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TGF-β induces PML SUMOylation, degradation and PML nuclear body disruption

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ABSTRACT

ProMyelocytic Leukemia (PML) protein is essential for the formation of nuclear matrix-associated organelles named PML nuclear bodies (NBs) that act as a platform for post-translational modifications and protein degradation. PML NBs harbor transiently and permanently localized proteins and are associated with the regulation of several cellular functions including apoptosis. There are seven PML isoforms, six nuclear (PMLI-VI) and one cytoplasmic (PMLVII), which are encoded by a single gene *via* alternative RNA splicing. It has been reported that murine PML-null primary cells are resistant to TGF-β-induced apoptosis and that cytoplasmic PML is an essential activator of TGF-β signaling. The role and the fate of interferon (IFN)-enhanced PML NBs in response to TGF-β have not been investigated. Here we show that IFNα potentiated TGF-β-mediated apoptosis in human cells. IFNα or ectopic expression of PMLIV, but not of PMLIII, enhanced TGF-β-induced caspase 8 activation. In response to TGF-β, both PMLIII and PMLIV were conjugated to SUMO and shifted from the nucleoplasm to the nuclear matrix, however only PMLIV, *via* its specific C-terminal region, interacted with caspase 8 and recruited it within PML NBs. This process was followed by a caspase-dependent PML degradation and PML NB disruption. Taken together, these findings highlight the role of PML NBs in the enhancement by IFN of TGF-β-induced apoptosis and caspase 8 activation.

1. Introduction

PML (also named TRIM19 for TRIpartite Motif protein 19) is the organizer of small nuclear-matrix structures named nuclear bodies (NBs). In response to diverse stimuli, PML NBs recruit a growing number of proteins implicated in different cellular processes such as protein degradation, post-translational modifications, antiviral response and apoptosis [1–4]. PML NBs are dynamic structures harboring numerous transiently and permanently localized proteins [5]. Due to alternative splicing, seven PML isoforms are synthesized from a single gene, designated PMLI-VII (reviewed in [6,7]). They share the N-terminal region, which encodes the RBCC (RING finger, B boxes and coiled-coil), whereas they differ in their C-terminal region. Most of PML isoforms (PMLI to PMLVI), contain the nuclear localization signal (NLS) and are localized in the nucleus, whereas PMLVII lacks the NLS and is found in the cytoplasm [7]. PML isoforms are almost equally expressed in the different cell lines and have the potential to form homodimers *via* their coiled-coil domain [8]. In addition to the RBCC motif, a post-translational modification of PML by SUMO (Small Ubiquitin-like Modifier), an ubiquitin-like protein of 11 kDa, is another requirement to PML NB function. *PML* is directly induced in response to interferons (IFNs) through the IFN-response elements present in the PML promoter [9], resulting in the increase of the expression of PML isoforms and the number of PML NBs [10].

PML localization is intimately linked to its SUMOylation. Within the nucleus, most of PML, in transfected [11–14] or IFN-treated [15] cells, is expressed in the diffuse nuclear fraction of the nucleoplasm (RIPA soluble fraction) with only a small fraction in the matrix-associated NBs

Abbreviations: IFN, Interferon; NBs, Nuclear Bodies; Ni^{2+}-NTA, Nickel-NitriloTriacetic Acid; PML, ProMyelocytic Leukemia protein; SUMO, Small Ubiquitin-related MOdifier; TGF-β, Transforming Growth Factor beta; UBC9, Ubiquitin Conjugating Enzyme9

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(RIPA insoluble fraction). The transfer of PML from the nucleoplasm to PML NBs is associated with PML SUMOylation and is important for PML NB functions such as the recruitment of PML NB partners, nuclear protein degradation and antiviral defense [2,7]. Indeed, it has been reported that arsenic trioxide (As_2O_3) treatment [13–16] or picornavirus infection [17,18] results in the shift of PML from the nucleoplasm to the nuclear matrix and in the increase of nuclear PML SUMOylation.

Transforming growth factor beta (TGF-β) induces apoptosis in various cell types including hepatoma, epithelial and hematopoietic cells. Many cancers of epithelial or lymphoid origin develop resistance to the apoptotic effects of TGF-β, suggesting that these cancer cells undergo neoplastic transformation and escape from normal growth control through an altered response to the anti-growth effects of TGF-β [19,20]. Furthermore, *in vivo* and cell-culture experiments argue for a pivotal role of TGF-β-mediated apoptosis in the maintenance of B- and T-cell homeostasis [21,22].

Different reports have demonstrated that caspase 8 activation is an essential mediator of apoptosis induced by TGF-β [23,24]. In addition, caspase 8 was shown to be conjugated to SUMO at lysine 156 [25]. This modification alters its localization without affecting its activity since the non SUMOylated p55 caspase 8 form is essentially present in the cytoplasm whereas SUMOylated p75 caspase 8 is exclusively nuclear and retains the ability to be activated [25].

TGF-β signaling is initiated by the formation of an heterodimeric complex consisting of two types of transmembrane serine/threonine kinase receptors. TGF-β binding leads to SMAD2 and SMAD3 phosphorylation, a process that is facilitated by the TGF-β-receptor adapter SARA (SMAD Anchor for Receptor Activation) and that results in the translocation of SMADs to the nucleus and the activation of target genes [26]. Multiple components of TGF-β signaling such as TGF-β receptor I, SMAD3 and 4 can be targeted by the SUMO system [27]. Remarkably PML was identified as a key regulator of TGF-β signaling. It has been reported that cytoplasmic PML (cPML) facilitates the interaction of SARA with SMAD2 and SMAD3 and enhances TGF-β-induced SMAD2 and SMAD3 phosphorylation [28]. Also, TGF-β signaling is impaired in murine PML null primary cells, and this defect is rescued by the restoration of cPML but not by nuclear PML [28,29]. However, the role of IFN-enhanced PML NBs in TGF-β-induced apoptosis is unknown.

