Promyelocytic leukemia nuclear bodies are predetermined processing sites for damaged DNA

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Summary

The promyelocytic leukemia protein (PML) participates in several cellular functions, including transcriptional regulation, apoptosis and maintenance of genomic stability. A key feature of this protein is its ability to induce the assembly of nuclear compartments termed PML-nuclear bodies (PML-NBs). Here we show that these nuclear structures recruit single-stranded DNA (ssDNA) molecules in response to exogenous DNA damage. ssDNA was readily detected in PML-NBs within 1 hour following exposure of cells to UV light. Confocal real-time imaging of cells expressing YFP-tagged PML did not reveal de novo formation of new PML-NBs following UV-irradiation, which shows that ssDNA focus formation occurred within pre-existing PML-NBs. Moreover, siRNA-mediated depletion of PML prevented ssDNA focus formation and sensitized cells to UV-induced apoptosis. PML-dependent ssDNA focus formation was found to be particularly efficient during S-phase of the cell cycle, and PML-depleted cells became retarded in S-phase upon growth in the presence of etoposide. In addition, we found that caffeine and the poly(ADP-ribose) polymerase (PARP) inhibitor NU1027 enhanced UV-induced recruitment of ssDNA to PML-NBs. Together, our results show that PML-NBs have the capacity to accommodate DNA metabolic activities that are associated with processing of damaged DNA.

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Introduction

Promyelocytic leukemia nuclear bodies (PML-NBs) are distinct structures that are present within the nucleus of most mammalian cells. A single nucleus commonly contains between 5 and 30 of these bodies and they vary in size from 0.1 to 1.0 μm (Bernardi and Pandolfi, 2003; Ching et al., 2005; Maul et al., 2000). The PML protein has been shown to play a critical role in the assembly and integrity of PML-NBs. Consequently, cells that do not express PML do not contain these structures (Ishov et al., 1999; Zhong et al., 2000). Several functions attributed to PML may therefore also be ascribed to PML-NBs.

Several studies suggest an important role of PML and PML-NBs in tumor suppression. For instance, $PML^{-/-}$ mice have an increased risk of tumor development (Wang et al., 1998a), and loss of PML expression has been correlated with tumor progression in several types of cancer (Gambacorta et al., 1996; Gurrieri et al., 2004; Zhang et al., 2000). Furthermore, PML may play an important role in the pathogenesis of acute promyelocytic leukemia (APL). This is evident from the occurrence of a chromosomal translocation leading to fusion of the PML gene to the gene encoding retinoic acid receptor α in APL cells (de The et al., 1990). An important role of PML in tumor biology is also underlined by several studies implicating its involvement in tumor suppression functions such as senescence (Ferbeyre et al., 2000; Pearson et al., 2000), apoptosis (Wang et al., 1998b), differentiation (Grignani et al., 1993) and maintenance of genomic stability (Zhong et al., 1999).

Recent reports have pointed at a role for PML and PML-NBs in the DNA damage response. For instance, a number of proteins with known functions in DNA repair and checkpoint signaling have been found to be localized within PML-NBs (Barr et al., 2003; Bischof et al., 2001; Mirzoeva and Petrini, 2001; Yang et al., 2002), and PML has been established as a target for the checkpoint kinases Chk2 and ATR (Bernardi et al., 2004; Yang et al., 2002). Furthermore, the PML protein has been found to be required for ionizing irradiation (IR)-induced stabilization and re-localization of the DNA damage response protein TopBP1 (Xu et al., 2003), and $PML^{-/-}$ cells exhibit an increased rate of sister chromatid exchange (Zhong et al., 1999). In addition, PML and PML-NBs have been implicated in recombinational processes that support telomere maintenance in ALT (for alternative lengthening of telomeres) cells. In a sub-population of such cells, nuclear structures termed ALT-associated PML-NBs (APBs) can be identified that contain PML, telomere repeat sequences, telomere-binding proteins, and several proteins with known functions in recombinational DNA repair (Yeager et al., 1999). Finally, several DNA viruses have been shown to position their DNA replication centers in close proximity to PML-NBs. The underlying reason for this may in part be due to an increased

incidence of replication-induced DNA damage caused by the replicating viral DNA (reviewed by Everett, 2006).

We showed in a previous study that PML-NBs sequester DNA molecules in response to extrachromosomal large T-antigen-dependent DNA replication, indicating a direct role of these bodies in DNA processing events associated with DNA synthesis (Jul-Larsen et al., 2004). In the present study we show that PML-NBs support ssDNA focus formation in response to exogenous DNA damage. Formation of these ssDNA foci depends on pre-established PML-NBs and appears to be enhanced during S-phase progression. We also show that depletion of PML sensitizes cells to UV-induced apoptosis and causes inhibition of S-phase progression in the presence of DNA damage caused by etoposide. Combined, our results show that PML-NBs have the capacity to accommodate DNA metabolic activities that aid in the repair of replication-induced DNA damage.

Results

ssDNA focus formation in response to UV-irradiation

To investigate the formation of PML-NB-associated ssDNA foci, cells were metabolically labeled with BrdU for more than one cell doubling (24 hours). They were then treated with UV light (or left untreated) and subsequently incubated in the presence or absence of caffeine for 1 or 2 hours prior to fixation. Sites of BrdU-containing ssDNA were then detected by an anti-BrdU antibody using an immunofluorescence labeling procedure that did not include denaturing of chromosomal DNA (Raderschall et al., 1999).

In non-irradiated GM-847 cells, nuclear foci that contained both ssDNA and PML were detected in 1-5% of the cells examined (Fig. 1A,B). Typically, The PML-NBs observed in these cells were fewer in number and larger in size compared to the other cells. Since GM-847 is an ALT cell line (Yeager et al., 1999), we suspected that these particular PML-NBs represented APBs, which are nuclear compartments containing telomere sequences, telomere-binding proteins and several protein components with known functions in recombinational DNA repair (Henson et al., 2002). To verify this, we performed double immunofluorescence labeling using antibodies against BrdU and the telomere-binding protein TRF2. In cells where ssDNA-containing foci were detected, TRF2 was found to concentrate within the same nuclear structures in more than 80% of the cases (Fig. 1A,C). Furthermore, double labeling of BrdU-containing ssDNA and PML revealed a high degree of co-localization between ssDNA foci and PML-NBs in nearly 100% of the cells examined (Fig. 1A). Thus, the low percentage of non-irradiated GM-847 cells, which contained ssDNA foci, represented cells with APBs.

UV-treatment of GM-847 cells resulted in an increase of cells that contained ssDNA foci (Fig. 1B). Furthermore, if UV-irradiated cells were grown in the presence of caffeine, a drug known to inhibit DNA-damage-induced checkpoint activation and DNA repair, an even more pronounced increase was detected (Fig. 1B). The UV-induced ssDNA-foci were similar in size and shape to those detected in non-irradiated cells, and they were found to co-localize with PML (Fig. 1A). However, in contrast to the ssDNA-containing structures detected in non-irradiated cells, the UV-induced foci did not contain detectable TRF2 (Fig. 1A,C), indicating that the ssDNA foci that formed in response to UV-damage were not telomere-associated. UV-irradiation and subsequent caffeine treatment did not lead to detectable alterations in PML-NB morphology, and we were not able to detect a significant increase or decrease in the number of PML-NBs per cell following this treatment (data not shown).

