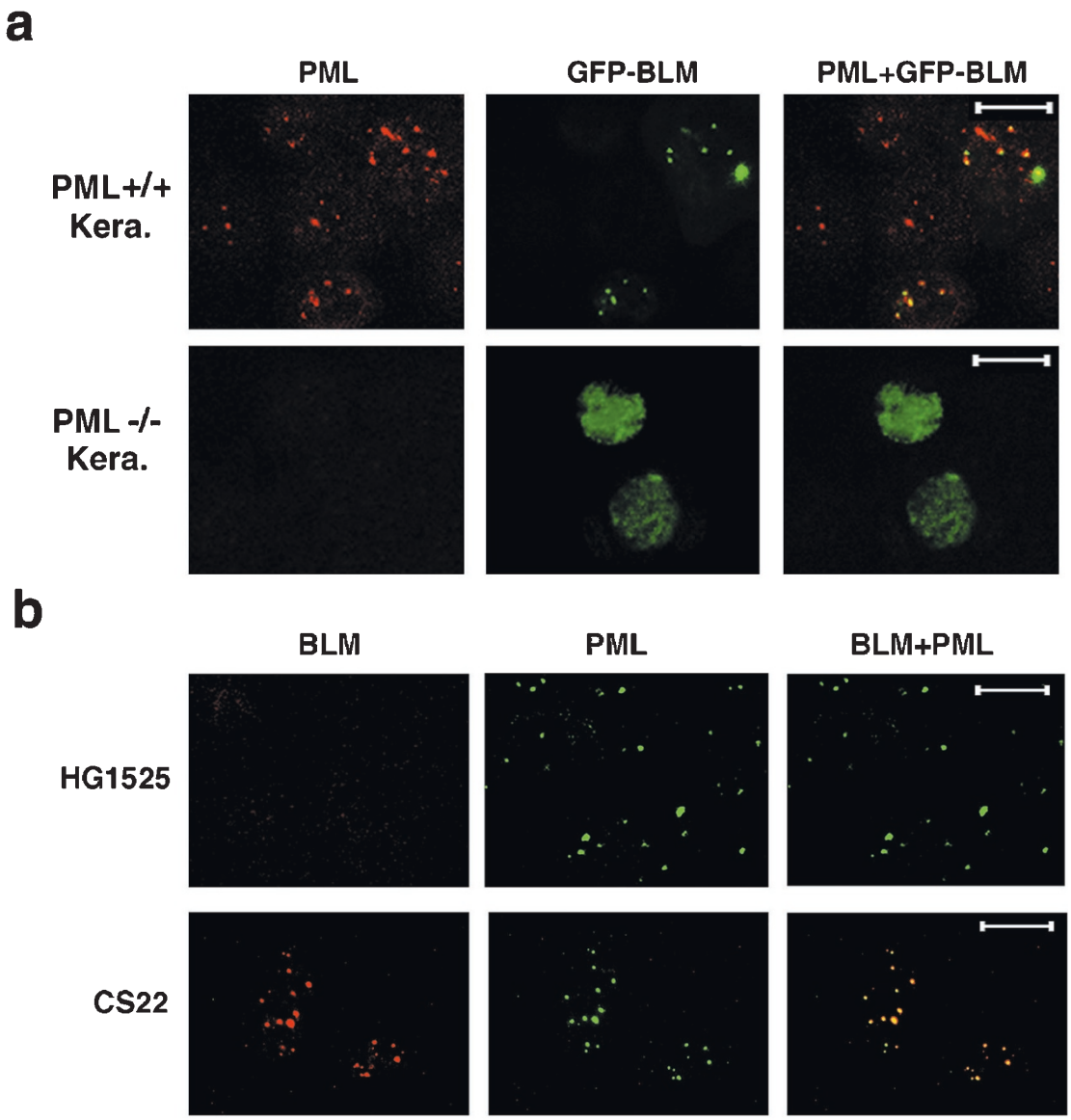

Figure 3 Localization of BLM in the absence of PML and localization of PML in the absence of BLM. (**a**) Distribution of transfected GFP-BLM in PML+/+ and PML−/− primary keratinocytes. Transfected GFP alone displayed a homogeneous distribution throughout the cell (not shown). To detect murine PML a rabbit polyclonal antibody was used. In PML+/+ cells, GFP-BLM co-localized with PML in nuclear speckles, whereas in PML−/− cells, GFP-BLM was nuclear but diffuse. (**b**) PML localization in BS cells. Experiments performed with HG1525 and CS22 cells showing that in the absence of BLM, PML remains localized to the NB. The distribution pattern of PML in HG1525 cells is indistinguishable from that in the control cell line CS22. The bar represents 10 μM

Although the level of SCEs in PML−/− cells is not as high as that in BS cells (German *et al*., 1977), our findings are in agreement with the fact that PML−/− mice are tumor-prone when challenged with carcinogens such as DMBA (Wang *et al*., 1998a) that are known, among other things, to increase the spontaneous level of SCEs. It is therefore tempting to speculate that PML might exert its tumor suppressive activity, at least in part, through its ability to regulate BLM nuclear localization and function in the maintenance of genomic stability.