IFNs are pleiotropic cytokines that were first identified as antiviral agents secreted by infected mammalian cells, but have later been shown to be important regulators of cell growth [30]. Based on their structure and interaction with distinct receptor complexes, IFNs are classified in three distinct types, I, II and III. IFNs consist in multiple type I species (including IFNα and IFNβ), one type II (IFNγ), and four members of type III (IFNλ1-4) [31]. IFNs mediate their effects by binding to corresponding cell surface receptors, activating the JAK/STAT pathway and leading to the induction of the IFN-stimulated genes (ISGs). IFNs induce apoptosis after long-time treatment in multiple cell lines and, often *via* a caspase-dependent mechanism. Furthermore, gene microarray studies have identified ISGs such as PML and caspase 8 as directly involved in IFN-apoptotic functions [32]. In addition, RNAi silencing of PML blocks IFNα-induced apoptosis in myeloma cells [33].

To gain insights into the mechanisms integrating PML NBs in TGF-β-mediated apoptosis, we investigated the effects of IFNα and TGF-β on PML modification and localization as well as on caspase 8 activation. Here, we show that IFNα pretreatment of Burkitt lymphoma BL41 cells increased TGF-β-induced apoptosis and caspase 8 activation. TGF-β enhanced the conjugation of PMLIII and PMLIV to SUMO, induced their transfer from the nucleoplasm to the nuclear matrix where SUMOylated caspase 8 (p75) and its activated forms (p45, p20) were found. PMLIV, but not PMLIII, interacted with caspase 8. The specific C-terminal part of PMLIV was required for the interaction and the recruitment of caspase 8 within PML NBs. This process was followed by caspase-dependent PML degradation and PML NB disruption.

2. Materials and methods

2.1. Materials

Human recombinant IFNα2b was obtained from Schering-Plough (Kenilworth, NJ, USA) and used at 1000 international Units/ml. Recombinant human TGF-β, the proteasome inhibitor MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal-H) and rabbit anti-phospho SMAD2 (pSer250) antibodies were purchased from Sigma (USA). The caspase inhibitor, Z-VAD-fmk, was from Promega (France) and caspase 8 Inhibitor II (Z-IETD-FMK) was from Merck (USA), they were both used at a concentration of 50 μM. Rabbit polyclonal anti-PML (sc-5621), mouse monoclonal anti-PML (PGM3, sc-966) and rabbit polyclonal anti-Actin (sc-1615) antibodies were from Santa Cruz Biotechnology (USA). Monoclonal anti-αTubulin (DM1A) antibody was from Sigma (USA). Rabbit anti-histone H3, mouse monoclonal anti-caspase 8 (1C12), rabbit monoclonal anti-SUMO2/3 (18H8) and rabbit anti-SMAD2 (3122) antibodies were from Cell Signaling (USA). Rabbit anti-caspase 8 antibodies used for immunofluorescence were from Novus biologicals (USA). Secondary antibodies conjugated to Alexa Fluor were purchased from Molecular Probes (Thermo Fischer, France).

2.2. Cell cultures

The Burkitt lymphoma cells BL41 were obtained from DSMZ (Germany) and were maintained in RPMI medium containing 10% fetal calf serum. HEK293 and U373MG cells were grown at 37 °C in DMEM supplemented with 10% foetal calf serum. U373MG cells stably expressing PMLIII (U373MG-PMLIII) and PMLIV (U373MG-PMLIV) [12] were kept in medium supplemented with 0.5 mg/ml of G418. Cells were transfected, using the lipofectamine 2000 as previously described [12] with His_6-tagged (SUMO1, SUMO2 or SUMO3), PMLIII, PMLIV, PMLIV-Δ8b mutant or the empty vector (EV).

2.3. Western blot analysis of total cell extracts, RIPA soluble and insoluble fractions

Analysis of total cell extracts, cells were washed in PBS, scraped into Laemmli buffer, and boiled for 5 min. Analysis of the RIPA soluble and insoluble fractions, after the different cell treatment, the cytoplasmic and nucleoplasm fraction was extracted by incubating the pellet in 50 μl of RIPA buffer for 20 min on ice followed by centrifugation at 12000g for 15 min to separate the RIPA soluble fraction (R) from the pellet (P). The RIPA soluble fraction (R) was kept and the pellet, which represents the RIPA insoluble fraction (P), was suspended in 50 μl of PBS. 50 μl of 2X Laemmli buffer was added to each fraction, and the samples were boiled for 5 min before western blot analysis.

2.4. Immunofluorescence staining and confocal microscopy

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at 4 °C and permeabilised for 5 min with 0.1% Triton X-100 in PBS. They were then prepared for simple or double-immunofluorescence staining and analyzed by confocal microscopy. PML was stained with mouse monoclonal anti-PML antibody followed by mouse Alexa Fluor 594. Caspase 8 was detected with rabbit anti-caspase 8 antibodies followed by rabbit Alexa Fluor 488. Confocal laser microscopy was performed on a Zeiss LSM 710 microscope.

2.5. Purification of His_6-tagged SUMO PML conjugates

The purification of His-tagged conjugates, using Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA)-agarose beads allows the purification of proteins that are covalently conjugated to His_6-SUMO. 24 h after

transfection, HEK293 cells were separated into two flasks for each condition, then were left untreated or treated with 1 ng/ml of TGF-β for 15 h. Cells were lysed in denaturating buffer A (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 8.0 plus 5 mM imidazole and 10 mM β-mercaptoethanol) then sonicated twice for 30 s at medium power; the lysates were incubated with 50 µl of Ni^{2+}-NTA-agarose beads (Qiagen, France) for 3 h at room temperature. The beads were successively washed with buffer B (0.1% triton X100; 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 6.3, 10 mM β-mercaptoethanol), and subsequently eluted with 200 mM imidazole in 0.15 M Tris-HCl pH 6.7, 30% glycerol and 0.72 M β-mercaptoethanol. The eluates were then analyzed by western blot.

2.6. Immunoprecipitation assays

BL41 (10^7) cells were treated with 1000U/ml of IFNα for 24 h before addition of 1 ng/ml of TGF-β for 15 h and HEK293 cells, transfected with PMLIII, PMLIV, PMLIV-Δ8b or the empty vector, were untreated or treated with TGF-β for 15 h. Next, cells were incubated for 30 min at 4 °C in 0. 5 ml of non-denaturing buffer containing 20 mM Tris-HCl (pH 7.4), 1 M NaCl, 5 mM $MgCl_2$, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NEM. After cell lysis, 50 µl were saved for the input and 2.4 ml of 20 mM Tris-HCl (pH 7.4) were added to the remaining 450 µl. The anti-caspase 8 antibody (Cell Signaling) was added, and the samples were incubated overnight at 4 °C. Protein G beads in immunoprecipitation buffer (IB) (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) were then added, and the samples were mixed for 2 h at room temperature. The beads were collected and washed four times with IB, and the bound proteins were subjected to western blotting.