In addition to GM-847 cells, several other cell lines were tested for their ability to form ssDNA-containing foci that associate with PML (Table S1 and Fig. S1 in supplementary material). Among the cell lines tested, several SV40-transformed cell lines, including GM-847, GM-637 and WI-38/VA-13 were found to be relatively efficient in this activity (between 10 and 40% foci-positive cells following UV-irradiation and subsequent incubation in the presence of 4 mM

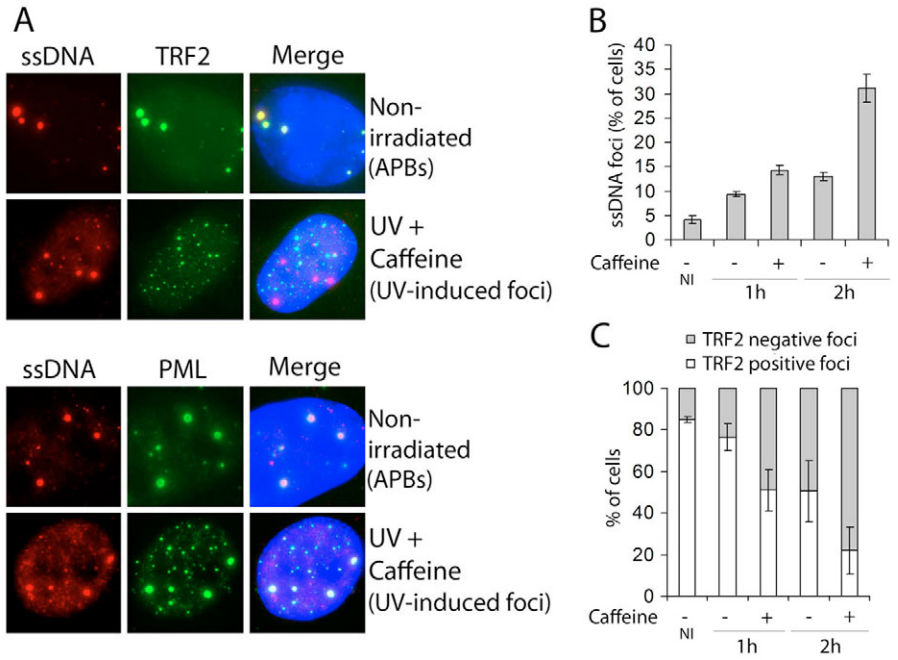


Fig. 1. Formation of ssDNA foci in GM-847 cells in response to UV-irradiation. GM-847 cells metabolically labeled for 24 hours with BrdU were subjected to 10 J/m^2 UV-irradiation and subsequently incubated for 1 or 2 hours in the presence or absence of 4 mM caffeine. (A) Representative immunofluorescence images of non-irradiated cells containing ssDNA-positive APBs and UV-irradiated and caffeine-treated cells containing UV-induced ssDNA foci. (B) Quantitative determination of cells containing ssDNA-foci. For each sample, more than 500 cells were scored. Average of two independent experiments ± standard deviation (s.d.) is shown. (C) Quantitative determination of cells exhibiting co-localization between TRF2 and ssDNA-containing foci. For each sample, 50 cells that contained ssDNA foci were evaluated. Average of two independent experiments ± s.d. is shown. (NI) Non-irradiated.

caffeine; Table S1 in supplementary material). We were not able to detect ssDNA focus formation in HeLa, MCF-7 or 293 cells (Table S1 in supplementary material), whereas other tumor cell lines, including U2OS, GaMg and U373 cells, exhibited a moderate ability to support ssDNA focus formation (5-10% of UV-irradiated cells contained ssDNA foci following incubation in the presence of 4 mM caffeine for 2 hours; Table S1 in supplementary material). Some focus formation was detected in primary fibroblasts and primary human umbilical vein endothelial cells following UV-irradiation albeit at low levels (less than 1% of the cells were positive after treatment; Table S1 in supplementary material). The variability among different cell lines to support PML-associated ssDNA focus formation may be attributed to varying capacity of these cells to repair UV-induced DNA lesions and/or to activate DNA damage-induced cell cycle arrest (see Discussion).

Caffeine, which was used in the experiments described above to enhance ssDNA focus formation following UV-irradiation, is known for its ability to sensitize mammalian cells to agents that cause DNA damage. It is thought that this drug exerts its effect either by its capacity to inactivate multiple DNA damage-responsive cell cycle checkpoints (Cortez, 2003; Rowley, 1992; Schlegel and Pardee, 1986) or by its ability to directly inhibit DNA repair (Arlett et al., 1975; Asaad et al., 2000; Buhl and Regan, 1974; Wang et al., 2004). A widely held view is that caffeine inhibits cell-cycle checkpoint activation by targeting the checkpoint kinases ATM and ATR (Blasina et al., 1999; Sarkaria et al., 1999). For this reason, we assessed the ability of GM-847 cells treated with ATM and/or ATR-specific siRNAs to support UV-induced ssDNA focus formation. We were not able to demonstrate any increase in the ability of PML-NBs to sequester ssDNA following UV-irradiation in ATM and/or ATR-depleted cells (Fig. S2 in supplementary material). In fact, we noted a decrease in UV-induced ssDNA focus formation following transfection of cells with ATR-specific siRNAs (Fig. S2B in supplementary material). Furthermore, caffeine was found to stimulate ssDNA focus formation in ATR and ATM-depleted cells with efficiency comparable to that seen for control-depleted cells (Fig. S2C in supplementary material). This result showed that caffeine does not stimulate UV-induced ssDNA focus formation by inhibiting ATM or ATR-dependent checkpoint activation.

UV-induced ssDNA focus formation depends on PML-NB integrity

Even though the ssDNA foci that formed in response to UV-irradiation co-localized with PML-NBs, it was not clear whether their formation depended on these structures or if they required expression of the PML protein. To address this issue we transfected GM-847 cells with PML-specific siRNA duplexes. This led to a significant reduction in PML expression accompanied by ablation of PML-NB formation within 30 to 72 hours following transfection (Fig. 2A,B). We found that the siRNA-mediated depletion of PML in GM-847 cells led to an inhibition of UV-induced ssDNA focus formation (Fig. 2B,C). A similar dependency of UV-induced ssDNA focus formation on PML expression was also shown for GM-637 cells (Fig. S3 in supplementary material).

To further assess if the UV-induced ssDNA foci represented newly formed structures, or if the ssDNA accumulated in pre-existing PML-NBs, we analyzed the movement of PML-NBs in GM-847 cells transiently transfected with a YFP-PML-expressing plasmid (Molenaar et al., 2003). The YFP-PML fusion protein was readily detected in PML-NBs at 1 day following transfection, and UV-irradiation combined with subsequent caffeine treatment for 90 minutes led to ssDNA focus formation in more than 20% of the YFP-positive cells (Fig. 2D,E). Using a spinning disc confocal microscope, we analyzed the PML-NB movement within more than 50 individual cell nuclei, each for a 90 minute time period, immediately following treatment with UV light and addition of growth medium containing 4 mM caffeine. During the recording period, some of the PML-NBs moved extensively within a restricted area of the nucleus, some of the bodies fused to one another and, in a few cases, budding of small bodies from larger ones was detected (see supplementary material Movie 1). These observations are generally in agreement with previous reports describing PML-NB movements in living cells (Eskiw et al., 2003; Muratani et al., 2002). Importantly, however, we did not detect de novo formation of new PML-NBs in any of the cells that were analyzed. Our results therefore show that the observed ssDNA focus formation in response to UV-irradiation occurred within pre-existing PML-NBs.

PML-dependent ssDNA focus formation is not connected to apoptotic DNA processing

Following UV-treatment and caffeine exposure, apoptotic cell death was observed at two hours post irradiation (Figs 3 and 8). Based on this observation and previous reports demonstrating a role of PML in apoptotic activation (Salomoni et al., 2005; Wang et al., 1998b), we suspected that the ssDNA detected within PML-NBs may not originate from the damaged DNA, but instead, may be associated with apoptotic DNA fragmentation. To clarify this issue, we analyzed the chromosomal DNA in irradiated and non-irradiated cells for the presence or absence of DNA fragments ranging in size from 50 to 300 kb. Such DNA fragments are known to reflect the early cleavage of chromosomal DNA by DFF40/CAD or endonuclease G during apoptosis (Widlak and Garrard, 2005).

Apoptotic DNA fragmentation in GM-847 cells was readily detected at 2 hours post-irradiation when irradiated cells were grown in the presence of caffeine (Fig. 3A, lower panel). Notably, this treatment also resulted in efficient formation of PML-NB-associated ssDNA foci (Fig. 3A, upper panel). However, the presence of the caspase inhibitor zVAD in the growth medium, following irradiation, prevented apoptosis of the UV-treated cells (data not shown) and abrogated formation of the 50-300 kb DNA fragments (Fig. 3A, lower panel) without affecting PML-dependent ssDNA focus formation (Fig. 3A, upper panel).

