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Promyelocytic Leukemia Nuclear Bodies Support a Late Step in DNA Double-Strand Break Repair by Homologous Recombination

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ABSTRACT

The PML protein and PML nuclear bodies (PML-NB) are implicated in multiple cellular functions relevant to tumor suppression, including DNA damage response. In most cases of acute promyelocytic leukemia, the *PML* and retinoic acid receptor alpha (*RARA*) genes are translocated, resulting in expression of oncogenic PML-RARα fusion proteins. PML-NB fail to form normally, and promyelocytes remain in an undifferentiated, abnormally proliferative state. We examined the involvement of PML protein and PML-NB in homologous recombinational repair (HRR) of chromosomal DNA double-strand breaks. Transient overexpression of wild-type PML protein isoforms produced hugely enlarged or aggregated PML-NB and reduced HRR by ~2-fold, suggesting that HRR depends to some extent upon normal PML-NB structure. Knockdown of PML by RNA interference sharply attenuated formation of PML-NB and reduced HRR by up to 20-fold. However, PML-knockdown cells showed apparently normal induction of H2AX phosphorylation and RAD51 foci after DNA damage by ionizing radiation. These findings indicate that early steps in HRR, including recognition of DNA double-strand breaks, initial processing of ends, and assembly of single-stranded DNA/RAD51 nucleoprotein filaments, do not depend upon PML-NB. The HRR deficit in PML-depleted cells thus reflects inhibition of later steps in the repair pathway. Expression of PML-RARα fusion proteins disrupted PML-NB structure and reduced HRR by up to 10-fold, raising the possibility that defective HRR and resulting genomic instability may figure in the pathogenesis, progression and relapse of acute promyelocytic leukemia. J. Cell. Biochem. 113: 1787–1799, 2012.

KEY WORDS: ACUTE PROMYELOCYTIC LEUKEMIA; PML NUCLEAR BODIES; DNA REPAIR; HOMOLOGOUS RECOMBINATION; ALL-TRANS RETINOIC ACID; ARSENIC TRIOXIDE

Promyelocytic leukemia nuclear bodies (PML-NB) are non-membrane-bound organelles within the nuclei of mammalian cells. PML-NB are also known as PML-oncogenic domains (PODs), nuclear domains-10 (ND10), and Kremer (Kr) bodies [Melnick and Licht, 1999]. PML-NB are not present in all tissue cell types and are not essential for cell survival, but they are implicated in many critical cellular processes relevant to tumor suppression, including induction of apoptosis, cell cycle checkpoints and senescence, transcriptional regulation, hormone signaling, protein degradation and post-translational modification, replication of chromosomal and viral DNA, chromatin remodeling, and DNA damage response and repair [Bernardi and Pandolfi, 2007; Lallemand-Breitenbach and de The, 2010]. Complete or partial loss of PML protein expression or PML-NB has been reported in several other tumor types in addition to acute promyelocytic leukemia [Gurrieri et al., 2004; Vincenzi et al., 2010].

PML protein is the primary structural component of PML-NB [Ishov et al., 1999]. The *PML* gene comprises nine exons that are alternatively spliced to yield seven groups of PML protein isoforms (PML-I to PML-VII). All PML isoforms share an N-terminal portion that includes the RBCC region: a RING-finger domain, two B-boxes and an α-helical coiled-coil domain. The RBCC region is required for PML multimerization and hence PML-NB formation, as well as the growth suppressing, pro-apoptotic and antiviral activities of PML

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[Jensen et al., 2001]. Differences among PML isoforms in the central and C-terminal regions confer specific protein binding and functional activities [Jensen et al., 2001]. All isoforms undergo sumoylation, and isoforms PML-I to PML-V also bind SUMO non-covalently via a SUMO-interacting motif.

The *PML* gene was originally cloned from breakpoints of the characteristic t(15;17) chromosomal translocations that occur in over 95% of acute promyelocytic leukemia (APL) patients [Kakizuka et al., 1991]. The translocated *PML* gene becomes fused to the retinoic acid receptor α gene (*RARA*), leading to expression of PML-RARα fusion proteins. Typically only one chromosome 15 and one chromosome 17 are translocated, and wild-type PML and RARα proteins continue to be expressed from the unaffected homologs. The leukemogenic effects of PML-RARα fusion proteins are thus dominant-negative [Brown et al., 1997]. In APL cells, PML-RARα fusion proteins form homodimers that bind retinoic acid response elements in chromosomal DNA. These protein-DNA interactions recruit corepressors, leading to blockage of target gene transcription [Vitoux et al., 2007]. PML-RARα fusion proteins also competitively inhibit binding between wild-type PML proteins, leading to disruption of discrete PML nuclear bodies into a characteristic pattern of dispersed nuclear "microspeckles." One normal activity of PML-NB is to mediate transcriptional control of differentiation by retinoic acid [Wang et al., 1998]. PML-RARα fusion proteins thus inhibit differentiation of promyelocytes, allowing their continued proliferation [Brown et al., 1997; Wang and Chen, 2008].

Many proteins involved in DNA damage response and DNA repair localize to PML-NB either constitutively or conditionally, including the DNA damage-sensing proteins ATM, ATR, BRCA1, Chk2, p53, MRN complex (MRE11, RAD50, and NBS1) and topoisomerase II binding protein-1 (TopBP1), as well as multiple proteins that participate in homologous recombinational repair (HRR) of DNA double-strand breaks, including the single-strand DNA binding protein RPA, the strand transferase RAD51, the RecQ-family helicases WRN and BLM, topoisomerase 3α (TOP3a) and the recombinational mediators RAD52 and BRCA2 [Yeager et al., 1999; Henson et al., 2002; Dellaire et al., 2003; Xu et al., 2003; Spardy et al., 2008]. PML-NB are required for regulation of the p53-induced apoptotic pathway after DNA damage [Bernardi et al., 2004], and PML-deficient cells show elevated frequencies of sister chromatid exchange [Zhong et al., 1999], indicating increased replication stress (or altered response to it). PML-NB increase in number and change their subnuclear distribution in response to DNA damage, and this response is dependent upon early DNA break-sensing proteins, leading to the suggestion that PML-NB may act to transduce DNA damage signaling [Dellaire et al., 2006a]. After DNA damage by ultraviolet radiation, foci of single-stranded DNA become co-localized with PML-NB [Bøe et al., 2006]. Because of these and other observations, PML-NB have been proposed to assist in repair of DNA double-strand breaks caused by stalled replication forks or exogenous DNA damage [Dellaire et al., 2006b]. PML-NB are also associated with alternative lengthening of telomeres (ALT), a homologous recombinational pathway used by some cells to maintain their telomeres independent of telomerase activity. In ALT cells, a subset of PML-NB colocalize with telomeric DNA, together with the telomere-binding proteins TRF1 and TRF2, and many of the HRR proteins listed above [Bryan et al., 1995].

Despite this growing body of correlative observations, it has been unclear whether or to what extent HRR (or DNA double-strand break repair more generally) is dependent upon PML protein or PML-NB. In this study, we used direct assays for HRR of a specifically induced chromosomal DNA double-strand break to investigate the involvement of PML protein and PML-NB. We perturbed PLM function in three ways: by depleting PML protein through RNA interference, by over-expressing wild-type PML isoforms, and by expressing PML-RARα fusion proteins cloned from APL patients. In each case, formation of PML-NB was altered and HRR was compromised, albeit to differing extents. Depletion of PML protein or expression of PML-RARα fusion proteins had comparably severe effects, and the deficit in HRR that accompanied PML-RARα expression was not effectively rescued by treatment of cells with all-trans retinoic acid (ATRA) or arsenic trioxide (ATO), two front-line drugs in therapy for APL. Our results are direct confirmation that fully efficient HRR is dependent upon PML proteins and/or PML nuclear bodies, and have implications for the pathogenesis and treatment of APL.

RESULTS

PML PROTEIN AND/OR NORMALLY FORMED PML-NB ARE REQUIRED FOR FULLY EFFICIENT HRR

To interrogate the involvement of PML protein and PML-NB in HRR, we used a previously established recombination assay in the human cell line HT1885 [Lio et al., 2004]. HT1885 was derived from the fibrosarcoma line HT1080, by stable chromosomal integration of an HRR reporter construct, which is diagrammed in Figure 1. The reporter construct consists of two defective puromycin-resistance (*Puro*) genes arranged so that homologous recombination between them can reconstruct a fully functional gene, conferring puromycin resistance on the cell. The assay system uses transient expression of the site-specific endonuclease *I-SceI* to initiate HRR, by causing a DNA double-strand break within the reporter construct. Frequencies of HRR after expression of *I-SceI* are three to four orders of magnitude greater than background so that, in practice, essentially all detected HRR events are specifically induced by *I-SceI* cleavage.