2.7. Apoptosis

Cell apoptosis was assessed using Annexin V-FITC/PI Kit (BD Biosciences). Briefly, untreated and treated BL41 cells were collected, resuspended in 100 µl of PBS and stained with Annexin V for 15 min at 4 °C, followed by PI staining. Cells were analyzed by flow cytometry on a Canto II (BD Biosciences).

3. Results

3.1. IFNα pretreatment enhances TGF-β-mediated apoptosis in BL41 cells

First, to investigate the ability of IFNα to influence BL41 cell death induced by TGF-β, cells were treated with 1000U/ml of IFNα for 24 h before adding 1 ng/ml of TGF-β for 40 h. The results shown in Fig. 1A revealed that pretreatment of the cells with IFNα significantly increased TGF-β-mediated apoptosis. Since cPML has been shown to modulate TGF-β signaling by facilitating SMAD phosphorylation [28], we tested whether cPML is implicated in the increase by IFNα of TGF-β-induced apoptosis. Western blot analysis of phosphorylated SMAD2 revealed that IFNα pretreatment did not alter TGF-β-induced SMAD2 phosphorylation (Fig. 1B), thus suggesting that cPML was not implicated in this process. Next, we tested whether the increase by IFNα of TGF-β-induced apoptosis in BL41 cells was accompanied by a higher caspase 8 activation. Western blot analysis for caspase 8 expression in extracts of BL41 cells treated with IFNα, TGF-β or their combination is shown in Fig. 1C. In accordance with a previous report [34] showing that the *caspase 8* gene is regulated through an IFN-stimulated response element (ISRE), we confirmed that IFNα increased p55 caspase 8 expression (Fig. 1C).

The activation of caspase 8 was demonstrated by the detection of cleaved p45 and p20 kDa fragments. The p45 and p20 cleaved forms of caspase 8 were not increased in IFNα-treated cells, they were enhanced in response to TGF-β and their intensity was further increased when cells were pretreated with IFNα before the addition of TGF-β (Fig. 1C). These findings indicate that the capacity of IFNα to increase TGF-β-induced apoptosis was mediated by an increase of the caspase 8 activity.

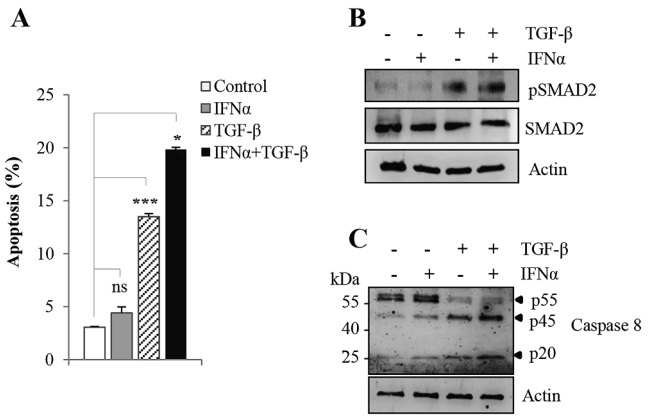

Fig. 1. IFNα increases TGF-β-mediated apoptosis and caspase 8 activation. (A) BL41 cells were treated with 1000U/ml of IFNα for 24 h before addition of 1 ng/ml of TGF-β for 40 h. Apoptosis was determined by flow cytometry. The results are representative of at least 3 independent experiments. Student *t* test was performed to determine the *p* value. The asterisks indicate a significant difference compared to control (**p < 0.01, *** p < 0.001), ns: not significant. (B/C) BL41 cells were untreated or treated with 1000U/ml of IFNα for 24 h before addition of 1 ng/ml of TGF-β for 40 h and their extracts were analyzed by western blot for SMAD2, pSMAD2, Actin (B), caspase 8 and Actin (C).

3.2. TGF-β induces caspase-dependent PML degradation leading to PML NB disruption.

As PML NBs play a role in mediating IFN-induced biological responses [2,4], we studied the fate of nuclear PML isoforms following TGF-β treatment. To determine the effects of TGF-β on PML protein levels, BL41 cells were treated with IFNα for 24 h before adding TGF-β for 24, 40 or 48 h (Fig. 2A and 2B). As previously reported [10], different forms of PML were upregulated in response to IFNα (Fig. 2A). The numerous bands detected, most likely represent different isoforms of PML resulting from alternative splicing of the single gene. The apparent molecular weights of nuclear PML isoforms (PMLI to PMLVI) are between 70 and 113 kDa, whereas cytoplasmic PMLVII has an apparent molecular weight of 50 kDa. This has been shown by analyzing PML by western blot in cells stably expressing each PML isoform [11,12,35–38]. In addition, all the nuclear PML isoforms have been shown to be SUMOylated [35], therefore, some bands could also represent modified PML isoforms in IFN-treated BL41 cells (Fig. 2A). The addition of TGF-β to IFN-treated BL41 cells resulted in a decrease of nuclear PML forms migrating at molecular weights ranging between 70 and 113 kDa (Fig. 2A and B) that was detected at 24 h (Fig. 2A) and enhanced 40 h and 48 h post-treatment (Fig. 2B). This decrease was accompanied by the appearance of cleaved forms of PML (Fig. 2A). In addition, immunofluorescence analyses performed on BL41 cells 40 h post TGF-β treatment, revealed a loss of PML staining in PML NBs with a delocalization of PML to the cytoplasm both in untreated and IFNα-treated cells (Fig. 2C). As expected, in the IFNα-treated cells, PML expression was upregulated leading to an increase in the number of PML NBs and TGF-β-mediated PML NB disruption was more pronounced (Fig. 2C).