We also assessed the ability of PML-NBs to recruit ssDNA in response to treatment with cyclohexamide and tumor necrosis factor α (TNFα), a combination of drugs that is known to induce apoptosis through receptor-mediated pathways. Treatment with these drugs induced apoptosis and formation of the 50-300 kb DNA fragments (Fig. 3B, lower panel), but did not lead to an increase in ssDNA focus formation within PML-NBs (Fig. 3B, upper panel).

Since we were not able to detect any relationship between apoptotic DNA fragmentation and PML-dependent ssDNA

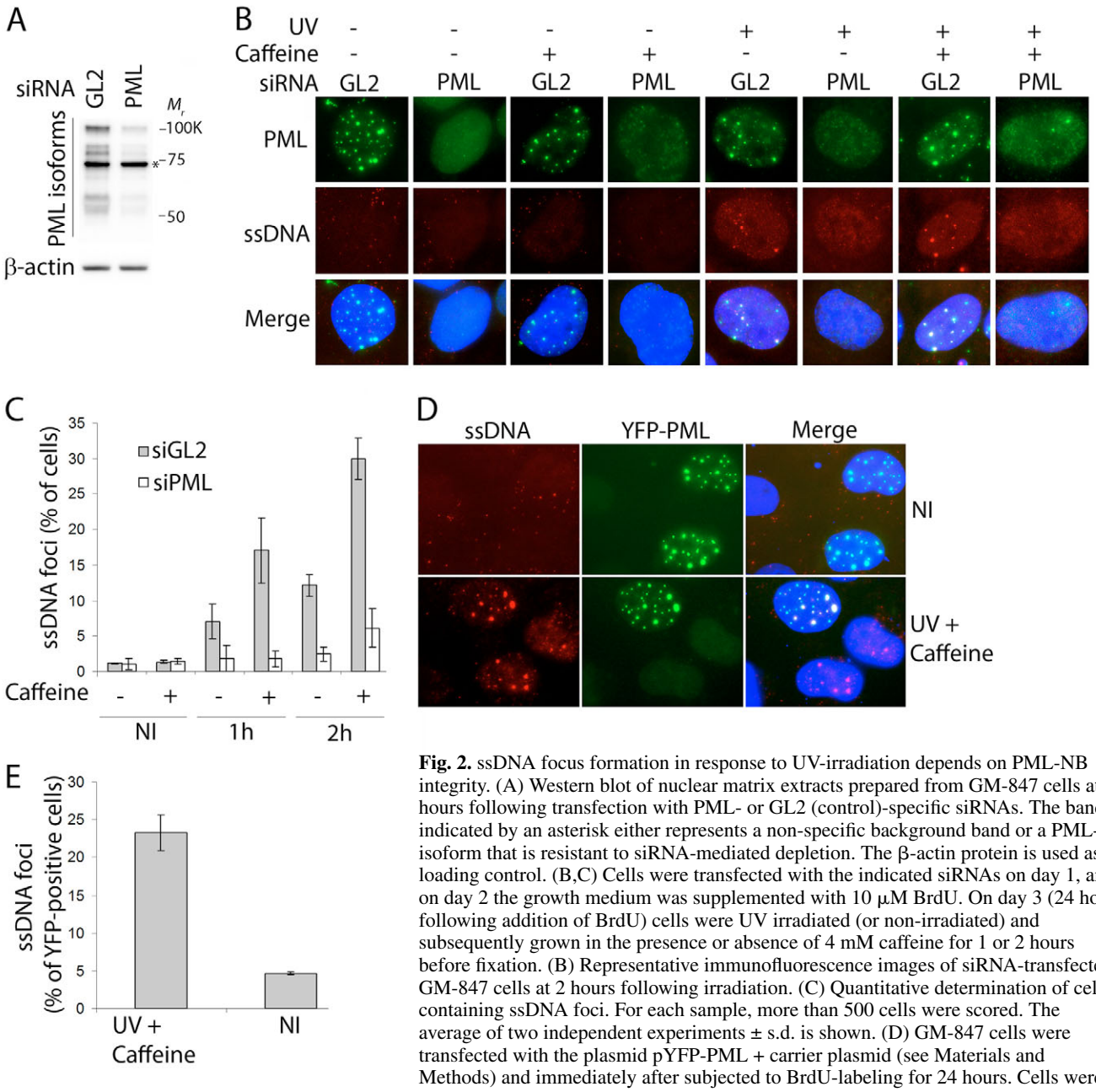


Fig. 2. ssDNA focus formation in response to UV-irradiation depends on PML-NB integrity. (A) Western blot of nuclear matrix extracts prepared from GM-847 cells at 48 hours following transfection with PML- or GL2 (control)-specific siRNAs. The band indicated by an asterisk either represents a non-specific background band or a PML-isoform that is resistant to siRNA-mediated depletion. The β-actin protein is used as a loading control. (B,C) Cells were transfected with the indicated siRNAs on day 1, and on day 2 the growth medium was supplemented with 10 μM BrdU. On day 3 (24 hours following addition of BrdU) cells were UV irradiated (or non-irradiated) and subsequently grown in the presence or absence of 4 mM caffeine for 1 or 2 hours before fixation. (B) Representative immunofluorescence images of siRNA-transfected GM-847 cells at 2 hours following irradiation. (C) Quantitative determination of cells containing ssDNA foci. For each sample, more than 500 cells were scored. The average of two independent experiments ± s.d. is shown. (D) GM-847 cells were transfected with the plasmid pYFP-PML + carrier plasmid (see Materials and Methods) and immediately after subjected to BrdU-labeling for 24 hours. Cells were then treated with UV light (or left non-irradiated) followed by incubation in the presence of caffeine for 90 minutes prior to fixation. The YFP signal and the immunofluorescently labeled BrdU are shown. (E) Quantitative determination of YFP-positive cells that contain ssDNA foci following UV-irradiation and caffeine treatment (same experiment as D). For each sample, 100 YFP-positive cells were scored. The average of two independent experiments ± s.d. is shown. (NI) Non-irradiated.

focus formation, we concluded that the observed accumulation of ssDNA within PML-NBs is not associated with apoptotic DNA processing. The UV induced ssDNA foci are therefore most likely directly associated with damaged DNA.

We were also able to induce efficient PML-dependent ssDNA focus formation using conditions that did not lead to extensive cell death by apoptosis. For example, treatment of GM-847 cells with 2.5, 5 or 10 μM etoposide for 24 hours stimulated formation of PML-dependent ssDNA foci in more than 30% of the cell examined (Fig. 7A) but did not lead to increased apoptotic cell death as determined by lack of apoptotic fragmentation, or by visual inspection of cell morphology (data not shown). Thus, formation of detectable PML-associated ssDNA foci within the nuclei following DNA damaging events may not always coincide with apoptotic cell death.

PML-dependent ssDNA focus formation is stimulated by S-phase progression

In a previous study we showed that PML-NBs sequester DNA molecules in response to large T-antigen-dependent extrachromosomal DNA replication. For this reason, we hypothesized that the ssDNA focus formation seen in UV damaged cells may be associated with replication of