To assess the importance of PML protein for HRR, we reduced PLM levels through RNA interference. A lentiviral vector expressing short hairpin-forming RNA against PML message was used to generate stably transduced clones of HT1885. The same vector without an shRNA insert was used to generate control clones. Stably transduced clones were assessed for PML knockdown in two ways: by immunofluorescence microscopy to visualize PML-NB formation and by western blotting to determine relative protein levels. Empty vector transduction did not affect PML-NB formation, as compared to parental, non-transduced HT1885 cells (data not shown). Results for two representative knockdown clones, designated shPML-1 and shPML-2, and one empty-vector control clone, shEV, are shown in Figure 2. In shPML-1 cells, PML-NB were visible, but their numbers and size were reduced relative to controls (Fig. 2A). In shPML-2 cells, the knockdown effect was more pronounced, so that no PML-NB were visible in most cells. Correspondingly, PML protein levels were reduced in both knockdown clones relative to controls, but to a

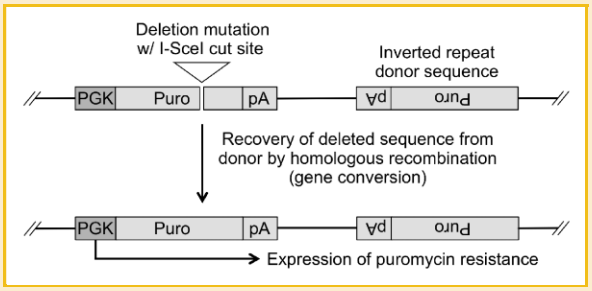

Fig. 1. Cellular assay for homologous recombinational repair (HRR). HT1885 cells are a clonal derivative of the HT1080 human fibrosarcoma cell line, in which the HRR reporter construct shown here has been chromosomally integrated. The reporter construct comprises two defective copies of a puromycin resistance gene (*Puro*) configured as inverted repeats. Each *Puro* repeat is coupled to the polyadenylation signal region of SV40 virus. The left *Puro* gene is under transcriptional control of the murine *PGK* enhancer/promoter, but does not produce a functional protein because 80 bp of coding sequence have been deleted and replaced with an 18 bp recognition sequence for the site-specific endonuclease *I-SceI*. The right *Puro* gene has an intact coding sequence but no promoter, and is transcriptionally silent. *I-SceI* endonuclease is transiently expressed from a transfected plasmid vector, and makes staggered cuts within its unique recognition site. The resulting DNA double-strand break can be repaired by homologous recombination, using the inverted repeat as a source of sequence information, to reconstruct a complete, active *Puro* gene. Cells in which the *Puro* gene has been reconstructed are then scored by formation of puromycin-resistant colonies in selective medium. The assay is specific for repair by homologous recombination. Repair of the *I-SceI* site double-strand break by single-strand annealing is precluded by the inverted configuration of the repeats. The break can be repaired by non-homologous end joining, but these repair events cannot recover the deleted coding sequence or reconstruct a functional *Puro* gene, and are not detected by the assay.

greater extent in shPML-2 cells than in shPML-1 cells (Fig. 2B). *I-SceI* endonuclease expression vector was transfected into shEV and shPML cells, and HRR frequencies were measured by puromycin-resistant colony formation. The frequency of break-induced HRR was reduced by about 3-fold in shPML-1 cells, and by 21-fold in shPML-2 cells, as compared to shEV cells (Fig. 2C). We infer that the more severe HRR reduction in shPML-2 cells than in shPML-1 cells is related to stronger suppression of PML protein expression and/or PML-NB formation. The reduced HRR in cells depleted of PML is evidently not caused by reduced viability or cell cycle arrest. Parallel measurements of cloning efficiency revealed no significant difference between shEV and shPML cells (data not shown). Comparison of cell cycle phase distributions in the shEV and shPML-2 clones by flow cytometry revealed only a modest shift in the deeply depleted shPML-2 cells, with G1, S, and G2/M fractions of 61.6%, 18.1%, and 19.5%, as compared to 51.5%, 23.8%, and 24.3% in shEV cells (Supplementary Fig. 2). These results indicate that depletion of PML protein reduces formation of PML-NB in HT1885 cells and concomitantly inhibits DNA double-strand break repair by homologous recombination.

The normal formation of PML-NB can be critical for the functional activity of PML itself and PML-NB interacting proteins. Overexpression of PML has been shown to cause formation of abnormal PML bodies [Beech et al., 2005], and we wished to determine whether this disturbance also affects HRR. We tested two representative PML isoforms, IV and VI. Of the six nuclear isoforms (PML-I to PML-VI), PML-IV was chosen because it specifically interacts with p53, and this interaction is critical for MDM2-mediated p53 degradation upon DNA damage [Fogal et al., 2000; Bernardi et al., 2004]. The p53 protein is a major effector of DNA damage response and known to influence HRR [Gatz and Wiesmüller, 2006]. PML-VI is the shortest of the nuclear isoforms of PML, the only one lacking the SUMO-interacting motif encoded by exon 7a, and presumably has the most restricted repertoire of interactions with other nuclear proteins.

Expression vectors for HA-tagged PML-IV or PML-VI were transiently transfected into HT1885 cells, and formation of PML-NB was examined by immunofluorescence microscopy. Anti-PML and anti-HA antibodies were used to detect all PML proteins or only the exogenous HA-PML, respectively. Overexpression of either PML isoform IV or VI resulted in PML-NB that were reduced in number but hugely enlarged, and endogenous PML protein was sequestered into the enlarged PML bodies. Representative images for cells overexpressing PML-VI are shown in Figure 3A. We then tested whether PML overexpression affects the efficiency of HRR. Expression vectors for PML-IV or PML-VI, or empty vector, were co-transfected with I-SceI expression vector into HT1885 cells and HRR frequencies were measured. The expression of I-SceI endonuclease was not altered by PML overexpression (Supplementary Fig. S1). Overexpression of either PML-IV or PML-VI was accompanied by a small (~1.5-fold) but reproducible and statistically significant drop in the frequency of HRR, as compared to empty-vector controls (Fig. 3B). The data shows that PML-IV and PML-VI overexpression alters PML-NB morphology and these changes may in fact inhibit HRR, though not as severely as PML knockdown. The SUMO-interacting motif encoded by exon 7a is not necessary for this effect, as it is not present in PML-VI. Taken together, our results suggest that the process of HRR depends to some extent upon normal PML-NB structure, and not merely on the presence of PML protein.

PML PROTEIN AND NUCLEAR BODIES ARE NOT REQUIRED FOR H2AX PHOSPHORYLATION OR RAD51 FOCI FORMATION AFTER DNA DAMAGE

We sought to learn whether PML plays a role in the initial sensing of DNA double-strand breaks, by examining the effects of PML knockdown on phosphorylation of histone H2AX after DNA damage. Twenty-four hours after plating on coverslips, cells were exposed to 8 Gy of X-irradiation, allowed to recover for 30 min, and then fixed. The formation of damage-induced foci was probed by staining with phospho-specific anti-H2AX antibodies and visualized by immunofluorescence microscopy. Individual cells were visually scored as positive for γH2AX foci if there was any clear γH2AX fluorescent signal within the nucleus (defined by DAPI staining), or negative for γH2AX foci if no fluorescent signal at all could be seen (Fig. 4A). Depletion of PML protein did not affect spontaneous H2AX phosphorylation, as the proportion of cells with γH2AX foci prior to irradiation was comparable between parental HT1885 and shEV, and shPML-2 cells (data not shown). H2AX phosphorylation can be induced by double-strand breaks resulting

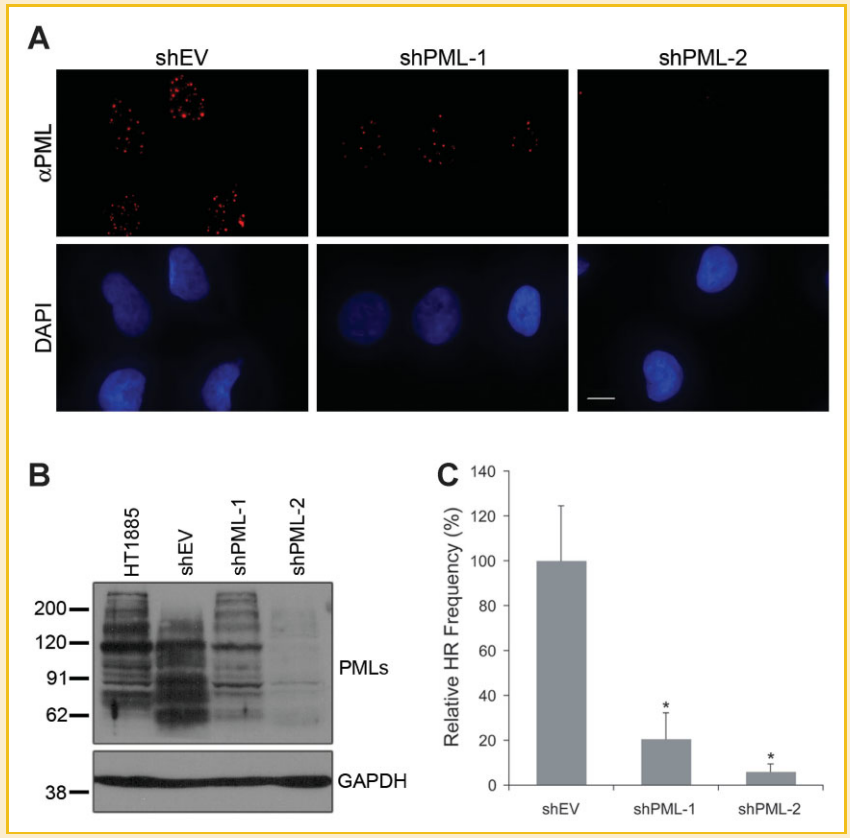

Fig. 2. PML knockdown by shRNA expression impairs the formation of PML-NB and sharply inhibits break-induced HRR. A: HT1885 cells were transduced with either parental pLL3.7 lentiviral vector (empty vector, shEV) or pLL3.7 expressing shRNA against PML (shPML). Transduced clones were isolated and analyzed by immunofluorescent staining with anti-PML monoclonal antibody, followed by Alexa-Fluor 594-conjugated secondary antibody (red). DAPI staining shows the nuclei. Scale bar: 10 μm. B: Protein status of PML in isolated clones was determined by western blotting. Proteins of whole cell lysates from parental HT1885, shEV, shPML1, and shPML2 cells were separated by SDS-PAGE and transferred to nitrocellulose membrane. All PML isoforms were detected with anti-PML polyclonal antibodies, followed by HRP-conjugated secondary antibody. GAPDH was detected as a loading control. C: Recombination frequencies in shEV, shPML1 and shPML2 cells. Expression vector for I-SceI endonuclease was transfected into shEV, shPML1 and shPML2 cells, and HRR frequencies were measured by scoring formation of puromycin-resistant colonies. Values shown are means (±SD) of two independent experiments, with each experiment performed in triplicate. HRR frequencies for shEV cells were normalized to 100%. *t*-Tests were performed to compare shPML1 and shPML2 cells to shEV; asterisk (*) indicates $P < 0.00001$.

from replication fork collapse during S-phase; so the small minority of non-irradiated cells that display γH2AX foci are likely S-phase cells (Fig. 4A). After radiation exposure, there was no obvious difference in γH2AX foci formation within PML-depleted cells as compared to parental HT1885 cells, as essentially all cells were γH2AX foci-positive in both populations (Fig. 4B). To quantitate intracellular levels of γH2AX, non-irradiated and irradiated cells (after 30 min recovery) were trypsinized, fixed and stained as above, then analyzed by flow cytometry. γH2AX levels in the shEV and shPML-2 cell populations were identical, and neither population was significantly different from parental HT1885 cells (Fig. 4C). It appears that PML protein and nuclear bodies are not required for the phosphorylation of H2AX in response to either spontaneous or IR-induced DNA breaks.