To test whether a decrease in PML expression is due to its degradation *via* proteasome- or caspase-dependent pathways, TGF-β was added to IFNα-treated BL41 cells in the presence of either the proteasome inhibitor MG132 or the caspase inhibitor Z-VAD-FMK, a caspase inhibitor with broad specificity. Western blot analyses revealed that MG132 treatment did not reverse TGF-β-induced decrease of PML

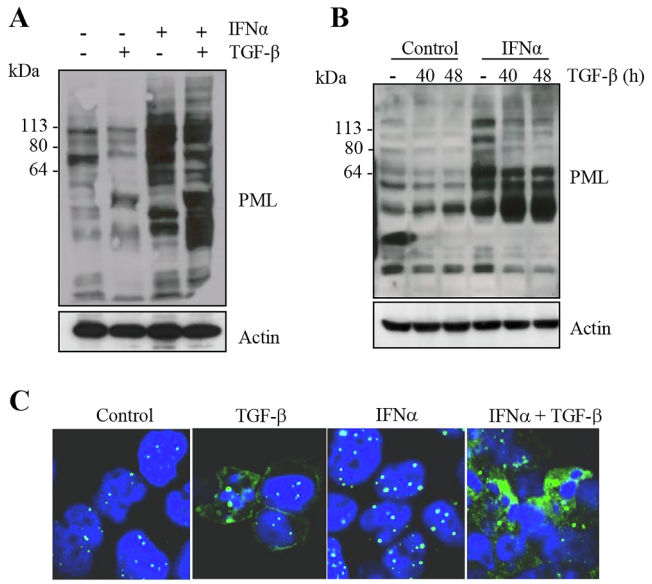

Fig. 2. TGF-β targets nuclear PML. (A/B) BL41 cells were untreated or treated with 1000 U/ml of IFNα for 24 h before addition of 1 ng/ml of TGF-β for 24 h (A), 40 h or 48 h (B). Western blot was performed and revealed with anti-PML and anti-Actin antibodies. (C) TGF-β disrupts PML NBs. BL41 cells were pretreated for 24 h with 1000 U/ml of IFNα before addition of TGF-β (1 ng/ml). 40 h later, immunofluorescence analysis was performed using anti-PML antibodies and DAPI staining.

protein (Fig. 3A). In contrast, addition of Z-VAD-FMK abolished TGF-β-induced PML protein decrease (Fig. 3B), both in untreated and IFNα-treated cells, thus suggesting that TGF-β induced caspase-dependent PML degradation and consequently PML NB disruption. In addition, TGF-β-induced PML degradation in IFNα-treated cells was reversed when cells were treated with caspase 8 inhibitor Z-IETD-FMK (Fig. 3C), thus suggesting that caspase 8 specifically targeted PML.

3.3. TGF-β induces a shift of PML to the nuclear matrix and PML conjugation to SUMO.

In the nucleus of untreated and IFNα-treated cells, PML is localized mainly in the nucleoplasm with a small fraction in the matrix-associated PML NBs [15]. Apart from PMLVII, all PML isoforms (PMLI to PMLVI) contain an NLS and are therefore found in the nucleus. The fact that the addition of TGF-β for 40 h in IFNα-treated BL41 cells leads to the degradation of nuclear PML isoforms (Fig. 3), prompted us to determine whether PML protein localization and modification were altered at an early time following TGF-β treatment such as 15 h. This hypothesis was tested in BL41 cells treated with IFNα for 24 h and in U373MG cells expressing PMLIII or PMLIV, the most studied PML isoforms in induction of apoptosis [3,7]. In addition, we have previously shown that the expression of PMLIII and PMLIV is enhanced in response to IFN [12]. To biochemically detect whether TGF-β treatment alters PML localization by inducing its transfer from the nucleoplasm to the nuclear matrix, we have analyzed as previously described [15,18] by western blot the RIPA soluble (the cytoplasm and most of the nucleoplasm) and insoluble (the nuclear matrix and some chromatin components) fractions from IFNα-treated BL41cells (Fig. 4A), U373MG cells overexpressing PMLIII (Fig. 4B) or PMLIV (Fig. 4C), untreated or treated for 15 h with TGF-β. Analysis by immunoblotting of the distribution of the RIPA soluble fraction marker Tubulin and the RIPA insoluble fraction marker Histone H3 showed that the degree of cross-contamination between these fractions was minimal. In IFNα-treated cells, PML was found mainly in the RIPA soluble fraction (Fig. 4A). Remarkably, upon TGF-β addition, the RIPA soluble PML was completely lost and was found in the RIPA-insoluble fractions with the appearance of PML-SUMO conjugated forms (Fig. 4A). In agreement with our previous report [38], we confirm that IFN enhanced cellular SUMO2/3 modification (Fig. 4A) and we show that SUMOylated proteins were also found in the RIPA-soluble fraction and were shifted, like PML, to the RIPA-insoluble fraction upon the addition of TGF-β (Fig. 4A). Also, PML and SUMO2/3 immunoblots revealed that TGF-β induced the transfer of both PML and its SUMOylated forms to the nuclear matrix in cells stably expressing PMLIII (Fig. 4B) or PMLIV (Fig. 4C), the lower bands of PML in Fig. 4B and 4C represent the unmodified PML and the upper bands represent the modified PMLs. In other words, TGF-β treatment shifted PML from the nucleoplasm to the nuclear matrix and promoted the appearance of higher molecular weight products representing the PML-SUMO conjugated forms.

Analysis of caspase 8 expression in extracts from PMLIII- and PMLIV-expressing cells untreated or treated with TGF-β (Fig. 4B and C), revealed that caspase 8 (p55) was found mainly in the RIPA-soluble fractions, whereas, different forms of caspase 8 were revealed in the insoluble fractions of TGF-β-treated PMLIV-expressing cells (Fig. 4C). These forms include activated caspase 8 (p45 and p20) and p75 caspase 8 that has been reported previously to be the SUMOylated form of caspase 8 [25]. Interestingly, PMLIV, but not PMLIII, enhanced in the nuclear matrix TGF-β-induced caspase 8 activation (Fig. 4B and C).

Taken together these results show that i- TGF-β induced the transfer of PML from the nucleoplasm to the nuclear matrix in cells expressing PMLIII or PMLIV and in IFNα-treated BL41 cells, ii- SUMOylated p75 caspase 8 and activated caspase 8 (p45 and p20) were present in the nuclear matrix of TGF-β-treated PMLIV-expressing cells, and iii- This process preceded PML degradation and PML NB disruption, a step that was observed at a later stage of TGF-β treatment.