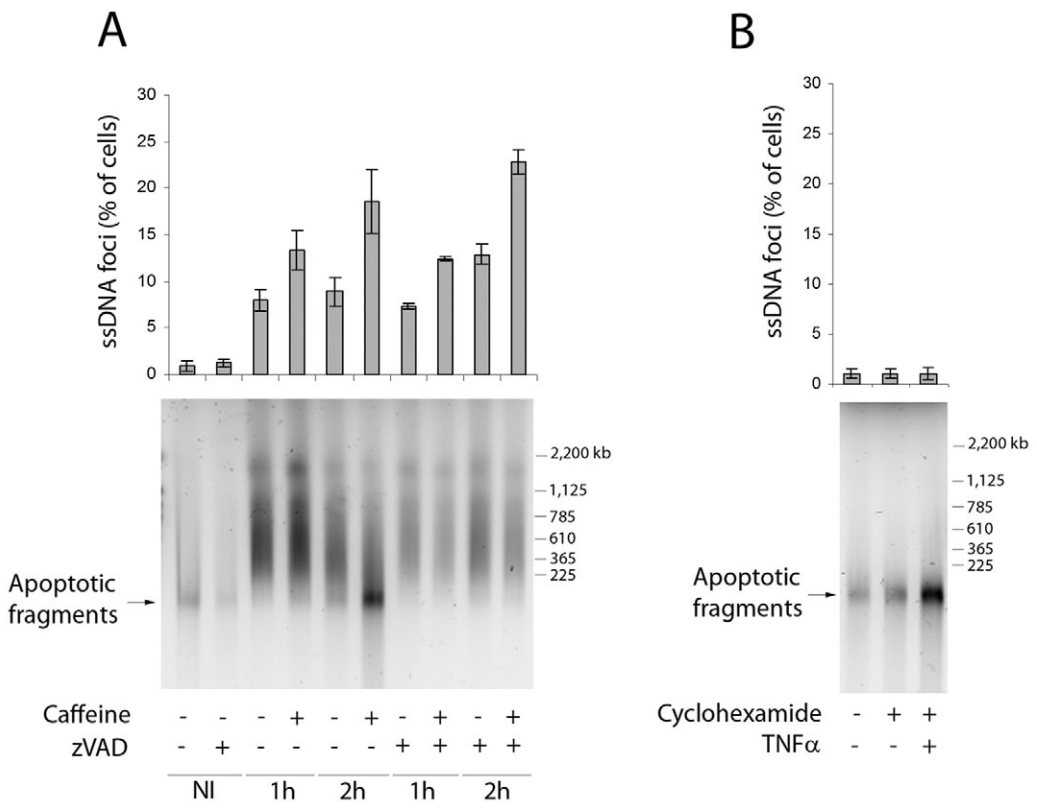


Fig. 3. ssDNA focus formation within PML-NBs is not associated with apoptotic DNA fragmentation. (A) Cells were treated with BrdU for 24 hours and subsequently irradiated with 10 J/m^2 UV light or non-irradiated. Cells were then incubated in the presence or absence of caffeine for 1 or 2 hours before analysis by immunofluorescence (upper panels) or pulse field gel electrophoresis (lower panels). Bars in upper panels represent quantification of cells containing ssDNA foci. For each sample, more than 400 cells were evaluated. The average of two independent experiments ± s.d. is shown. (B) GM-847 cells were incubated in the presence of BrdU for 24 hours and then treated with the drugs indicated for 3 hours. Following treatment, cells were analyzed as in panel A by immunofluorescence labeling (upper panel) and pulse field gel electrophoresis (lower panel).

chromosomal DNA. Moreover, since caffeine has been shown to affect post-replication DNA repair activities following UV-irradiation (Arlett et al., 1975; Buhl and Regan, 1974), the stimulatory effect of this drug on the ability of PML-NBs to sequester ssDNA may be due to its capacity to enhance formation of replication-associated DNA lesions. Another class of drugs that have been proposed to sensitize tumor cells by inhibition of DNA repair mechanisms and activation of replication-induced DNA damage, are PARP inhibitors (Bryant et al., 2005; Farmer et al., 2005; Szuts and Krude, 2004; Yang et al., 2004). For this reason we assessed the potential of the PARP inhibitor NU1025 to induce PML-associated ssDNA focus formation in UV-irradiated cells. We found that the presence of NU1025 in the growth medium, after treatment with UV light, significantly enhanced ssDNA focus formation in a PML-dependent manner (Fig. 4A). We also observed a similar increase in UV-induced ssDNA focus formation following siRNA-mediated depletion of the PARP-1 protein (Fig. 4B,C). This result is consistent with a common role of caffeine and PARP inhibitors to stimulate PML-dependent ssDNA focus formation in response to UV-irradiation.

To further investigate a possible association of PML-dependent ssDNA focus formation and DNA synthesis, we analyzed the formation of ssDNA foci in cells irradiated at different time-points following synchronization in mitosis by nocodazole treatment and mitotic shake-off. At 5 hours post-release, a time-point where the majority of the cells were found to be in the G1 phase (Fig. 5A), we detected UV-induced ssDNA foci in less than 5% of the cells examined (Fig. 5B). At 12 hours following release, when approximately 50% of the cells were found to be in S-phase (Fig. 5A), we detected ssDNA focus formation in nearly 20% of the cells examined (Fig. 5B,C). Thus, the UV-induced sequestration of ssDNA in PML-NBs is more efficient during S-phase than during the G1-phase of the cell cycle.

We next assessed the ability of PML-NBs to recruit ssDNA following treatment of PML-depleted and control-depleted GM-847 cells with 0.5 mM mimosine for 24 hours. This drug arrests cells in the G1 phase of the cell cycle close to the G1/S transition and, at the same time, induces the formation of chromosomal DNA breaks (Krude, 1999; Szuts and Krude, 2004). Following mimosine treatment, we noted an increase in the number of cells containing PML-NB-associated ssDNA foci (Fig. 6B). Interestingly, removal of mimosine from the growth medium, which causes a synchronous progression of cells through S-phase, led to a dramatic increase of cells containing ssDNA-positive PML-NBs (Fig. 6B). We subsequently performed a similar experiment by treating cells for 24 hours with hydroxyurea (HU), a drug that arrests cells in S-phase and may cause DNA breakage at stalled replication

forks. Treatment of cells with HU caused an increase in ssDNA focus formation, and similar to treatment with mimosine the ability of PML-NBs to sequester ssDNA was found to increase following release from the cell cycle block (Fig. 6C). Thus, the ability of both mimosine and HU to induce ssDNA focus formation appears to be enhanced by S-phase progression.

The topoisomerase type II inhibitor etoposide is known to cause DNA damage leading to cell cycle arrest during the G1, S or G2-phase. We found that treatment of GM-847 cells with this drug induced PML-dependent ssDNA focus formation in a dose-dependent manner (Fig. 7A). In order to assess a possible effect of PML-depletion on cell cycle progression in the presence of this drug, control and PML-depleted cells were treated for 24 hours with different concentrations of etoposide. The cells were then analyzed for the presence of histone H3 phosphorylation at serine 10, which is known to occur mainly during mitosis. In the absence of etoposide, no significant differences in the level of histone H3 phosphorylation could be observed between PML-depleted cells and cells transfected with the control siRNAs (Fig. 7B). By contrast, when cells were grown in the presence of etoposide, the amount of histone

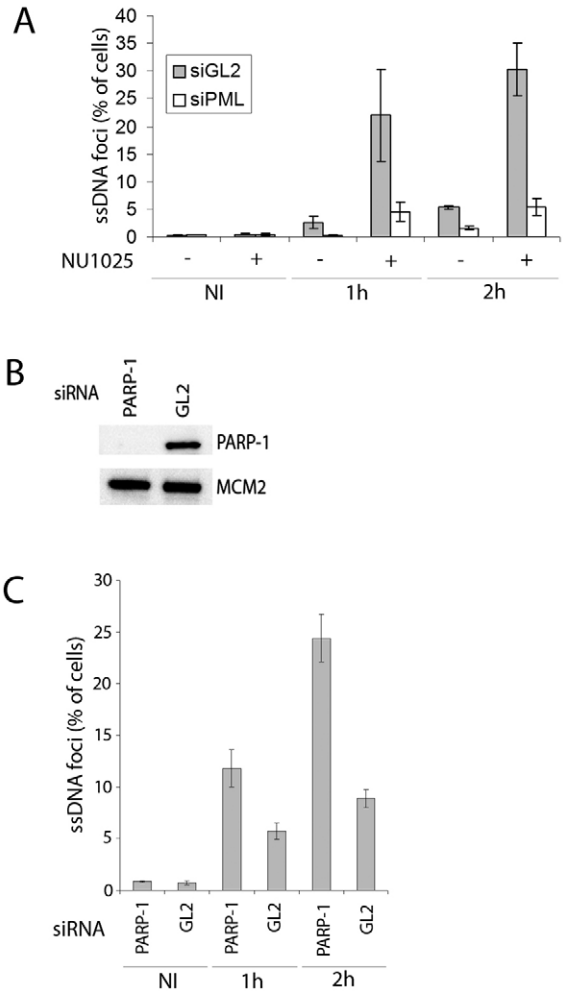


Fig. 4. Inhibition of PARP-1 stimulates UV-induced ssDNA focus formation. (A) GM-847 cells seeded on coverslips were transfected with PML-specific siRNAs or control siRNAs (GL2) as indicated. BrdU was added to the culture medium at 24 hours after transfection. At 48 hours post-transfection cells were treated with 10 J/m^2 UV light and subsequently incubated in the presence or absence of 0.5 mM NU1025 for 1 or 2 hours before fixation and immunofluorescence analysis. Data represent the percentage of cells containing ssDNA foci. For each sample more than 300 cells were evaluated. The data represent the average of two independent experiments ± s.d. (B) Western blot of extracts prepared from GM-847 cells at 72 hours following transfection with siRNAs specific for PARP-1 and GL2 (control). The MCM2 protein is used as a loading control. (C) GM-847 cells were transfected with the siRNAs indicated. BrdU was added to the growth medium at 48 hours following transfection. At 24 hours following addition of BrdU, cells were irradiated with 10 J/m^2 UV light or non-irradiated and subsequently incubated in normal growth medium for 1 or 2 hours. (NI) Non-irradiated.