The RAD51 protein plays an essential role in strand invasion during HRR, and damage-induced RAD51 nuclear foci are believed to represent RAD51-ssDNA filament formation at the processed ends of double-strand breaks [Pellegrini et al., 2002]. RAD51 foci have been reported to localize at PML-NB in ALT cells [Yeager et al., 1999]. We inquired whether PML knockdown affects RAD51 foci formation in HT1885 cells with or without DNA damage. Cells were X-irradiated, or mock-irradiated, then fixed and processed for immunofluorescence microscopy after intervals of recovery ranging from 30 min to 8 h, and RAD51 foci were visually scored. Cells containing five or more discrete RAD51 foci were scored as foci-positive (Fig. 5A and B). Without irradiation, the proportion of cells showing RAD51 foci formation was not reduced in shPML-2 cells, as compared to parental HT1885 cells or shEV controls, indicating that PML-NB are not required for the RAD51 foci normally formed during DNA replication (Fig. 5C). After X-ray exposure, an increase in the proportion of cells with RAD51 foci was apparent across all three cell populations by 30 min, and continued over the following several hours. However, the time course of this increase was similar among parental HT1885 cells, shEV control cells, and shPML-2 knockdown cells, and there were no significant differences in the proportion of RAD51 foci-positive cells at any time point up to 8 h. Even though depletion of PML caused significant reduction of HRR in our model system (Fig. 2C), RAD51 foci formation was not

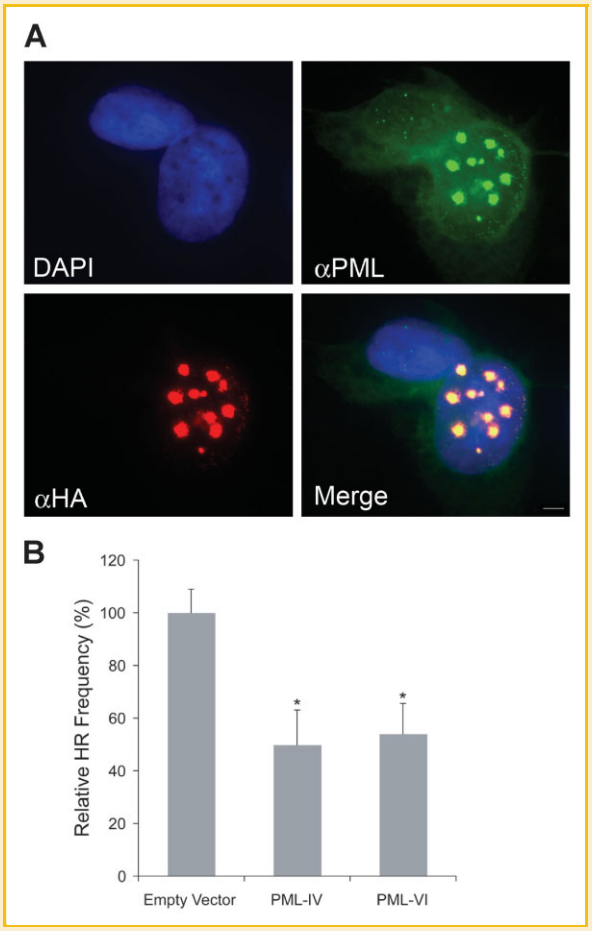

Fig. 3. PML overexpression induces grossly enlarged PML-NB and inhibits break-induced HRR. A: HT1885 cells were transiently transfected with HA-tagged PML isoform vectors and analyzed by immunofluorescent staining with anti-HA monoclonal antibody and anti-PML polyclonal antibody, and secondary antibodies conjugated with Alexa-Fluor 594 (red) or Alexa-Fluor 488 (green), respectively. DAPI staining shows the nuclei. Scale bar: 10 μm. B: HT1885 cells were co-transfected with I-SceI expression vector plus PML-IV or PML-VI expression vector, or empty vector, and HRR frequencies were measured by scoring formation of puromycin-resistant colonies. Values shown are means (±SD) of three independent experiments for PML-IV and six for PML-VI, each performed in triplicate. HRR frequencies for empty vector were normalized to 100%. *t*-Tests were performed to compare values for each isoform vector to equivalent numbers of empty vector determinations performed in parallel; asterisk (*) indicates $P < 0.005$.

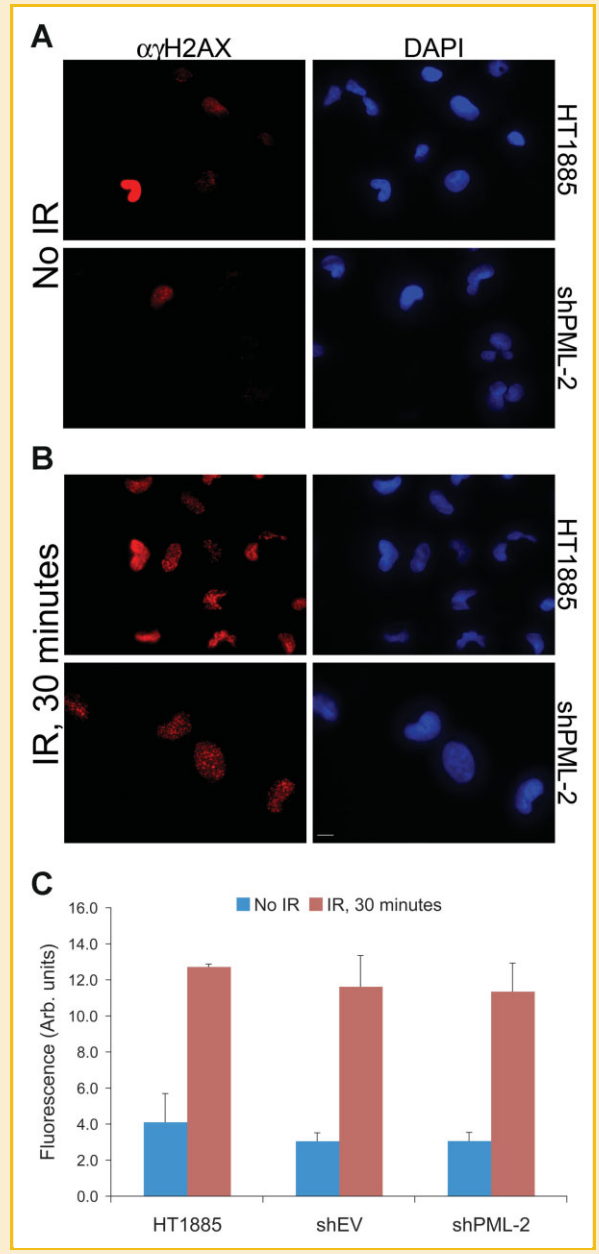

Fig. 4. PML knockdown does not affect the formation of γH2AX foci after X-irradiation. A, B: Immunofluorescence imaging of parental HT1885 and shPML2 cells were mock-exposed (A) or exposed to 8 Gy of X-irradiation followed by 30 min of recovery (B). Fixed cells were stained with anti-γH2AX monoclonal antibody followed by Alexa-Fluor 594-conjugated secondary antibody (red). Cell nuclei were stained with DAPI. Scale bar: 10 μm. C: Quantification of cellular γH2AX levels. Parental HT1885, shEV, and shPML-2 cells, were mock exposed or exposed and allowed to recover as above, then trypsinized, fixed, and stained with anti-γH2AX monoclonal antibody followed by Alexa-Fluor 594-conjugated secondary antibody. Cellular γH2AX levels were then measured by flow cytometry, with correction for background fluorescence (measured in control cells stained with secondary antibody only). Values shown are means (±SD) of three replicate experiments performed in parallel.

affected (Fig. 5C). Evidently, PML proteins and PML-NB are also not required for RAD51 foci formation after radiation-induced double-strand break damage. Together, the lack of change in γH2AX foci response and RAD51 foci response indicate that PML protein and nuclear bodies are not essential for the early events leading to repair of DNA double-strand breaks by HRR.