To further demonstrate that TGF-β induces PML SUMOylation, PMLIII or PMLIV were co-expressed in HEK293 cells with the pcDNA3, pcDNA3-His_6-tagged SUMO1, SUMO2 or SUMO3 and then untreated or treated with TGF-β for 15 h. Cell extracts were purified on Ni^{2+}-NTA-agarose beads and subjected to immunoblotting with anti-PML antibodies.

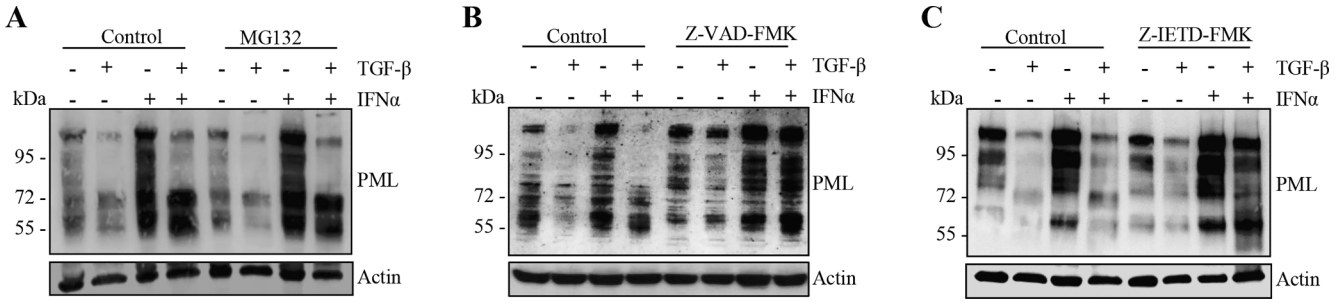

Fig. 3. TGF-β induces a caspase-dependent PML degradation. (A/B/C) BL41 cells were untreated or treated with IFNα for 24 h before addition of TGF-β for 40 h in the absence or presence of MG132 (1 μM) (A), Z-VAD-FMK (50 μM) (B) or Z-IETD-FMK (50 μM) (C). Extracts from untreated and treated cells were analyzed by western blot with anti-PML and anti-Actin antibodies.