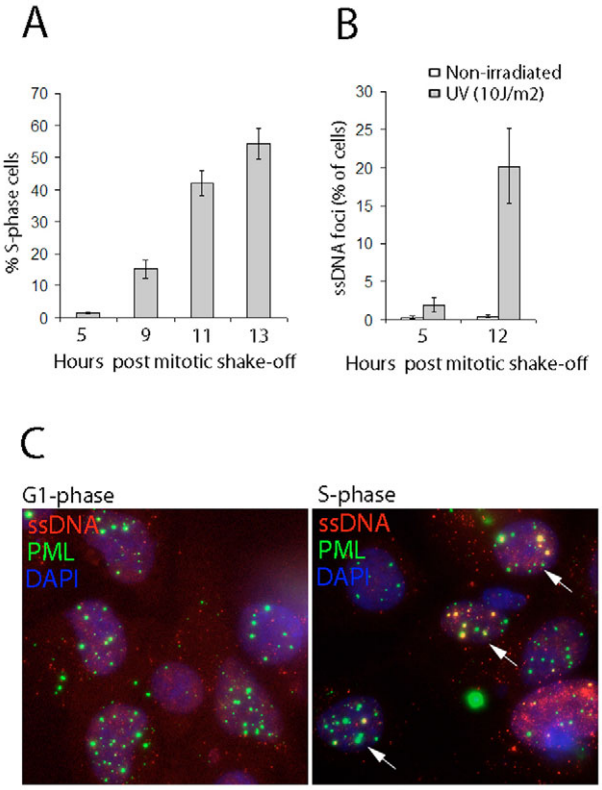
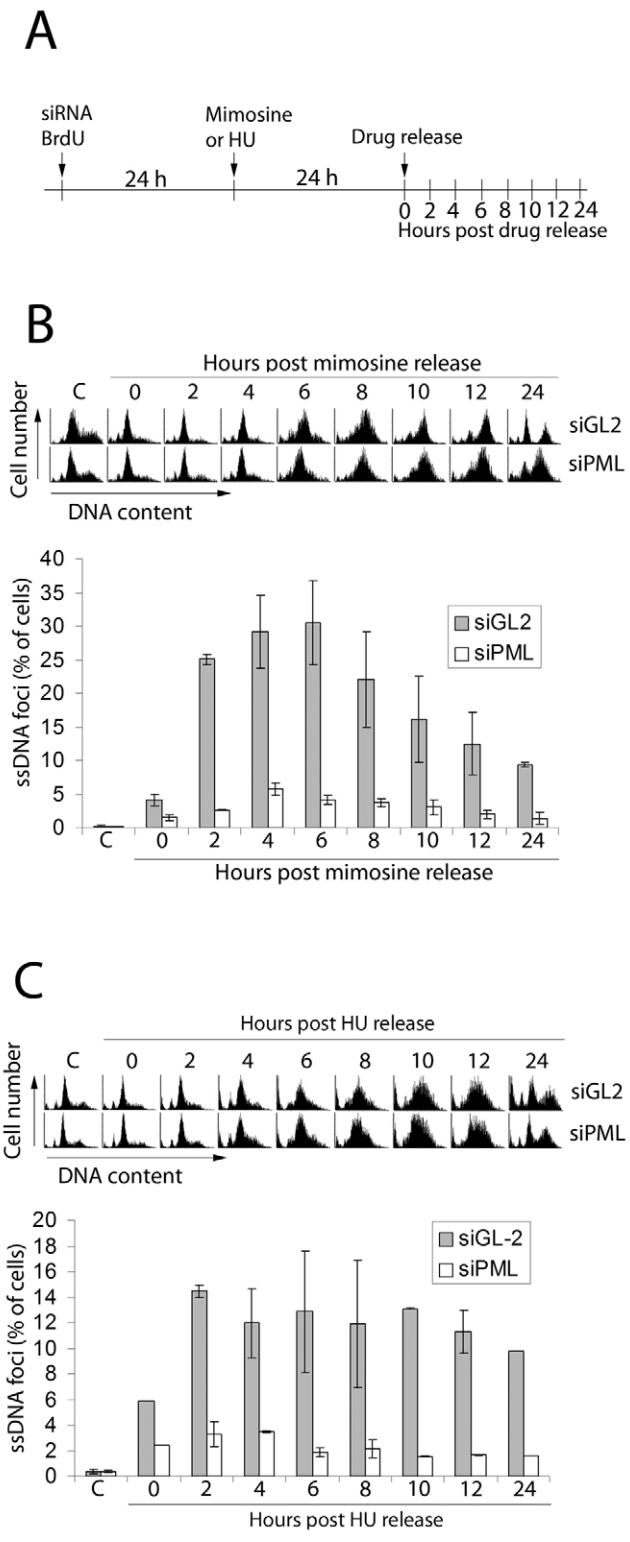


Fig. 5. UV-induced ssDNA focus formation is stimulated during S-phase. (A) GM-847 cells treated with nocodazole for 14 hours were subjected to mitotic shake-off and subsequently seeded on coverslips. At the indicated time-points, cells were pulsed with BrdU, and the number of cells in S-phase were determined by immunofluorescence labeling using an anti-BrdU antibody. For each sample more than 400 cells were scored. The data represent the average of two independent experiments ± s.d. (B) GM-847 cells were treated with BrdU for 24 hours and subsequently subjected to nocodazole treatment and mitotic shake-off in parallel with the experiment described in A. At the indicated time-points following mitotic shake-off, cells were UV-irradiated with 10 J/m^2, and subsequently incubated in the presence of 4 mM caffeine for 2 hours. The number of cells containing PML-associated ssDNA foci were determined by immunofluorescence labeling. For each sample more than 400 cells were scored. The data represent the average of two independent experiments ± s.d. (C) Representative images of UV-induced PML-associated ssDNA foci in G1 and S-phase cells formed by UV-irradation at 5 and 12 hours post mitotic shake-off, respectively.

H3 phosphorylation was considerably reduced in PML-depleted cells compared with control cells (Fig. 7B). This indicated that PML-depleted cells are less efficient than control cells in entering mitosis in the presence of etoposide. A similar result was obtained by analyzing the cell cycle profiles of PML-depleted and control cells using laser scanning cytometry (LSC). Cells transfected with control siRNAs showed primarily an arrest during the G2/M phase of the cell cycle upon treatment with etoposide (Fig. 7C). PML-depleted cells, however, showed a higher tendency for S-phase arrest (Fig. 7C). No apparent differences in cell cycle distribution between control cells and PML-depleted cells were noted in the absence of etoposide (Fig. 7C). These results indicate that the PML-depleted cells are less efficient than control cells in progressing through S-phase in the presence of DNA damage caused by etoposide. A similar effect of topoisomerase type II inhibitors on PML-dependent ssDNA focus formation and cell-cycle progression was also noted for GaMg cells (Fig. S4 in supplementary material).