PML-RARα FUSION PROTEINS FROM ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS EXERT DOMINANT-NEGATIVE EFFECTS ON HRR

In acute promyelocytic leukemia, chromosomal t(15;17) translocations typically involve one of three breakpoint cluster regions (bcr)

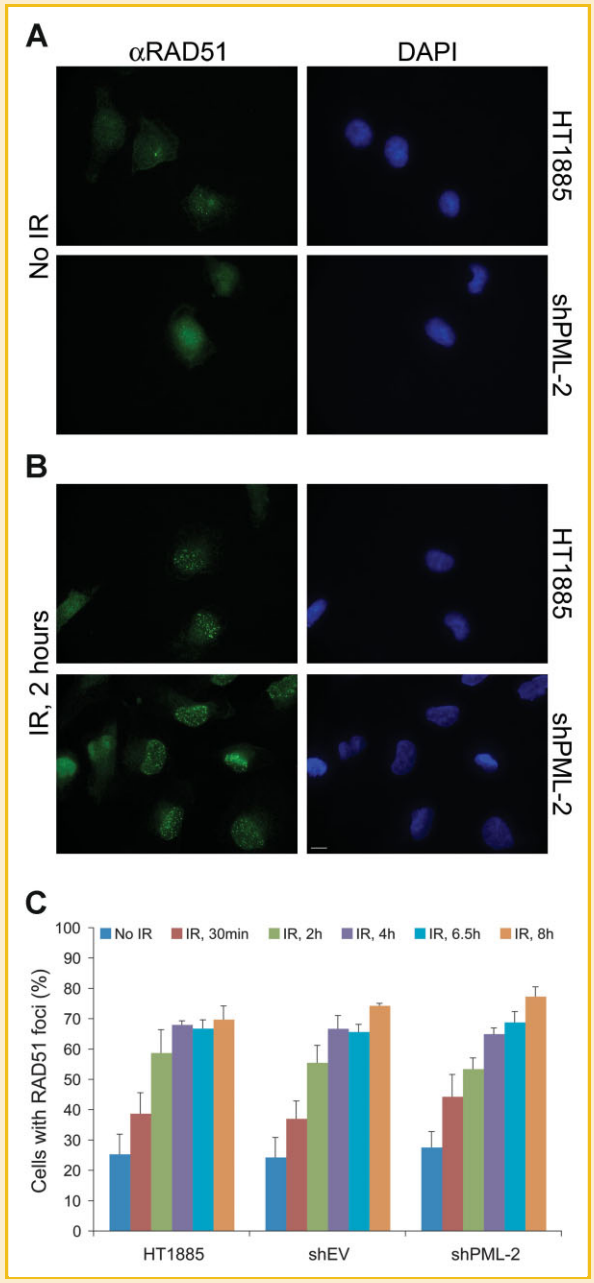

Fig. 5. PML knockdown does not affect the formation of RAD51 foci after X-irradiation. A, B: RAD51 foci were visualized in parental HT1885 cells and shPML-2 by immuno-fluorescent staining with anti-RAD51 polyclonal antibody, followed by Alexa-Fluor 488-conjugated secondary antibody. Cell nuclei were stained with DAPI. Cells in (B) were exposed to 8 Gy of X-irradiation followed by 2 h of recovery before fixation. Scale bar: 10 μm. C: Time course of RAD51 foci formation. Parental HT1885, shEV and shPML2 cells were fixed and stained without irradiation, or were exposed to 8 Gy of X-irradiation followed by recovery intervals of 2, 4, 6.5, or 8 h of recovery before fixation. Cells with at least five RAD51 foci were scored as RAD51 foci-positive. At least 300 cells were scored in each determination. Values graphed are means (±SD) of five to seven determinations for each time point.

within the *PML* gene on chromosome 15, whereas the breakpoint on chromosome 17 invariably disrupts the *RARA* gene within the second intron [Miller et al., 1992; Pandolfi et al., 1992]. The bcr1 and bcr3 correspond to *PML* introns 3 and 6, and the resulting two major isoforms of PML-RARα fusion protein are referred to as the short (S) and long (L) isoforms, respectively. The bcr2 spans *PML* exons 5 and 6, and the resulting PML-RARα fusions are referred to as V (variant) isoforms. L and S isoforms of PML-RARα fusion protein occur in approximately 55% and 40% of APL patients, respectively, while V isoforms occur in fewer than 5% of patients. Although differences between the two major isoforms in biological activity and APL phenotypes are subtle, early case reports indicated that patients with PML-RARα(S) fusions had higher rate of relapse and shorter durations of remission and survival compared to patients with L isoforms [Huang et al., 1993; Vahdat et al., 1994; Jurcic et al., 2001]. In a later study, PML-RARα(L), but not PML-RARα(S), directly promoted cell growth by altering chromatin structure and thereby activating the growth-promoting gene *c-fos* in response to mitogen stimulation [Tussie-Luna et al., 2006].

Because alteration of PML-NB in our system severely affected HRR, we hypothesized that disruption of PML nuclear bodies by PML-RARα fusion proteins might affect HRR as well. We first asked whether PML-RARα fusion protein expression disrupts PML-NB formation in HT1885 cells. HA-tagged PML-RARα(S) or PML-RARα(L) fusion protein expression vectors were transiently transfected into HT1885 cells, and PML-RARα fusion proteins were visualized by immunofluorescence microscopy. Both PML-RARα(S) and PML-RARα(L) fusion proteins disrupted PML-NB into a pattern of dispersed nuclear microspeckles (Fig. 6A) and endogenous PML was also dispersed into microspeckles (data not shown). To determine the effects of PML-RARα fusion proteins on HRR, and whether the short and long isoforms have differential effects, PML-RARα(S) or PML-RARα(L) expression vectors were transiently co-transfected with I-SceI expression vector into HT1885 cells, and HRR frequencies were measured (Fig. 6B). Both PML-RARα(S) and PML-RARα(L) reduced break-induced HRR by more than 5-fold compared to empty vector controls, and the HRR frequency reductions from the two isoforms were not significantly different. Even sharper reductions in HRR, of 10-fold or more, were measured in transfected clones of HT1885 cells selected for stable expression of PML-RARα fusions (Fig. 8). These results show that, although the short and long isoforms of PML-RARα may differentially affect cell survival and clinical course, they have essentially identical and strong dominant-negative effects on PML-NB formation and HRR. This is true even though wild-type PML protein is still present, which again suggests that HRR is to some extent dependent upon normally formed PML-NB rather than the presence of PML protein alone.

PML PROTEIN AND NUCLEAR BODIES ARE NOT REQUIRED FOR NON-HOMOLOGOUS END JOINING

Non-homologous end joining (NHEJ) is an alternative pathway to HRR for repair of chromosomal double-strand breaks, and is highly active in most mammalian cells. It is also involved in stable integration of transfected DNA into chromosomal DNA (non-homologous vector integration), presumably by joining the ends of

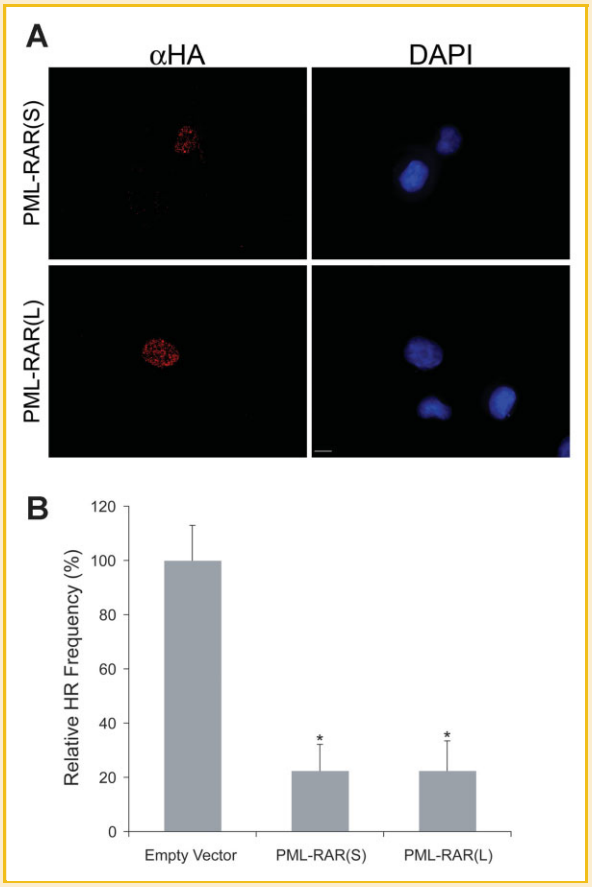

Fig. 6. PML-RARα fusion proteins disrupt PML-NB formation and inhibit break-induced HRR. A: HT1885 cells were transiently transfected with either HA-tagged PML-RARα(S) or HA-tagged PML-RARα(L) and analyzed by immunofluorescent staining with anti-HA monoclonal antibody, followed by Alexa-Fluor 594-conjugated secondary antibody (red). DAPI staining shows the nuclei. Scale bar: 10 μm. B: Recombination frequency in HT1885 cells transiently expressing PML-RARα fusions. PML-RARα(S) or PML-RARα(L) expression vector, or empty vector was co-transfected with I-SceI expression vector into HT1885 cells, and HRR frequencies were measured by puromycin-resistant colony formation. Values shown are means (±SD) of four independent experiments for PML-RARα(S) and eight for PML-RARα(L), each performed in triplicate. HRR frequencies for empty vector transfections were normalized to 100%. *t*-Tests were performed to compare HRR frequencies for each PML-RARα isoform to an equivalent number of empty-vector determinations performed in parallel. Asterisks (*) indicate $P < 0.000001$.