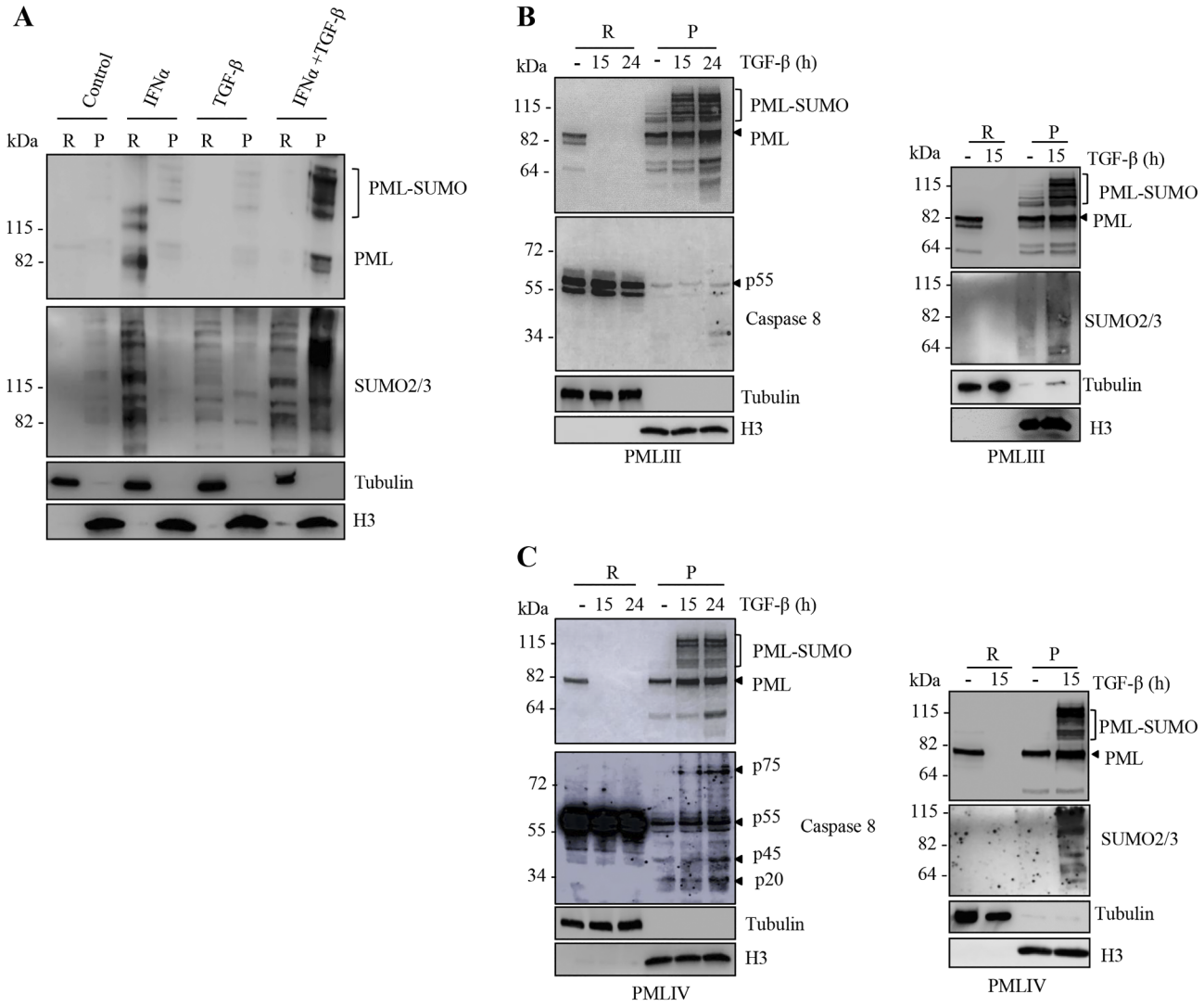

Fig. 4. TGF-β shifts PML from the nucleoplasm to the nuclear matrix. (A) TGF-β treatment shifted the IFN-enhanced PML towards the nuclear matrix. RIPA soluble (R) and insoluble (P) fractions from BL41 cells untreated or treated with IFNα for 24 h before addition of TGF-β for 15 h, were analyzed by western blot using a 4–12% gradient gel and revealed by anti-PML, anti-SUMO2/3, anti-Tubulin and anti-Histone H3 antibodies. (B/C) RIPA soluble (R) and insoluble (P) fractions from untreated or TGF-β-treated U373MG-PMLIII (B) or U373MG-PMLIV (C) cells were analyzed by western blot using a 4–12% gradient gel and revealed by anti-PML, anti-caspase 8, anti-Tubulin, anti-Histone H3 or anti-SUMO2/3 antibodies. Note that the RIPA-soluble PML (arrowhead) was lost in response to TGF-β and was found in the RIPA-insoluble fractions with PML-modified forms.

We did not detect PML modification by SUMO1 in Ni^{2+}-NTA–purified extracts from cells expressing His-SUMO1 (Fig. 5A and B). This observation is in line with the notion that SUMO1 conjugation is much more difficult to detect than SUMO2/3 conjugation [39]. In contrast, PML was found conjugated to SUMO2 and SUMO3, with a higher increase in PML SUMOylation in the presence of TGF-β (Fig. 5A and B). The modifications of PML by SUMO2 and SUMO3 were more apparent due to the capacity of SUMO-2/3 to form polymeric chains on PML [40].

Taken together, our results clearly demonstrate that TGF-β induced the shift of PMLIII and PMLIV from the nucleoplasm to the nuclear matrix and enhanced their conjugation to SUMO2 and SUMO3.

3.4. Caspase 8 interacts with PML and is recruited to PML NBs in response to IFNα and TGF-β

The fact that SUMOylated caspase 8 (p75) is associated with its nuclear localization [25] and that a number of proteins associated with PML are SUMOylated [41] prompted us to look for an association between caspase 8 and PML. Double immunofluorescence studies were performed in BL41 cells pretreated with IFNα before the addition of TGF-β for 15 h because at that time (Fig. 4), PML was shifted to the nuclear matrix and was not cleaved in response to TGF-β. The anti-caspase 8 antibodies used did not recognize the cytoplasmic caspase 8 and revealed caspase 8 in the nucleus. Double immunofluorescence staining for caspase 8 and PML revealed that caspase 8 was increased in IFNα-treated cells and was found localized in the nucleus outside PML NBs (Fig. 5C). Interestingly, treatment with TGF-β alone induced a recruitment of caspase 8 to PML NBs, this recruitment was further enhanced by IFNα pretreatment, which is due to the upregulation of PML within the NBs. Given that caspase 8 and PML proteins colocalized in the NBs in response to IFNα and TGF-β, we investigated whether they interacted using co-immunoprecipitation assays (Fig. 5D). BL41 cells were pretreated with IFNα before the addition of TGF-β for 15 h. The cell extracts were immunoprecipitated with anti-caspase 8 antibodies and analyzed by western blot using anti-PML antibodies. Caspase 8 antibodies precipitated PML only in extracts of cells treated with IFNα and TGF-β. These results demonstrate that in response to IFNα and TGF-β, PML interacted with caspase 8 and recruited it within PML NBs. PML co-immunoprecipitated with caspase-8 had an apparent molecular