Depletion of PML sensitizes cells to UV-induced apoptosis

Since formation of ssDNA-containing foci was induced by UV exposure and further enhanced following caffeine treatment or inhibition of PARP-1, we wanted to determine the effect of PML-depletion on UV-induced apoptotic cell death. For this purpose, GM-847 cells were transfected with the PML-specific siRNAs or control siRNAs. At day three following transfection, cells were UV-irradiated and subsequently incubated in the presence or absence of caffeine for 2 hours. Apoptotic cell death was then quantified by visual inspection of chromosome condensation (Fig. 8A,B) or by analysis of apoptotic DNA fragmentation using pulse field gel electrophoresis (Fig. 8C,D). Interestingly, apoptotic cell death was found to be dramatically increased in PML-depleted cells compared with control cells following UV-irradiation. These results show that PML contributed to survival of GM-847 cells following UV-induced DNA damage. A similar effect of PML-depletion on UV-induced apoptosis was also demonstrated using GM-637 cells (Fig. S5 in supplementary material).

Discussion

In the present study a role for PML-NBs in the compartmentalization of DNA processing activities is highlighted by the observation that these nuclear structures support ssDNA focus formation in response to chromosomal DNA damage. Moreover, the importance of this activity in cellular DNA metabolic processes is underscored by our findings showing that PML is needed for efficient S-phase progression in a DNA damaging environment, and the finding that PML-depleted cells are sensitized to UV-induced

Fig. 6. ssDNA focus formation is stimulated by S-phase progression. (A) GM-847 cells were transfected with the indicated siRNAs, and BrdU was immediately added to the growth medium. At 24 hours following transfection, BrdU was removed from the cells and mimosine or HU were added. 24 hours thereafter, drugs were removed and the cells were incubated in normal growth medium until fixation at the indicated time-points. (B) ssDNA focus formation following release from mimosine block. The upper panel shows laser scanning flow cytometry analysis of TO-PRO-3-labeled cells. The lower panel shows quantitative determination of cells containing BrdU-labeled ssDNA foci. Bars represent the percentage of cells containing ssDNA foci. For each sample, more than 200 cells were evaluated. Data represent the average of two independent experiments ± s.d. (C) ssDNA focus formation following release from HU block. The same experiment as in B, except HU was used instead of mimosine. 'C' indicates samples where cells have not been treated with mimosine or HU.

apoptosis. ssDNA represents an intermediate in several DNA repair mechanisms and is thought to play a crucial role in checkpoint signaling. It is therefore possible that the observed ability of PML-NBs to support ssDNA focus formation represents an integral feature of this nuclear compartment that aids in the process of maintaining genomic integrity.

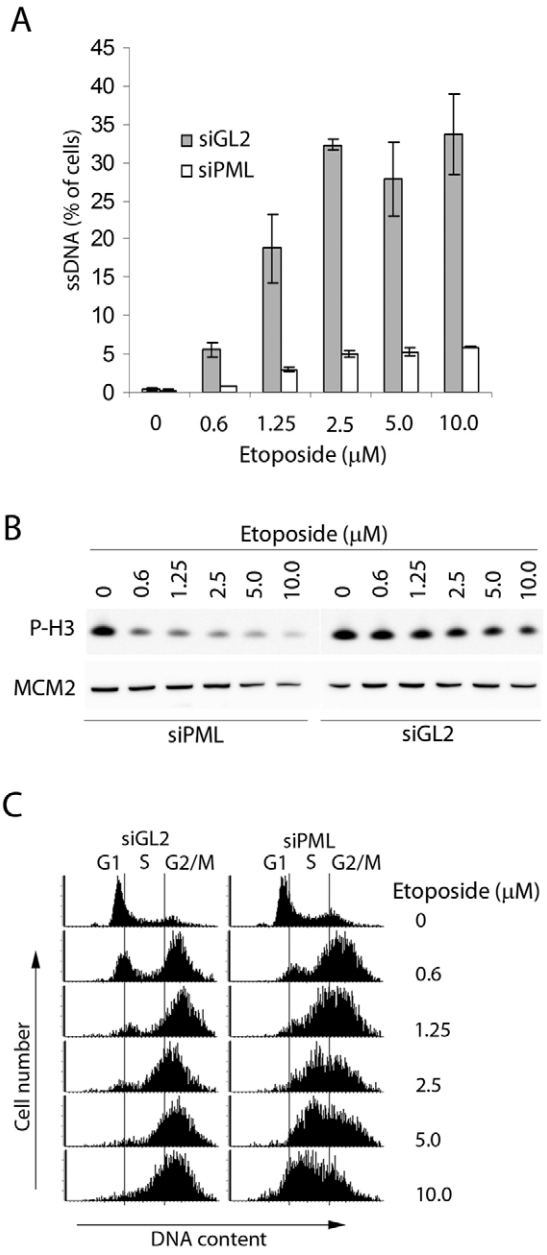


Fig. 7. Effect of PML depletion on cell cycle progression in the presence of DNA damage caused by etoposide. GM-847 cells were transfected with control siRNAs (GL2) or PML-specific siRNAs, and at 24 hours following transfection the culture medium was supplemented with 10 μM BrdU. At 48 hours post-transfection the BrdU-containing medium was removed and cells were incubated for another 24 hours in medium containing the indicated concentrations of etoposide. (A) Quantitative determination of cells containing ssDNA foci within PML-NBs. For each sample, more than 500 immunofluorescence labeled cells were evaluated. Bars represent the average of two independent experiments ± s.d. (B) Western blot analysis of soluble cell extracts using an antibody specific for histone H3 phosphorylated on Ser10. The MCM2 protein is used as loading control. (C) Analysis of To-Pro-3-stained cells by laser scanning cytometry.

A characteristic feature of cells that contain damaged DNA is the presence of discrete nuclear structures that harbor DNA repair complexes as well as proteins involved in checkpoint signaling pathways. The best characterized of these DNA repair foci are probably those that are formed in response to double-strand DNA breaks (DSBs). In this case, nuclear structures are formed at presumptive sites of DNA damage, and protein complexes that participate in the DNA repair process assemble at these focal sites in a coordinated manner (Haaf et al., 1995; Lisby et al., 2004; Lukas et al., 2004). Based on our observations that PML-NBs sequester ssDNA in response to DNA damage, and taking into account previous studies that demonstrate the presence of several DNA repair proteins within this compartment, the PML-NBs may also be regarded as a type of DNA repair structure. However, unlike the previously characterized DSB-induced foci, which appear to be formed transiently in response to DNA damage, the PML-NBs represent permanent nuclear structures that are also present in most cells during a normal unperturbed cell cycle. The experiments presented here, showing that ssDNA focus formation occurs within pre-existing PML-NBs, therefore may imply a hitherto unexplored intrinsic capacity of these compartments to recruit damaged DNA and to organize DNA processing events following DNA damage. Alternatively, instead of recruiting ssDNA from other nuclear sites, the PML-NBs may harbor specific regions of chromosomal DNA that are processed into ssDNA subsequent to DNA damage.

Although our study points to a role of PML and PML-NBs in DNA processing subsequent to DNA damage, the precise nature of the molecular mechanisms involved is not clear. However, several observations made in the present study suggest that the PML-dependent ssDNA focus formation is particularly efficient during S-phase progression. For example, cell-cycle synchronization experiments using nocodazole revealed that ssDNA focus formation was less efficient in cells residing in the G1-phase compared with cells in S-phase. Furthermore, cell cycle blockage using HU or mimosine revealed an increase in PML-dependent ssDNA focus formation following release of cells from the block, suggesting that PML-NBs sequester ssDNA more efficiently in cells that support active DNA synthesis. Lastly, the presence of etoposide in the cell culture medium resulted in a more prominent S-phase arrest in PML-depleted cells compared with control cells, indicating that PML is needed for efficient S-phase progression in the presence of DNA damage induced by this drug.