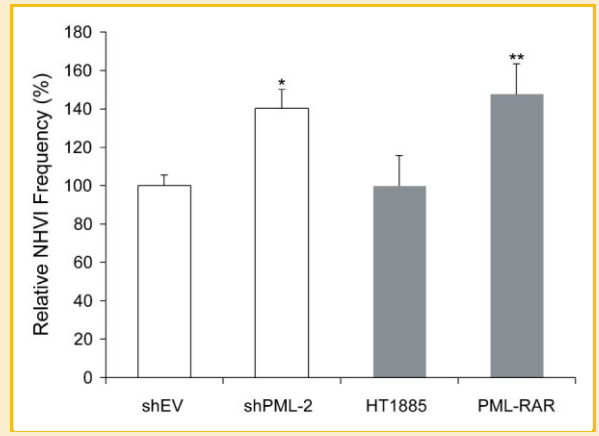

Fig. 7. PML-NB are not required for non-homologous end joining. Non-homologous vector integration (NHVI) frequencies in shEV vs. shPML2 cells, and in parental HT1885 cells vs. cells stably expressing PML-RARα(S). A plasmid vector with no sequence homology to the human genome, pMC1neo-polyA, was linearized with Xmn-I endonuclease and transfected into each cell population. Frequencies of NHVI were measured by scoring formation of neomycin (G418)-resistant colonies. Values shown are means (±SD) of three replicate transfection and plating experiments performed in parallel for each cell line. Mean NHVI frequency for shEV cells or parental HT1885 cells was normalized to 100%. *P* values: * < 0.01 for shPML2 compared to shEV; ** < 0.001 for PML-RARα(S) compared to parental HT1885 (*t*-tests).

linearized vector DNA to spontaneous chromosomal breaks [Hromas et al., 2008]. To determine whether PML nuclear bodies play any part in DNA repair by NHEJ, we assayed non-homologous vector integration in HT1885 cells stably transfected for PML knockdown or expression of PML-RARα fusion proteins. A small plasmid vector, pMC1neo-polyA, which has no homology to the human genome, was linearized and transfected into HT1885 cells, and integration frequencies were measured by scoring formation of neomycin (G418)-resistant colonies. As shown in Figure 7, vector integration was not inhibited by either PML knockdown or PML-RARα(S) fusion protein expression. Instead, both PML knockdown and fusion protein expression were accompanied by slightly increased vector integration (1.4 and 1.5-fold, respectively). The lack of any negative effect on vector integration suggests that PML protein and PML-NB have little or no part in repair of chromosomal double-strand breaks by NHEJ.

ALL-TRANS RETINOIC ACID (ATRA) AND ARSENIC TRIOXIDE (ATO), TWO EFFECTIVE THERAPIES FOR ACUTE PROMYELOCYTIC LEUKEMIA, FAIL TO RESCUE HRR IN CELLS EXPRESSING PML-RARα FUSION PROTEINS

ATRA has been used to treat APL since 1985. ATRA treatment restores PML-NB formation and induces promyelocyte differentiation [Wang and Chen, 2008]. Our data suggest disruption of PML-NB structure is related to reductions in HRR efficiency, and so we hypothesized that ATRA treatment might also rescue HRR inhibition by PML-RARα fusions. To test this, recombination assays were done in HT1885 cells that had been stably transfected and selected for sustained expression of PML-RARα(S). Cells were pre-treated overnight with 1.0 μM ATRA or solvent (DMSO) alone, and then I-SceI expression vector was transfected, with ATRA/DMSO treatment continuing until cells were replated for colony formation on the following day. As shown in Figure 8A, stable expression of PML-RARα(S) reduced HRR frequency by ~10-fold as compared to parental HT1885 cells (when both received DMSO alone). With ATRA treatment, cells expressing PML-RARα(S) still showed ~4-fold reduction of HRR compared to parental HT1885 cells treated with DMSO alone, and ~3-fold reduction relative to parental HT1885 treated with ATRA. Treatment with ATRA reduced the frequency of HRR in parental HT1885 cells slightly (~1.2-fold), which may reflect some degradation of endogenous PML upon ATRA treatment [Isakson et al., 2010]. Thus, ATRA treatment gave,

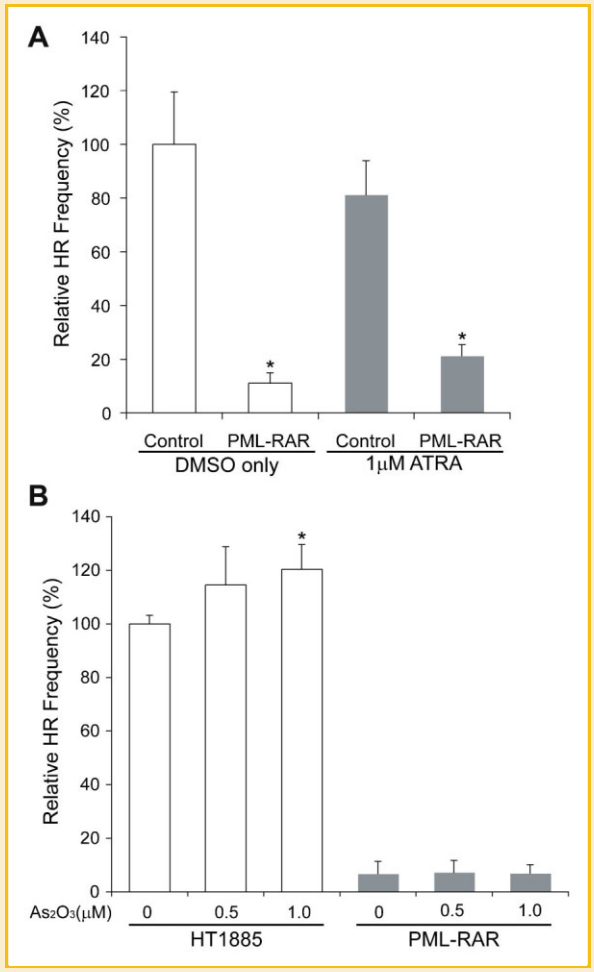

Fig. 8. Inhibition of HRR in cells expressing PML-RARα fusion protein is not effectively rescued by ATRA or arsenic trioxide. A: Frequencies of break-induced HRR in HT1885 cells stably expressing PML-RARα(S) fusion protein were compared those of parental HT1885 cells, with and without ATRA treatment. Cells were pretreated for 16 h with 1.0 μM ATRA or an equivalent amount of solvent (DMSO), then transfected for transient expression of I-SceI endonuclease, with continuing ATRA/DMSO treatment for another 24 h until cells were replated for colony formation. Relative HRR frequencies graphed are means (±SD) of two independent experiments, each done in triplicate. Values for parental HT1885 cells treated with DMSO alone were normalized to 100%. HRR frequency was reduced in cells stably expressing PML-RARα(S) relative to parental HT1885, in the presence of ATRA or DMSO alone. Asterisk (*) indicates $P < 10^{-6}$ (*t*-test). In cells stably expressing PML-RARα(S), HRR was reduced about 2-fold less with ATRA treatment as compared to DMSO alone ($P < 0.002$, *t*-test), but was still nearly 4-fold lower than HRR in parental HT1885 cells. B: Frequencies of break-induced HRR in HT1885 cells stably expressing PML-RARα(S) fusion protein were compared those of parental HT1885 cells, with and without arsenic trioxide (ATO) treatment. Cells were treated for 2 h with 0.5 μM ATO or 1.0 μM ATO or mock-treated with an equivalent volume of solvent (0.1 N NaOH), then transfected for transient expression of I-SceI endonuclease, with continuing ATO treatment for another 24 h until cells were replated for colony formation. Relative HRR frequencies graphed are means (±SD) of two independent experiments, each done in triplicate. Values for parental HT1885 cells treated with solvent alone were normalized to 100%. In cells stably expressing PML-RARα(S), HRR frequency was not increased by ATO treatment as compared to solvent alone. In parental HT1885 cells treated with 1.0 μM ATO, HRR frequency was increased about 20% relative to solvent alone. Asterisk (*) indicates $P < 0.005$ (*t*-test).

at best, a weak partial rescue of the lost HRR proficiency in cells expressing PML-RARα fusions.

Arsenic trioxide, As_2O_3 (ATO), was reported as a highly successful treatment for APL by Chinese clinicians in the mid-1990s. ATO causes rapid degradation of PML-RARα fusion proteins followed by partial differentiation and/or apoptosis of APL cells [Chen et al., 1996]. HT1885 cells stably expressing PML-RARα(S) fusion protein were tested to see whether ATO might rescue HRR. Treatment with ATO at 0.5 or 1.0 μM was begun 2 h prior to transfection with I-SceI expression vector and continued until cells were replated for selection of recombinants on the following day. In parental HT1885 cells treated with ATO, HRR frequency was modestly increased, and the difference became significant in cells treated with 1.0 μM. ATO. However, ATO treatment produced no improvement of the depressed HRR levels in cells stably expressing PML-RARα(S).

DISCUSSION

HRR DEPENDENCE UPON PML

In this study, we have shown that normal HRR function is dependent upon PML protein and/or structurally normal PML-NB. This conclusion was arrived at using three approaches: RNA interference to deplete PML protein levels, over-expression of wild-type PML isoforms, and expression of dominant-negative PML-RARa fusion proteins cloned from APL patients.

Intracellular levels of PML protein affect the formation of PML-NB, and either depletion or elevation of PML can affect the functions of NB-localizing proteins [Li et al., 2000; Haupt et al., 2009]. Depletion of all PML protein isoforms is arguably the most straightforward test of PML involvement in HRR, and our knockdown results indicate clearly that PML protein has an important function that supports HRR (Fig. 2), though this function may well be indirect and exerted through its role in PML-NB.

Transient overexpression of PML isoforms induces formation of PML-NB that are hugely enlarged but fewer in number than normal PML-NB (compare Figs. 2A and 3A) and in this sense, interrogates the importance of normal PML-NB structure [Beech et al., 2005]. Proteins that normally localize to PML-NB can become sequestered within enlarged PML-NB, inhibiting their normal activities [Li et al., 2000; Lin et al., 2003]. Transcriptional repression activity of DAXX, for example, can be attenuated by PML overexpression in this way [Li et al., 2000]. The comparatively mild effects on HRR we measured with PML overexpression (Fig. 3B) might reflect partial sequestration of one or more HRR proteins.