Furthermore, we show that the presence of the PML-RARα oncoprotein disrupts the normal localization of BLM in the APL cell line NB4. Although there is compelling evidence demonstrating the leukemogenic role of PML-RARα both *in vitro* and *in vivo*, PML-RARα seems to be necessary but not sufficient for leukemogenesis in transgenic mice (He *et al*., 1997, 1999; Brown *et al*., 1997; Grisolano *et al*., 1997). This is supported by the very long latent period which precedes leukemia development in these murine models of APL (He *et al*., 1997, 1999; Brown *et al*., 1997;

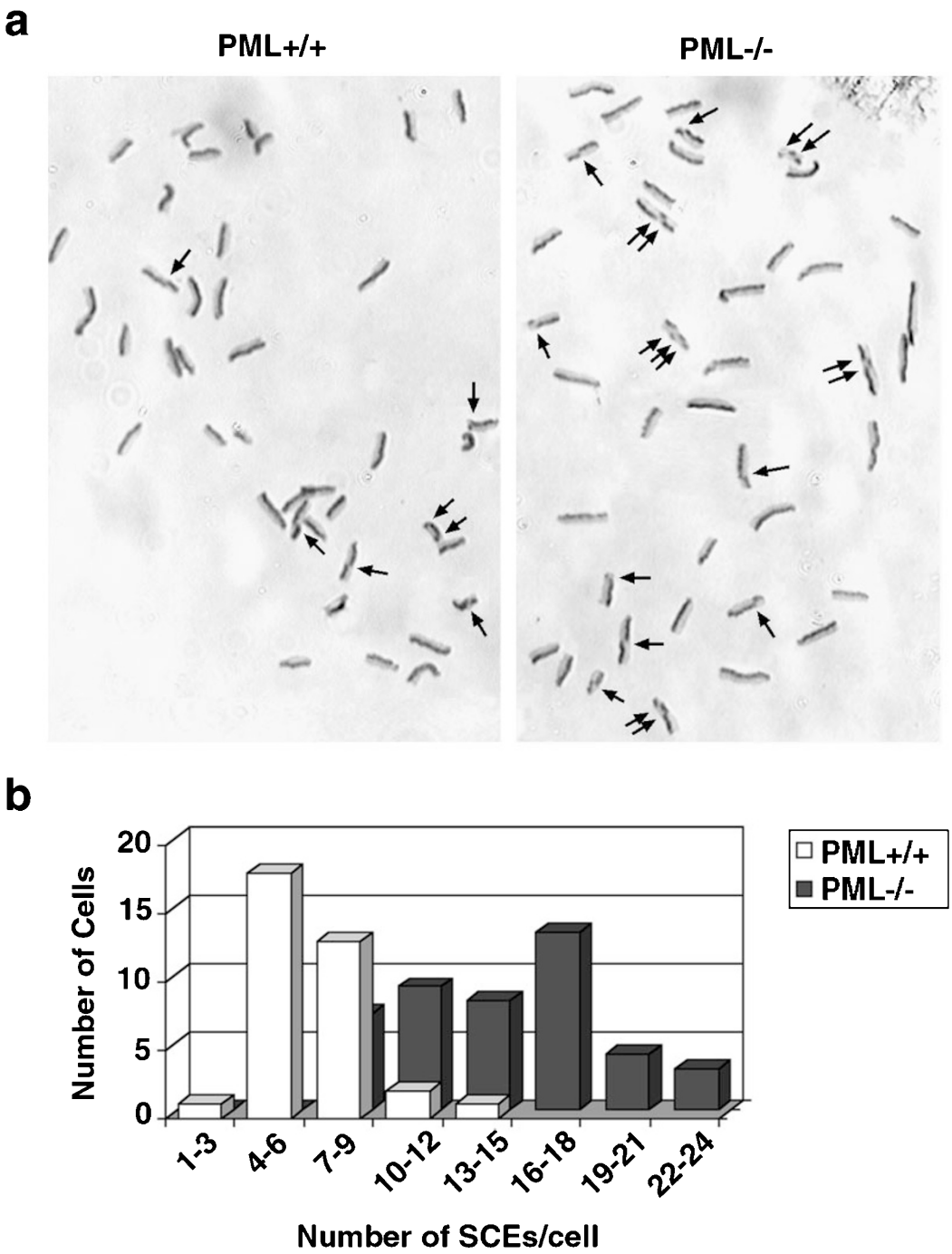

Figure 4 PML−/− cells exhibit higher levels of SCEs than PML+/+ MEF cells. (**a**) Representative pictures of SCEs in PML+/+ and PML−/− metaphases. The arrows point to the points of exchange between sister chromatids. (**b**) A histograph that shows the distribution of SCEs in primary PML+/+ and PML−/− cells. SCE analysis was performed in metaphase MEFs (35 PML+/+ cells and 44 PML−/− cells). PML+/+ cells have 6.5±2.3 SCEs/cell, whereas PML−/− cells have 14.2±4.2 SCEs/cell. The average numbers of chromosomes in both PML+/+ and PML−/− cells were 40