A connection between PML-NBs and chromosomal DNA synthesis may also represent the underlying reason why caffeine and the PARP-inhibitor NU1025 both have a stimulatory effect on UV-induced PML-dependent ssDNA focus formation. For example, previous studies have shown that the radiosensitizing effect of caffeine on mammalian cells is particularly pronounced during S-phase (Kuhlmann et al., 1968; Rauth, 1967). In addition, this drug is known to inhibit the repair of gaps that form in daughter strand DNA during chromosomal DNA replication following UV-irradiation (Arlett et al., 1975; Buhl and Regan, 1974), an effect of

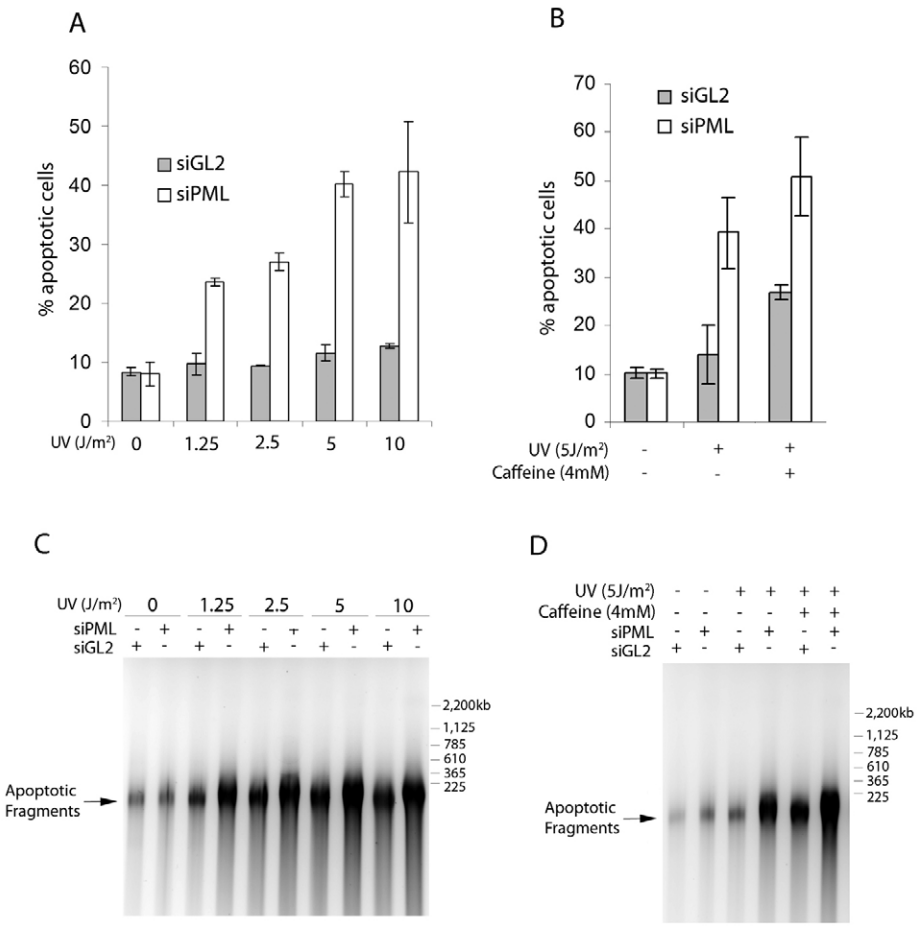


Fig. 8. Effect of PML depletion on UV-induced apoptosis. GM-847 cells were transfected with PML-specific siRNAs or control siRNAs (GL2) and subjected to the indicated doses of UV irradiation on day 3 following transfection. Subsequent to irradiation, cells were incubated in the absence or in the presence of 4 mM caffeine for 2 hours prior to analysis. (A,B) Cells were fixed, stained with Hoechst and evaluated by immunofluorescence microscopy for the presence or absence of condensed chromatin. For each sample, more than 500 cells were evaluated. The data represents the mean ± s.d. of two independent experiments. (C,D) Analysis of apoptotic DNA fragmentation by pulse field gel electrophoresis. The 50-300 kb apoptotic DNA fragments are indicated.

caffeine that is particularly enhanced in cells lacking functional DNA pol η (Cleaver et al., 1979; Lehman et al., 1975; Limoli et al., 2000). Similarly to caffeine, PARP inhibitors are also thought to sensitize cells to DNA damaging agents by interfering with DNA repair activities (Tong et al., 2001). It is therefore possible that both of these components enhance UV-induced PML-dependent focus formation due to a common ability to interfere with the processing of replication-induced DNA damage.

The difference seen among different cell lines in the capacity to form detectable PML-associated ssDNA foci may be attributed to differences in the ability to repair UV-damaged DNA and/or to activate DNA-damage-induced checkpoints. For example, SV-40-transformed cells, which were found in the present study to be particularly efficient in PML-dependent ssDNA focus formation, are known to be deficient in multiple DNA repair pathways (Bowman et al., 2000; Digweed et al., 2002; Wu et al., 2004). It is also of interest to note that studies in yeast have shown that defects in checkpoint activation or DNA repair mechanisms frequently result in the formation of abnormally long stretches of ssDNA at stalled replication forks (Lopes et al., 2006; Sogo et al., 2002).

A role of PML-NBs in the processing of replication-induced DNA damage is also in agreement with our previous work showing that these structures accumulate ssDNA molecules following infection of cells with the polyomaviruses simian virus 40 or polyomaviurs BK (Jul-Larsen et al., 2004). In this case, accumulation of DNA within PML-NBs was found to be dependent on active replication of viral DNA molecules. A direct link between PML and the process of viral DNA synthesis has also been demonstrated in cells infected by herpes simplex type 1 (HSV-1). Following infection with this virus, virally induced nuclear compartments were formed, and the recruitment of PML to these nuclear structures depended on components of the viral DNA synthesis machinery (Burkham et al., 1998). It is also worth mentioning that the PML protein has been shown to functionally interact with TopBP1 (Xu et al., 2003), a protein that appears to play an important role in maintaining genomic integrity during S-phase of the cell cycle (Kim et al., 2005; Makiniemi et al., 2001). In addition, PML-NBs have been noted in a previous study to be associated with sites of DNA synthesis during middle-late S-phase (Grande et al., 1996). Lastly, a resent study demonstrated an increasing number of PML-NB fission events during S-phase of the cell cycle, and that the newly formed fission products exhibited an increased capability of associating with chromatin (Dellaire et al., 2006).

Our finding that PML-depleted cells enter apoptosis more readily than non-depleted cells following UV-irradiation was surprising in light of previous studies showing that PML$^{-/-}$ mouse embryonic fibroblasts in general are more resistant than wild-type cells to apoptotic cell death (reviewed by Bernardi and Pandolfi, 2003). This discrepancy between our study and previous reports may be attributed to differences in experimental settings and/or differences in the cell types used. It is plausible, for example, that PML-dependent processing of

damaged DNA is more critical for cell survival in certain tumor cells or transformed cell lines than in normal primary cells. It should also be noted that the PARP-1 protein exhibits pro-apoptotic activities in normal cells, whereas inhibition of PARP-1 in tumor cells or transformed cell lines leads to increased sensitivity to DNA damaging agents (Tong et al., 2001).

Despite the accumulating evidence showing that PML participates in the cellular response to DNA damage, it is clear from our and previous studies that PML-NBs do not represent nuclear structures that are created as a result of exogenous DNA damage. In fact, several DNA repair proteins have been shown to relocate from the PML-NBs to other nuclear sites following treatment of cells with γ-irradiation (Barr et al., 2003; Mirzoeva and Petrini, 2001; Yang et al., 2002). This has led several investigators to speculate that this nuclear compartment exerts its function in DNA damage response primarily by functioning as a protein storage depot, assembly factories for protein complexes, or as a nuclear compartment that supports enzymatic post-translational modifications of protein components. Our finding that PML-NBs have the capacity to accommodate ssDNA within 1 hour following UV-irradiation suggests that these structures contribute directly as DNA repair compartments. The presence of DNA repair proteins such as Rad51, BLM and RPA in PML-NBs of unperturbed cells may further suggest that these structures are active in DNA metabolic processes also in the absence of exogenous DNA damaging stimuli. Thus, the persistence of these nuclear structures in most mammalian cells during a normal cell cycle may reflect the need for PML-NBs for secure procession of damaged DNA that may arise during DNA metabolic activities such as transcription or DNA replication.