Expression of dominant-negative PML-RARα fusion proteins disrupts PML-NB structure in a different way. The fusion proteins form heterodimers and/or aggregates with wild-type PML isoforms, competitively inhibiting normal PML interactions and causing dispersion of PML-NB into smaller nuclear "microspeckles" [Melnick and Licht, 1999]. Dominant-negative expression is thus a test for the importance of structure, in the sense that endogenous wild-type PML protein is still present, but normally formed PML-NB are reduced or absent. Inhibition of HRR by PML-RARα fusions (Fig. 6) may reflect both disruption of PML-NB structure and

competitive inhibition of normal interactions between PML and HRR proteins.

Together, this series of results indicate that fully competent HRR is dependent on PML protein, but that it also depends upon the higher-order structure of normally formed PML-NB. The challenge ahead will be to dissect the mechanistic role(s) of PML isoforms and PML-NB in HRR. We consider that it may be an indirect one, and reflective of a more general function for PML and PML-NB in governing the timely recruitment and/or removal of many key catalytic or regulatory nuclear proteins at critical stages of the molecular pathways they participate in.

FUNCTIONAL SPECIFICITY OF PML-NB IN DNA DOUBLE-STRAND BREAK REPAIR

In this study, PML knockdown did not impair non-homologous integration of transfected vector DNA into chromosomal DNA (Fig. 7), and this implies that PML protein and PML-NB are not required for repair of chromosomal breaks by non-homologous end joining. The modest increases seen in vector integration may reflect impaired healing of chromosomal breaks by HRR, which could make chromosomal break ends more readily available for joining to vector ends. Previous studies have shown that inhibiting expression of NHEJ proteins increases the frequency of HRR, which indicates that an equilibrium normally exists between the two repair pathways [Allen et al., 2003; Shrivastav et al., 2009]. Our results suggest the corresponding possibility that cells impaired for HR tend to compensate through increased reliance on NHEJ. The lack of a requirement for PML in NHEJ suggests that the functions of PML protein or PML-NB in DNA double-strand break repair are specific to the HRR pathway.

PML proteins and PML-NB were not required for the initial sensing of chromosome breaks at the level of H2AX phosphorylation, as formation γH2AX foci after X-irradiation appeared normal in PML knockdown cells (Fig. 4). This is somewhat surprising, because a number of proteins involved in early response to DNA double-strand breaks associate with PML-NB: the ATM and ATR kinases, RPA, the MRN complex, TopBP1 [Xu et al., 2003]. However, the lack of effect on H2AX phosphorylation does not preclude a role for PML protein or PML-NB in other aspects of damage response that involve these co-localizing proteins.

Neither was PML required for the initial steps of HRR, as reflected by damage-induced formation of RAD51 foci. The percentage of PML-knockdown cells with RAD51 foci was comparable to parental HT1885 cells up to 8 h after X-irradiation (Fig. 5). This is remarkable because other studies in which both HRR efficiency and RAD51 foci response were measured have generally found that the two endpoints correlate, i.e., reduced HRR is accompanied by reduced induction of RAD51 foci [Lu et al., 2005; Takizawa et al., 2010]. We are aware of no previously reported exceptions to this trend. However, RAD51 foci are widely presumed to represent the formation of ssDNA/RAD51 nucleoprotein filaments, which is an early step in HRR. Subsequent steps, including homology searching, strand invasion of a duplex DNA donor molecule, extension of 3' ends by DNA synthesis and resolution of the resulting heteroduplex DNA intermediates, are all necessary to consummate the repair process. Our results should therefore not be taken to discredit RAD51 foci as marker for HRR. Rather, they make a cautionary point about relying on RAD51 foci response alone as an unequivocal indicator of cellular competence for HRR. While it is likely true that cells deficient in RAD51 foci response are invariably compromised for HRR, it is now clear that the converse is not true: an apparently normal RAD51 foci response does not assure full competence for HRR.

Our results indicate that one or more later steps of HRR, occurring after the initial formation of ssDNA/RAD51 nucleoprotein filaments, is facilitated by PML protein and/or PML nuclear bodies. Several previous observations lend support to this interpretation. The BLM and WRN proteins are members of the widely conserved ReqQ family of helicases, which function during HRR by promoting resolution of heteroduplex DNA intermediate structures into completed repair products. Both proteins can physically interact with PML and with RAD51 [Bischof et al., 2001; Eladad et al., 2005; Otterlei et al., 2006; Liu et al., 2011]. BLM becomes heavily concentrated in PML-NB during replication and in response to radiation-induced double-strand breaks [Zhong et al., 1999; Bischof et al., 2001]. WRN redistributes after DNA damage, moving from a predominantly nucleolar localization into the nucleoplasm, where it partially colocalizes with PML-NB [Liu et al., 2011]. In PML−/− mouse embryonic fibroblasts, the frequency of sister chromatid exchange in metaphase cells was increased more than 2-fold relative to PML+/+ controls [Zhong et al., 1999], suggesting a defect in normal resolution of replication-associated HRR intermediates, analogous to that seen in Bloom syndrome [Wu and Hickson, 2003; Heyer, 2004].

IMPLICATIONS FOR APL

Defects in HRR and resulting genomic instability can be major drivers of tumorigenesis. Early-onset familial breast cancer associated with *BRCA1* or *BRCA2* mutation is the best-known example of this, but disturbances of HRR are known or presumed to contribute to several other cancer predisposition syndromes as well, including Bloom syndrome (*BLM* mutation) Werner syndrome (*WRN* mutation), Nijmegen breakage syndrome and AT-like syndrome (mutations of the MRN complex proteins NBS1 and MRE11, respectively) [Helleday, 2010]. Fanconi anemia predisposes affected children to acute myelogenous leukemia, and illustrates the susceptibility of myeloid lineage cells to deficiency in HRR. Mutations in any of the 13 Fanconi anemia (*FANC*) genes identified so far cause defects in repair of DNA interstrand crosslinks (which involves HRR), and two of the Fanconi genes encode critical HRR proteins, i.e., BRCA2 and PALB2 [D'Andrea, 2010].

Compromised ability to maintain genome stability has been suggested as part of APL pathogenesis on the basis of studies in cells from APL patients. The immortalized APL cell line NB4 [Bischof et al., 2001], isolated from a patient after second relapse, displays sub-tetraploid and highly variable karyotypes, with chromosome numbers ranging from ~ 60–90 [Lanotte et al., 1991]. Our results suggest the possibility that HRR deficiency induced by PML-RARα fusion proteins might have some part in APL leukemogenesis, progression and relapse.

Therapy with all-trans retinoic acid (ATRA) and/or arsenic trioxide (ATO) has been effective in driving APL into remission

[Wang et al., 2011]. ATRA acts as ligand of PML-RARα, driving dissociation of transcriptional corepressor complexes and recruitment of coactivator complexes, and hence restores transactivation of RARα target genes. In this way, ATRA promotes promyelocyte maturation by inducing G1 arrest and terminal differentiation [Wang et al., 2006]. ATO induces promyelocyte differentiation and/or apoptosis by directly binding to PML and PML-RARα fusion proteins, leading to their sumoylation and aggregation [Zhang et al., 2010]. Both ATRA and ATO treatments induce degradation of PML-RARα fusion proteins by autophagy [Isakson et al., 2010]. However, in HT1885 cells expressing PML-RARα fusions, treatment with ATRA produced only a weak partial reversal of HRR deficiency (Fig. 8A). The concentration of ATRA used, 1 μM, has been effective in restoring PML-NB formation, cellular differentiation and growth control in previous cell culture studies with the NB4 cell line [Lanotte et al., 1991; Duprez et al., 1996]. Treatment with ATO at 0.5 or 1.0 μM failed to rescue HRR deficiency in any measure (Fig. 8B), even though these concentrations are sufficient to halt growth of NB4 cells [Chen et al., 1996].

If blast cells in APL patients are also deficient in HRR, therapy with ATRA or ATO would likely not correct this. Ongoing HRR deficiency in any residual population of cells surviving ATRA or ATO treatment would tend to accelerate accumulation of further mutations, ultimately increasing the likelihood of relapse with resistance to therapy. However, if HRR deficiency can be confirmed in blast cells of APL patients, it may also present an opportunity to augment current therapies with drugs that exploit this weakness, including poly-ADP-ribose polymerase (PARP) inhibitors and DNA interstrand-crosslinking agents. Our findings call for re-examination of the pathogenesis of APL and its relapse after primary treatment, with new attention to the potential contribution of HRR deficiency and resulting genomic instability.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

HT1885 human fibrosarcoma cells and HEK293FT lentiviral packaging cells were grown at 37°C with 5% CO_2 in Alpha-MEM medium (αMEM) (Life Technologies, Inc.) supplemented with 9% fetal bovine serum (FBS) (Serum Source International, Inc.). Cell transfections were done using TransIT-LT1 transfection reagent (Mirus Biotech, Inc.), except for lentiviral packaging, which was done by calcium phosphate co-precipitation. Antibodies used in this study were the monoclonal PG-M3 and polyclonal H-238 antibodies against PML (Santa Cruz Biotechnology), the polyclonal anti-RAD51 antibody H-92 (Santa Cruz Biotechnology), the monoclonal anti-phospho-histone H2AX antibody JBW301 (Millipore, Inc.), the monoclonal anti-hemagglutinin (HA) epitope antibody 12CA5 (Roche, Inc.), the horseradish peroxidase-conjugated GAPDH antibody ab9385 (Abcam, Inc), and anti-rabbit IG (Novus, Inc.) and anti-mouse IgG (Santa Cruz Biotechnology) secondary antibodies.