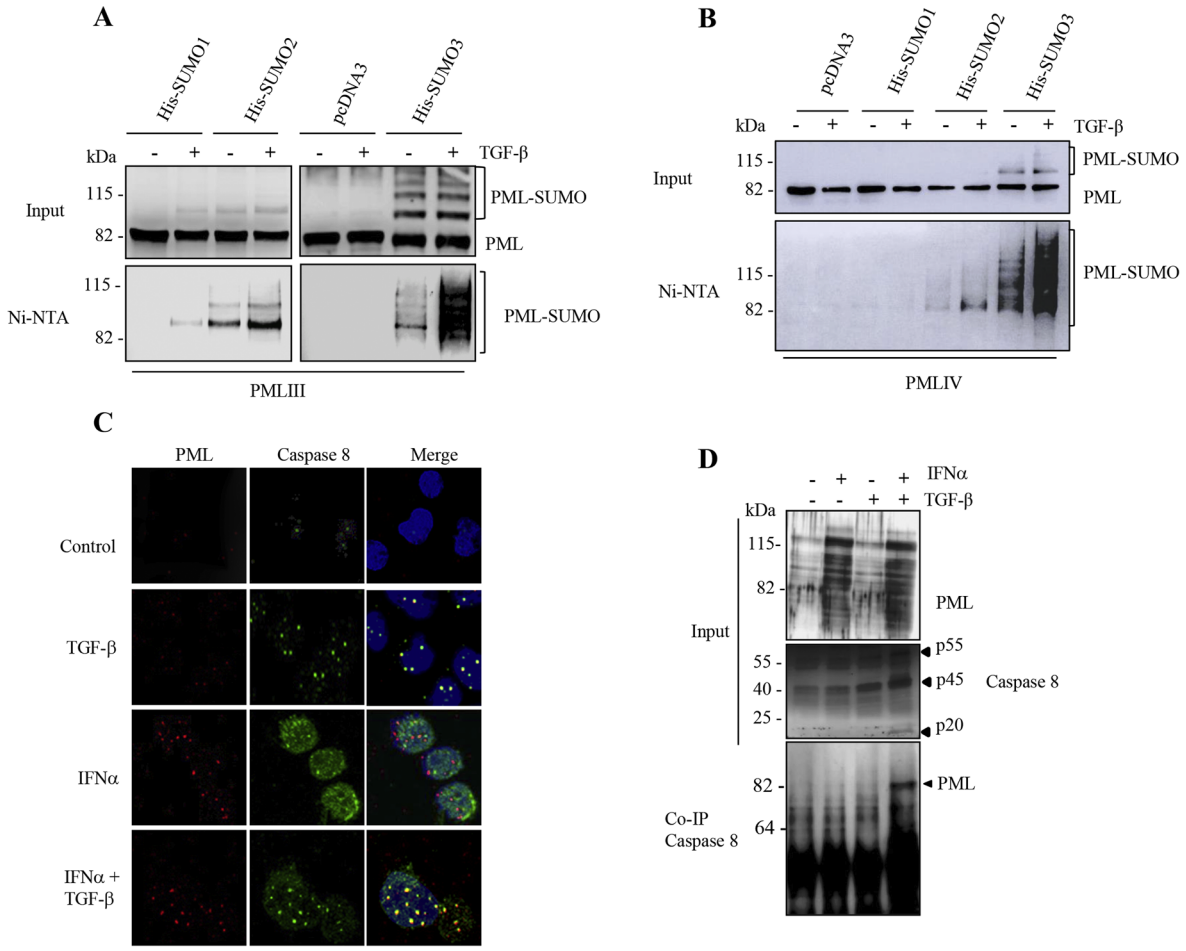

Fig. 5. TGF-β increases the conjugation of PML to SUMO and induces the recruitment of caspase 8 to PML NBs. (A/B) HEK293 cells were co-transfected with PMLIII (A) or PMLIV (B) expressing vector and pcDNA3, His_6-tagged SUMO1, SUMO2 or SUMO3 and cell extracts from untreated and TGF-β-treated cells for 15 h were purified on Ni^{2+}-NTA-agarose beads. The inputs and the purified extracts were analyzed by western blot using a 4–12% gradient gel and revealed by anti-PML antibodies. (C) BL41 cells treated with IFNα for 24 h before the addition of TGF-β for 15 h, were analyzed for double immunofluorescence staining using anti-caspase 8 and anti-PML antibodies. (D) BL41 cells were prepared as in (C), whole-cell lysates were immunoprecipitated with anti-caspase 8 antibodies and analyzed by western blot using anti-PML antibodies.

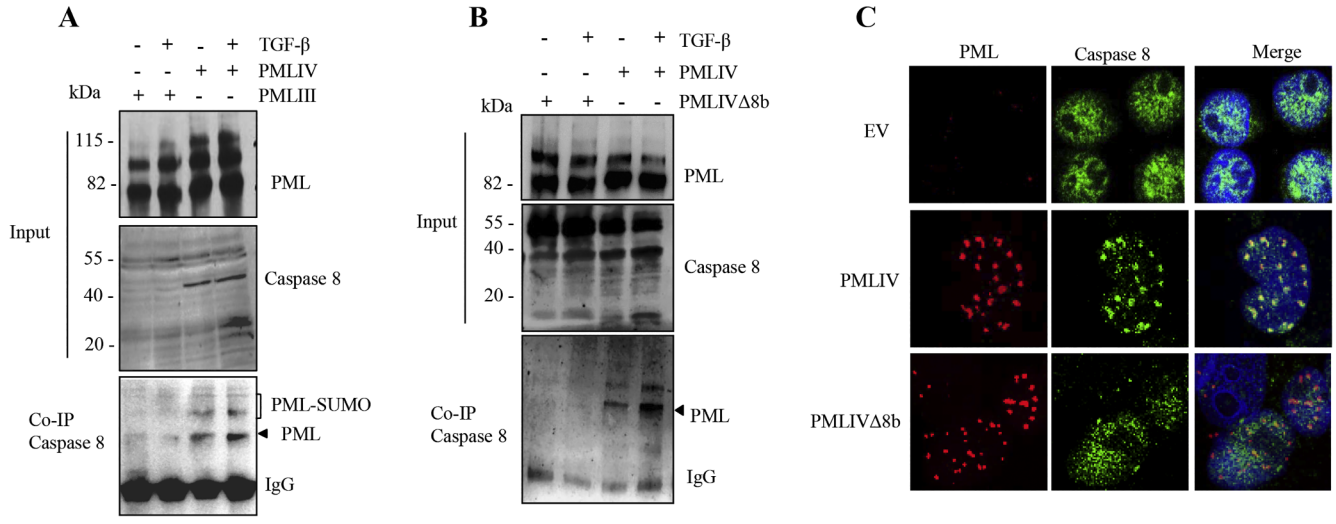

Fig. 6. PMLIV *via* its C-terminal region interacts with caspase 8 and recruits it within PML NBs. (A) U373MG-PMLIII and U373MG-PMLIV cells were untreated or treated with TGF-β for 15 h. (B) U373MG cells expressing PMLIV or PMLIV-Δ8b were untreated or treated with TGF-β for 15 h. Whole-cell lysates were immunoprecipitated with anti-caspase 8 antibodies and analyzed by western blot using anti-PML antibodies. (C) Cells expressing the empty vector (EV), PMLIV or PMLIV-Δ8b treated with TGF-b for 15 h, were analyzed for double immunofluorescence staining using anti-caspase 8 and anti-PML antibodies.

weight of 80 kDa (Fig. 5D). The apparent molecular weights of PMLI and II are 113 kDa, PMLIII and IV are 80 kDa, PMLV 72 kDa, PMLVI 65 kDa and PMLVII 50 kDa, which have been validated by several reports [12,35,36,42]. Therefore, we tested the interaction between PMLIII or PMLIV with caspase 8.

3.5. The C-terminal region of PMLIV is required for the interaction and the recruitment of caspase 8 within PML NBs

Co-immunoprecipitation analysis assessing interactions between caspase 8 and PMLIII or PMLIV revealed that only PMLIV interacted with caspase 8 and enhanced TGF-β-induced caspase 8 activation (Fig. 6A). PMLIV differs from the other five nuclear PML isoforms by the presence of exon 8b in its C-terminal region. To map the region of PMLIV involved in the interaction with caspase 8, we used PMLIV mutant with exon 8b deleted (PMLIV-Δ8b) (Fig. 6B). The coimmunoprecipitation assays showed that unlike PMLIV, the PMLIV-Δ8b was unable to interact with caspase 8 demonstrating that the C-terminal region of PMLIV is needed for the specific interaction with caspase 8. In addition, double immunofluorescence staining revealed that caspase 8 colocalized with PMLIV but not with PMLIV-Δ8b mutant (Fig. 6C), indicating that exon 8b of PMLIV is essential for recruitment of caspase 8 into PML NBs.