Materials and Methods

Cell culture and drugs

All cell lines except human umbilical endothelial cells (HUVECs) were grown in Iscove's modified Dulbeccos medium (IMDM) (Bio Wittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS). HUVECS were grown as described previously (Jul-Larsen et al., 2004). Caffeine (Calbiochem, San Diego, CA) was used at a final concentration of 4 mM. The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD) was obtained from Alexis Corporation (Lauzanne, Switzerland) and used at a final concentration of 50 μM. The PARP inhibitor NU1025 was purchased from Calbiochem and used at a final concentration of 0.5 mM. Hydroxyurea and mimosine was from Calbiochem and used at a final concentration of 4 and 0.5 mM, respectively. Etoposide was from Bristol-Myers Squibb Company (New York, NY). TNFα and cyclohexamide were obtained from Sigma-Aldrich (St Louis, MO) and used at a final concentration of 20 ng/ml and 1 μg/ml, respectively. Nocodazole was from Calbiochem and used at a final concentration of 0.1 μg/ml.

SiRNA transfection

RNA oligonucleotides used to prepare siRNA duplexes were obtained from Eurogentec (Seraing, Belgium). The following siRNA target-sequences were used: PML, 5′-gcaucuacugccgaggaug-3′ (Jul-Larsen et al., 2004); PARP-1, 5′-gccuccgcuccugaacaat-3′; ATM, 5′-cauacuacucaaagacauu-3′; ATR, 5′-aaccucccgugauguugcuuga-3′; control siRNA against the firefly luciferase gene (GL2), 5′-cuuacgcugaguacuucga-3′. Transfection of cells with siRNA duplexes was carried out by using the oligofectamine reagent from Invitrogen (Carlsbad, CA).

Antibodies

Primary antibodies used were mouse monoclonal anti-BrdU (RPN20; Amersham, Pharmacia Biotech, Uppsala, Sweden), rabbit anti-PML (H-238; Santa Cruz Biotechnology, CA), mouse monoclonal anti-TRF2 (Upstate, Waltham, MA); rabbit anti-PARP (Upstate); rabbit anti-phospho histone H3 (Upstate); goat anti-MCM2 (N-19; Santa Cruz Biotechnology), goat anti-β-actin (I-19; Santa Cruz Biotechnology), goat anti-ATM (H-248; Santa Cruz Biotechnology) and goat anti-ATR (N-19; Santa Cruz Biotechnology).

ssDNA detection, UV-irradiation and immunofluorescence labeling

In order to detect ssDNA, cells seeded on coverslips were grown in the presence of 10 μM BrdU for 22 hours. Cells were then washed twice in normal medium and subsequently grown for another 2 hours in the absence of BrdU prior to treatment with UV light or drugs. UV-irradiation was carried out using a hand-held UVG-54 lamp from UVP (Upland, CA), which emitted light at a wavelength of 254 nm. Calibration of the lamp was achieved using a UV-meter. Immediately prior to irradiation, cells were overlaid with a thin layer of pre-warmed PBS, and growth medium with or without drugs was added immediately after irradiation. Fixation of cells and immunofluorescence labeling was carried out as previously described (Jul-Larsen et al., 2004). Scoring of cells containing ssDNA foci was carried out using a 40× oil immersion lens attached to a Leica DMRXA microscope. For each sample, the cells within 6 to 10 randomly selected fields of view were evaluated. Acquisition of fluorescence microscopic images was performed using a Leica DMRXA microscope as previously described (Jul-Larsen et al., 2004). Laser scanning microscopy images were obtained using a 63× oil immersion objective attached to a Zeiss LSM 510 Meta microscope (Jena, Germany). Determination of the degree of ssDNA labeling that overlapped the PML signal was obtained using LSM 5 Image Examiner software. A fluorescence intensity value of 50 (see diagrams in supplementary material Fig. S1) was used as threshold for these measurements. For each analysis two parallel experiments were performed each scoring 4-8 representative nuclei.

Protein extracts and western blotting

Protein extracts were prepared from cells grown in 6 or 10 cm culture dishes. Cells were detached from the plates by trypsin treatment and subsequently washed twice in PBS followed by suspension in lysis buffer (20 mM Tris, pH 7.5, 0.42 M KCl, 25% glycerol, 0.1 mM EDTA, 5 mM MgCl, 0.5% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Following incubation on ice for 30 minutes, samples were centrifuged at 12,000 ***g*** for 10 minutes, and the supernatant obtained was referred to as soluble protein extract. The resulting pellet was re-suspended in digestion buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 25% glycerol, 0.1 mM EDTA, 5 mM MgCl, 0.5% Triton X-100, 100 μg/ml DNase I, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin), and incubated for 20 minutes at room temperature. Subsequently, ammoniumsulphate was added to a final concentration of 250 mM followed by incubation for 5 minutes at room temperature and centrifugation at 12,000 ***g*** for 10 minutes. The resulting pellet was suspended in disassembly buffer (8 M urea, 1% 2-mercaptoetanol, 1 mM PMSF), incubated for 20 minutes at room temperature followed by centrifugation at 12,000 ***g*** for 10 minutes. The supernatant was referred to as nuclear matrix extract. Western blotting was performed using NuPAGE 4-12% gradient Bis-Tris gels from Invitrogen.

Live cell imaging

GM-847 cells were seeded in 35 mm glass bottom microwell dishes from MatTek Corporation (Ashland, MA). The day after, cells were transfected with the plasmid pYFP-PML (kindly provided by Wiesmeijer and Dirks at Leiden University Medical Center in the Netherlands) (Molenaar et al., 2003) using the FuGENE6 reagent from Roche (Indianapolis, IN). To avoid over-expression of the PML-YFP fusion protein, which would result in abnormally large and deformed PML-NBs, we transfected cells with 7.5 ng pYFP-PML per dish and balanced the amount of total DNA molecules transfected using 1.5 μg of the plasmid pCMV-HA (Clontech, Palo Alto, CA) as a carrier DNA. Following transfection, cells were grown in the presence of 10 μM BrdU until the time of live cell imaging the subsequent day. Immediately following UV exposure of transfected cells, pre-warmed growth medium containing 4 mM caffeine was added, dishes were sealed with parafilm and subsequently placed on top of a 63× oil immersion lens situated in a 37°C incubation chamber. The lens was attached to an Ultraview RS spinning disc confocal microscope from Perkin Elmer (Wellesley, MA). 3D images were acquired every 30 seconds during a total recording period of 90 minutes. Between 5 and 8 optical sections separated by 0.5 μm were acquired for each time-point, and the exposure time for each of the sections was 50 milliseconds. Images were viewed as maximum intensity projections of each time point.

Apoptosis assays

For scoring of chromatin condensation, cells grown on glass cover slips in 24-well plates were fixed by adding an equal volume of 4% paraformaldehyde containing Hoechst 33342 (10 μg/ml) to the growth medium. Evaluation of the cells was carried out using an Axiovert 200M fluorescence microscope from Zeiss (Oberkochen, Germany) equipped with a 20× objective.

For analysis of chromosomal DNA fragmentation by pulse field gel electrophoresis, cells grown in 6 cm dishes were released by trypsin, and subsequently embedded in 1% agarose plugs (two plugs per cell culture dish). The plugs were treated with a buffer containing 100 mM EDTA, pH 8.0, 1% sodium N-lauroyl-sarcosinate and 0.5 mg/ml proteinase K for 24 hours, and subsequently washed 4 × 20 minutes in a buffer containing 20 mM Tris, pH 8.0 and 50 mM EDTA. The plugs were loaded on a 1% agarose gel (Megabase agarose from Bio-

Rad, Hercules, CA) and separation was performed for 20 hours on a Bio-Rad CHEF-DR III pulse field gel electrophoresis apparatus at 120° field angle, 240 seconds switch time and 4 V/cm^3. The chromosomal DNA was stained with SYBR Green (Molecular Probes, Eugene, OR) overnight and analyzed using the LAS-3000 imaging system (Fujifilm).

Laser scanning flow cytometry

To generate cell cycle profiles of cells grown on glass cover slips, we stained cells with the TO-PRO-3 reagent from Molecular Probes, and subsequently analyzed the cells using a laser scanning flow cytometer (CompuCyte, Cambridge, MA).

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