PLASMID EXPRESSION VECTORS

Isoforms of human PML and PML-RARα cDNA used in this study were originally isolated from human bone marrow [Kakizuka et al., 1991]. Vectors for HA-PML-VI, HA-PML-RARα(S) and HA-PML-RARα(L) contain an optimized N-terminal translation start site and an HA tag (MDYPYDVPDY) linked to the indicated PML sequences. All PML and PML-RARα cDNA constructs were cloned in the pCMX vector, except PML-IV in the pCDNA3 vector. The lentiviral vector pLL3.7 was created by Dr. Van Parijs [Rubinson et al., 2003], and carries the mouse U6 promoter for shRNA expression and an enhanced green fluorescent protein (EGFP) cassette. The pLL3.7-shPML vector expresses shRNA against nucleotides 318–336 of the PML mRNA (5′-CGTCTTTTTCGAGAGTCTGTT-3′), which is present in all isoforms of PML message.

RNA INTERFERENCE

Lentiviral expression of shRNA was used to knock down the level of PML proteins. HEK293FT packaging cells were seeded in 100 mm plates at 1,000,000 cells/plate. 24 h later, cells were transfected with DNA-$CaPO_4$ precipitates of lentiviral vector and packaging vectors (pLP1, pLP2, and pLP-VSVG). After 16 h of incubation, transfected cells were washed twice with phosphate-buffered saline (PBS), then re-fed with fresh medium, and lentiviral particle supernatants were harvested 48 h afterward. For lentiviral infection, HT1885 cells were seeded at 500,000 cells/100 mm plate, and incubated with lentiviral supernatants for one day. Infected cells were washed twice with PBS, and re-fed with fresh medium. EGFP expression was used as an indicator of lentiviral infection. Infected cells were replated at low density in 100 mm plates for colony formation, and individual clones of EGFP-positive cells were isolated. Depletion of PML protein was confirmed by western blotting.

HOMOLOGOUS RECOMBINATION ASSAYS

HT1885 cells were used to measure frequencies of break-induced HRR, as described previously [Lio et al., 2004] and illustrated in Figure 1. HT1885 cells were seeded in 6-well plates at 300,000 cells/well. The next day, each well was co-transfected with 1 μg of I-SceI expression vector pCMV(3xNLS) I-SceI and 1 μg of treatment or control vector using TransIT-LT1 transfection reagent. One day after transfection, cells were replated in triplicate for puromycin selection at 100,000 cells/100 mm culture dish. Parallel platings to measure cloning efficiency were made in triplicate at 250 cells/100 mm dish, without selection. One day after replating, puromycin (Sigma-Aldrich, Inc.) was added into selection cultures at 1 μg/ml. Selection cultures were re-fed with fresh medium and puromycin 3 days after replating. After 10 days, puromycin-resistant colonies were fixed and stained with 1% crystal violet in methanol, and counted. Cloning efficiency cultures were incubated for 10 days without re-feeding, then fixed, stained and counted. Colonies of fewer than 50 cells were excluded. Cloning efficiency was calculated as the number of unselected colonies produced per 250 cells plated. HRR frequency was calculated as the number of puromycin-resistant colonies per 100,000 cells plated, divided by cloning efficiency. HR assay results graphed in the figures are means of 2 to 8 independent experiments, each conducted in triplicate, i.e., with three separate transfections and sets of platings per treatment group.

NON-HOMOLOGOUS VECTOR INTEGRATION ASSAYS

Non-homologous vector integration (NHVI) was used as a measure of repair by non-homologous end-joining [Hromas et al., 2008]. One day prior to transfection, HT1885 cells were seeded in a 6-well plate at 300,000 cells/well. The next day, each well was transfected with 2 μg of a linearized plasmid vector, pMC1neo-polyA, which carries a neomycin resistance gene, but has no sequence homology to the human genome. Vector DNA was linearized by digestion with *Xmn-I* endonuclease (New England Biolabs) and transfected into HT1885 using TransIT-LT1 transfection reagent (MirusBio). One day after transfection, cells were replated in triplicate for selection of stable transfectants at 100,000 cells/100 mm culture dish, and for measurement of cloning efficiency at 250 cells/100 mm dish. One day after replating, G418 (Geneticin, Life Technologies) was added to selection cultures at 400 μg/ml. Cells were re-fed twice with fresh medium and G418 at days 3 and 6 days after replating. After 10 days, G418-resistant colonies were fixed and stained with 1% crystal violet in methanol and counted. Cloning efficiency cultures were incubated for 10 days without re-feeding, then fixed, stained and counted. Colonies of fewer than 50 cells were excluded. Cloning efficiency was calculated as the number of unselected colonies produced per 250 cells plated. NHVI frequency was calculated as the number of G418-resistant colonies per 100,000 cells plated, divided by cloning efficiency. NHVI assay results graphed in Figure 7 are means of 3 independent experiments, each conducted in triplicate, i.e., with three separate transfections and sets of platings per treatment group.

WESTERN BLOTTING

Proteins from whole cell extracts in 2X SDS sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 1 nM bromophenol blue, 3.1% DTT) were separated on Tris-glycine SDS-PAGE gels, and transferred to nitrocellulose membranes using a Mini Trans-Blot transfer system (Bio-Rad). Endogenous PML proteins were probed with polyclonal anti-PML antibodies and HRP-conjugated secondary antibodies. As an internal control, GAPDH was detected using HRP-conjugated anti-GAPDH antibody. HRP-conjugated antibodies were visualized using HyGLO HRP chemiluminescent detection reagent (Denville Scientific).

IMMUNOFLUORESCENCE MICROSCOPY

Cells were seeded onto poly-D-lysine-coated coverslips in 6-well plates 1 day prior to fixation. For γH2AX and RAD51 foci formation, cells were X-irradiated with 8 Gy and allowed to recover for indicated times at 37°C before fixation. Cells were fixed with 4% paraformaldehyde, permeablized with 0.2% Triton-X in PBS, and blocked overnight with 1% BSA. Fixed cells were probed with indicated primary antibodies, and after washing, with Alexa Fluor 594 (red) and Alexa Fluor 488 (green)-conjugated goat anti-mouse or anti-rabbit secondary antibodies. Cell nuclei were counterstained with DAPI. The processed cover glasses were mounted on slides with Biomeda Gel-Mount aqueous medium (Electron Microscopy Sciences), and visualized using an Axiovert 200M inverted epifluorescence microscope (Zeiss). Images were captured and analyzed using Slidebook software (Olympus).

FLOW CYTOMETRY

Flow cytometry was used to determine cell cycle progression and measure levels of phosphorylated histone H2AX (γH2AX) in HT1885 cells. For cell cycle progression, asynchronously growing HT1885 cells were collected by trypsinization 18–24 h after passage, and then fixed with ice-cold 70% ethanol. Fixed cells were washed with PBS, and then resuspended in PBS with RNaseA and 1mg/ml of propidium iodide. For γH2AX detection, cells were X-irradiated with 8 Gy and allowed to recover for 30 min at 37°C before sample preparation. Cells were detached by trypsinization and washed once with 3% FBS/PBS. Cells were fixed using Cytofix/Cytoperm buffer, and permeablized with Cytoperm Plus buffer, both from a BD-Pharmingen FITC BrdU Flow Kit (BD Bioscience, catalog # 51-2354AK). Fixed cells were probed with anti-phospho-histone H2AX antibody JBW301 (Millipore, Inc.), and after washing, with Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies. After further washing, cells were pelleted and resuspended in 3% FBS/PBS. Cells were analyzed using a Cytomics FC500 flow cytometer, and data were analyzed using CXP Analysis software (Beckman Coulter, Inc.).

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REFERENCES

Allen C, Halbrook J, Nickoloff JA. 2003. Interactive competition between homologous recombination and non-homologous end joining. Mol Cancer Res 1:913–920.

Beech SJ, Lethbridge KJ, Killick N, McGlincy N, Leppard KN. 2005. Isoforms of the promyelocytic leukemia protein differ in their effects on ND10 organization. Exp Cell Res 307:109–117.

Bernardi R, Pandolfi PP. 2007. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat Rev Mol Cell Biol 8:1006–1016.

Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH, Pandolfi PP. 2004. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol 6:665–672.

Bischof O, Kim SH, Irving J, Beresten S, Ellis NA, Campisi J. 2001. Regulation and localization of the Bloom syndrome protein in response to DNA damage. J Cell Biol 153:367–380.

Bøe SO, Haave M, Jul-Larsen A, Grudic A, Bjerkvig R, Lønning PE. 2006. Promyelocytic leukemia nuclear bodies are predetermined processing sites for damaged DNA. J Cell Sci 119:3284–3295.

Brown D, Kogan S, Lagasse E, Weissman I, Alcalay M, Pelicci PG, Atwater S, Bishop JM. 1997. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci USA 94:2551–2556.

Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. 1995. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 14:4240–4248.

Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY, Chen Z. 1996. In vitro studies on cellular and molecular mechan-

isms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 88:1052–1061.

D'Andrea AD. 2010. Susceptibility pathways in Fanconi's anemia and breast cancer. N Engl J Med 362:1909–1919.

Dellaire G, Ching RW, Ahmed K, Jalali F, Tse KC, Bristow RG, Bazett-Jones DP. 2006a. Promyelocytic leukemia nuclear bodies behave as DNA damage sensors whose response to DNA double-strand breaks is regulated by NBS1 and the kinases ATM, Chk2, and ATR. J Cell Biol 175:55–66.

Dellaire G, Ching RW, Dehghani H, Ren Y, Bazett-Jones DP. 2006b. The number of PML nuclear bodies increases in early S phase by a fission mechanism. J Cell Sci 119:1026–1033.

Dellaire G, Farrall R, Bickmore WA. 2003. The Nuclear Protein Database (NPD): sub-nuclear localisation and functional annotation of the nuclear proteome. Nucleic Acids Res 31:328–330.

Duprez E, Lillehaug JR, Naoe T, Lanotte M. 1996. cAMP signalling is decisive for recovery of nuclear bodies (PODs) during maturation of RA-resistant t(15;17) promyelocytic leukemia NB4 cells expressing PML-RAR alpha. Oncogene 12:2451–2459.