Grisolano *et al*., 1997). However, in the pre-leukemic phase, PML-RARα transgenic mice display aberrant hemopoiesis, characterized by an indolent myeloproliferative disorder which results in the progressive accumulation of myeloid cells which are blocked at the promyelocytic stage of hemopoietic differentiation (He *et al*., 1997, 1999; Brown *et al*., 1997; Grisolano *et al*., 1997). Thus, other genetic events have to occur to transform this myeloproliferative disorder into full-blown leukemia. It is therefore possible that PML-RARα could destabilize the genome through its ability to delocalize both PML and BLM, favoring the accumulation of additional mutations in tumor suppressor genes and/or oncogenes that might participate, along with PML-RARα, to the leukemogenic process.

Although further studies are required to determine the role of BLM in APL pathogenesis, our findings clearly implicate PML and the NB in genomic stability and shed new light on the role of PML in tumor suppression.

Materials and methods

Cell cultures

The BS lymphoblastoid cell line HG1525, which is homozygous for the mutation BLMc.1784C>A causing a stop codon at residue 595, was obtained from Dr J German (Ellis *et al*., 1999). Two control normal lymphoblastoid cell lines, CS17 and CS22, were obtained from Dr M Sadelain. Mouse primary keratinocytes and MEFs were prepared and maintained as previously described (Filvaroff *et al*., 1990; Wang *et al*., 1998a). HeLa, HL60, and U937 were obtained from American Type Culture Collection. Culture medium and reagents were obtained from Life Technologies. The HeLa cells were maintained in Dulbecco's modified Eagle's medium with high glucose content and 10% fetal bovine serum (FBS). Cells that grow in suspension were maintained in RPMI 1640 with 2 mM L-glutamine and 15% v/v FBS.

Western blot analysis

HG1525 and CS22 cells were grown as described above and harvested by centrifuging at 1500 r.p.m. for 5 min. The protein extract was obtained by lysing the cells with the lysis buffer (0.2% NP40, 250 mM NaCl, 50 mM Tris, pH = 8, 10 mM EDTA, 1 mM DTT supplemented with complete protease inhibitor tablet (Roche)). Equal amount of protein was electrophoresed on a 6% SDS–PAGE. Western blot analysis was carried out with a rabbit polyclonal anti-BLM antibody (antibody were raised by Cocalico Biologicals, Inc. and purified by the method described in Ellis *et al*., 1999; Neff *et al*., 1999).

Plasmids and transfections

Full length BLM was subcloned in-frame in the pEGFP vector (Clontech). 4×10^5 primary keratinocytes per well in 6-well clusters were transfected with 1 μg of pEGFP vector or pEGFP-BLM using the SuperFect reagent (Qiagen). Twenty-four hours after transfection, cells were harvested for direct inspection under UV light or for indirect immunofluorescence analysis.

Indirect immunofluorescence

HeLa cells were grown as described above, and were trypsinized and cytospun for 5 min at 1000 r.p.m. directly onto glass slides. U937, HL60, HG1525, CS17 and CS22 cells were grown as described above and cytospun onto glass slides before staining. Cells were fixed in 4% paraformaldehyde for 20 min at RT and permeabilized in methanol for 20 min at −20°C. After washing three times in PBS and blocking in PBS containing 10% heat-inactivated goat serum (blocking buffer), the cells were incubated for 1 h at RT with the first antibody (rabbit polyclonal anti-BLM, mouse monoclonal anti-PML (PG-M3, Santa Cruz) and rabbit polyclonal anti-PML (Boddy *et al*., 1996)) diluted in the blocking buffer. For detection, fluorescein- or Texas red-conjugated horse anti-mouse IgG or goat anti-rabbit IgG antibodies were diluted 1 : 200 in blocking buffer containing 1 ng/ml DAPI. Cells were incubated with the appropriate secondary antibody for 1 h at RT, washed three times in PBS, and the slides were mounted with a coverglass in Vectashield mounting medium (Vector Laboratories, Inc.). Slides were viewed on an Olympus fluorescence microscope or analysed in the Sloan-Kettering Institute confocal microscopy core facility (Katia Manova, Laboratory Director).

SCE analysis

The differential staining of sister chromatids in passage 4 primary MEFs that had replicated for two cell cycles was done using a previously described protocol (German *et al*., 1977). The SCEs in intact metaphases were counted by light microscopy with a 100× objective.

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