Eladad S, Ye TZ, Hu P, Leversha M, Beresten S, Matunis MJ, Ellis NA. 2005. Intra-nuclear trafficking of the BLM helicase to DNA damage-induced foci is regulated by SUMO modification. Hum Mol Genet 14:1351–1365.

Fogal V, Gostissa M, Sandy P, Zacchi P, Sternsdorf T, Jensen K, Pandolfi PP, Will H, Schneider C, Del Sal G. 2000. Regulation of p53 activity in nuclear bodies by a specific PML isoform. EMBO J 19:6185–6195.

Gatz SA, Wiesmüller L. 2006. p53 in recombination and repair. Cell Death Differ 13:1003–1016.

Gurrieri C, Capodieci P, Bernardi R, Scaglioni PP, Nafa K, Rush LJ, Verbel DA, Cordon-Cardo C, Pandolfi PP. 2004. Loss of the tumor suppressor PML in human cancers of multiple histologic origins. J Natl Cancer Inst 96:269–279.

Haupt S, di Agostino S, Mizrahi I, Alsheich-Bartok O, Voorhoeve M, Damalas A, Blandino G, Haupt Y. 2009. Promyelocytic leukemia protein is required for gain of function by mutant p53. Cancer Res 69:4818–4826.

Helleday T. 2010. Homologous recombination in cancer development, treatment and development of drug resistance. Carcinogenesis 31:955–960.

Henson JD, Neumann AA, Yeager TR, Reddel RR. 2002. Alternative lengthening of telomeres in mammalian cells. Oncogene 21:598–610.

Heyer WD. 2004. A new deal for Holliday junctions. Nat Struct Mol Biol 11:117–119.

Hromas R, Wray J, Lee SH, Martinez L, Farrington J, Corwin LK, Ramsey H, Nickoloff JA, Williamson EA. 2008. The human set and transposase domain protein Metnase interacts with DNA Ligase IV and enhances the efficiency and accuracy of non-homologous end-joining. DNA Repair 7:1927–1937.

Huang W, Sun GL, Li XS, Cao Q, Lu Y, Jang GS, Zhang FQ, Chai JR, Wang ZY, Waxman S, Chen Z, Chen SJ. 1993. Acute promyelocytic leukemia: clinical relevance of two major PML-RAR alpha isoforms and detection of minimal residual disease by retrotranscriptase/polymerase chain reaction to predict relapse. Blood 82:1264–1269.

Isakson P, Bjoras M, Boe SO, Simonsen A. 2010. Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein. Blood 116:2324–2331.

Ishov AM, Sotnikov AG, Negorev D, Vladimirova OV, Neff N, Kamitani T, Yeh ET, Strauss JF 3rd, Maul GG. 1999. PML is critical for ND10 formation and recruits the PML-interacting protein Daxx to this nuclear structure when modified by SUMO-1. J Cell Biol 147:221–234.

Jensen K, Shiels C, Freemont PS. 2001. PML protein isoforms and the RBCC/TRIM motif. Oncogene 20:7223–7233.

Jurcic JG, Nimer SD, Scheinberg DA, DeBlasio T, Warrell RPJ, Miller WHJ. 2001. Prognostic significance of minimal residual disease detection and PML/RAR-alpha isoform type: long-term follow-up in acute promyelocytic leukemia. Blood 98:2651–2656.

Kakizuka A, Miller WHJ, Umesono K, Warrell RPJ, Frankel SR, Murty VV, Dmitrovsky E, Evans RM. 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. Cell 66:663–674.

Lallemand-Breitenbach V, de The H. 2010. PML nuclear bodies. Cold Spring Harb Perspect Biol 2:a000661.

Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. 1991. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 77:1080–1086.

Li H, Leo C, Zhu J, Wu X, O'Neil J, Park EJ, Chen JD. 2000. Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. Mol Cell Biol 20:1784–1796.

Lin DY, Lai MZ, Ann DK, Shih HM. 2003. Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential. J Biol Chem 278:15958–15965.

Lio Y-C, Schild D, Brenneman MA, Redpath JL. J. CD. 2004. Human RAD51C deficiency destabilizes XRCC3, impairs recombination and radiosensitizes S/G2-phase cells. J Biol Chem 279:42313–42320.

Liu J, Song Y, Qian J, Liu B, Dong Y, Tian B, Sun Z. 2011. Promyelocytic leukemia protein interacts with Werner syndrome helicase and regulates double-strand break repair in gamma-irradiation-induced DNA damage responses. Biochemistry (Moscow) 76:550–554.

Lu H, Guo X, Meng X, Liu J, Allen C, Wray J, Nickoloff JA, Shen Z. 2005. The BRCA2-interacting protein BCCIP functions in RAD51 and BRCA2 focus formation and homologous recombinational repair. Mol Cell Biol 25:1949–1957.

Melnick A, Licht JD. 1999. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood 93:3167–3215.

Miller WHJ, Kakizuka A, Frankel SR, Warrell RPJ, DeBlasio A, Levine K, Evans RM, Dmitrovsky E. 1992. Reverse transcription polymerase chain reaction for the rearranged retinoic acid receptor alpha clarifies diagnosis and detects minimal residual disease in acute promyelocytic leukemia. Proc Natl Acad Sci USA 89:2694–2698.

Otterlei M, Bruheim P, Ahn B, Bussen W, Karmakar P, Baynton K, Bohr VA. 2006. Werner syndrome protein participates in a complex with RAD51, RAD54, RAD54B and ATR in response to ICL-induced replication arrest. J Cell Sci 119:5137–5146.

Pandolfi PP, Alcalay M, Fagioli M, Zangrilli D, Mencarelli A, Diverio D, Biondi A, Lo Coco F, Rambaldi A, Grignani F, Rochette-Egly C, Gaube MP, Chambon P, Pelicci PG. 1992. Genomic variability and alternative splicing generate multiple PML/RAR alpha transcripts that encode aberrant PML proteins and PML/RAR alpha isoforms in acute promyelocytic leukaemia. EMBO J 11:1397–1407.

Pellegrini L, Yu DS, Lo T, Anand S, Lee M, Blundell TL, Venkitaraman AR. 2002. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. Nature 420:287–293.

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Zhang M, Ihrig MM, McManus MT, Gertler FB, Scott ML, Van Parijs L. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 33:401–406.

Shrivastav M, Miller CA, De Haro LP, Durant ST, Chen BP, Chen DJ, Nickoloff JA. 2009. DNA-PKcs and ATM co-regulate DNA double-strand break repair. DNA Repair 8:920–929.

Spardy N, Duensing A, Hoskins EE, Wells SI, Duensing S. 2008. HPV-16 E7 reveals a link between DNA replication stress, fanconi anemia D2 protein,

and alternative lengthening of telomere-associated promyelocytic leukemia bodies. Cancer Res 68:9954–9963.

Takizawa Y, Qing Y, Takaku M, Ishida T, Morozumi Y, Tsujita T, Kogame T, Hirota K, Takahashi M, Shibata T, Kurumizaka H, Takeda S. 2010. GEMIN2 promotes accumulation of RAD51 at double-strand breaks in homologous recombination. Nucleic Acids Res 38:5059–5074.

Tussie-Luna MI, Rozo L, Roy AL. 2006. Pro-proliferative function of the long isoform of PML-RARa involved in acute promyelocytic leukemia. Oncogene 25:3375–3386.

Vahdat L, Maslak P, Miller WHJ, Eardley A, Heller GASD, Warrell RPJ. 1994. Early mortality and the retinoic acid syndrome in acute promyelocytic leukemia: impact of leukocytosis, low-dose chemotherapy, PML/RAR-alpha isoform, and CD13 expression in patients treated with all-trans retinoic acid. Blood 84:3843–3849.

Vincenzi B, Perrone G, Santini D, Grosso F, Silletta M, Frezza A, Rossi S, Russo A, Rabitti C, Gebbia N, Badalamenti G, Casali P, Muda AO, Dei Tos AP, Tonini G. 2010. PML down-regulation in soft tissue sarcomas. J Cell Physiol 224:644–648.

Vitoux D, Nasr R, de Thé H. 2007. Acute promyelocytic leukemia: New issues on pathogenesis and treatment response. Int J Biochem Cell Biol 39:1063–1070.

Wang H, Chen XY, Wang BS, Rong ZX, Qi H, Chen HZ. 2011. The efficacy and safety of arsenic trioxide with or without all-trans retinoic acid for the treatment of acute promyelocytic leukemia: a meta-analysis. Leuk Res 35:1170–1177.

Wang JG, Barsky LW, Davicioni E, Weinberg KI, Triche TJ, Zhang XK, Wu L. 2006. Retinoic acid induces leukemia cell G1 arrest and transition into differentiation by inhibiting cyclin-dependent kinase-activating kinase binding and phosphorylation of PML/RARalpha. FASEB J 20:2142–2144.

Wang ZG, Delva L, Gaboli M, Rivi R, Giorgio M, Cordon-Cardo C, Grosveld F, Pandolfi PP. 1998. Role of PML in cell growth and the retinoic acid pathway. Science 279:1547–1551.

Wang ZY, Chen Z. 2008. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 111:2505–2515.

Wu L, Hickson ID. 2003. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature 426:870–874.

Xu ZX, Timanova-Atanasova A, Zhao RX, Chang KS. 2003. PML colocalizes with and stabilizes the DNA damage response protein TopBP1. Mol Cell Biol 23:4247–4256.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res 59:4175–4179.

Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, Zhang QY, Yang HY, Huang QH, Zhou GB, Tong JH, Zhang Y, Wu JH, Hu HY, de Thé H, Chen SJ, Chen Z. 2010. Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. Science 328:240–243.

Zhong S, Hu P, Ye TZ, Stan R, Ellis NA, Pandolfi PP. 1999. A role for PML and the nuclear body in genomic stability. Oncogene 18:7941–7947.