4. Discussion

Different functions could be attributed to different PML isoforms, which involvement in various cellular processes is due to their ability to interact with different partners [7]. Although PML isoforms may have related functions due to their common functional RBCC/TRIM domain, there is increasing evidence suggesting that PML isoforms possess distinct functions that are mediated by their specific C-terminal domain. Nuclear PML is the organizer protein of the PML NBs and all six human nuclear PML isoforms (PMLI to PMLVI) are able to form PML NBs when expressed in PML-negative cells [43].

The first reports revealing a function for cPML showed that overexpression of cPML positively regulates TGF-β signaling by enhancing TGF-β-induced SMAD2 phosphorylation [44]. Here, we report that in BL41 cells, pretreatment with IFNα, which enhanced all PML isoforms, promoted apoptosis mediated by TGF-β without enhancing SMAD2 phosphorylation, thus suggesting that cPML was not implicated. In addition, IFNα potentiated TGF-β-induced caspase 8 activation. Also, we show that the addition of TGF-β during 40 h targeted nuclear PMLs by inducing their caspase-dependent degradation resulting in PML NB disruption. Caspase 8 was shown to be responsible for the decrease of nuclear PML levels in TGF-β-treated cells, since this process is reversed in the presence of a specific caspase 8 inhibitor.

Furthermore, TGF-β treatment during 15 h, a time that preceded TGF-β-induced apoptosis and PML degradation, revealed that TGF-β altered PML localization by inducing its shift from the nucleoplasm to the nuclear matrix. We have shown recently that IFN enhances global cellular SUMOylation [38] and we report here that SUMOylated proteins in response to IFN were found in the RIPA-soluble fraction and were transferred, like PML, to the nuclear matrix upon the addition of TGF-β.

Interestingly, TGF-β enhanced PMLIII and PMLIV conjugation to SUMO2 and SUMO3. However, only PMLIV interacted with caspase 8 and enhanced TGF-β-induced caspase 8 activation. Remarkably, TGF-β treatment induced the shift of both PMLIV and caspase 8 to the nuclear matrix where SUMOylated caspase 8 (p75) [25], activated caspase 8 (p45 and p20) and SUMOylated PML were found. In response to IFNα and TGF-β PML interacted with caspase 8, recruited it within PML NBs and promoted TGF-β-induced apoptosis.

The specific C-terminal domain of PMLIV, which is encoded by exon 8b of the *PML* gene, differentiates this isoform from the other six PML isoforms. The variability in the COOH-terminal region leads to the specific functions of some PML isoforms (reviewed in [1,7]). PMLIV is the most studied PML isoform that has been implicated in antiviral defense and apoptosis. Indeed, PMLIV *via* its C-terminal part sequesters viral proteins within PML NBs, therefore blocking virus propagation [12,45]. Also, PMLIV specifically recruits and activates p53 in PML NBs and enhances apoptosis following various stimuli [1,46–48]. We show here that the C-terminal portion of PMLIV is essential for the interaction with caspase 8 and its recruitment within PML NBs since PMLIV-Δ8b

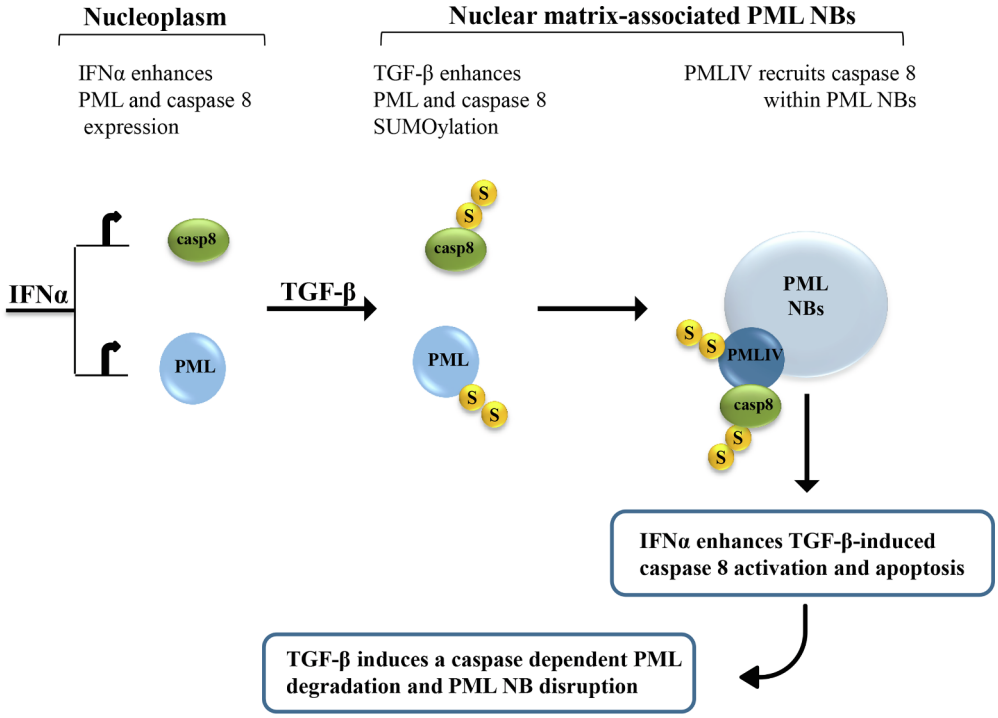

Fig. 7. IFNα *via* nuclear PML enhances TGF-β-induced caspase 8 activation and apoptosis.

mutant neither colocalized nor interacted with caspase 8 and failed to activate caspase 8.

This process is followed by caspase 8-dependent PML degradation and PML NB disruption. Accordingly, it has been shown that PML contains a caspase cleavage site at amino acid 552 [49] that is present in all nuclear PML isoforms and is missing in PMLVII. Regulation of apoptosis is controlled by caspases, which are divided into two classes: initiator caspases (2, 8, 9 and 10) and effector caspases (3, 6 and 7). PML has been associated with multiple apoptosis pathways including caspase 1 and caspase 3 activation upon exposure to different stimuli such as Fas, TNF, ceramide or IFNs [50]. The most direct link between PML and caspases is being provided by the observation that caspase 2 colocalizes with PML in PML NBs [1,51]. Our results provide evidences that nuclear PML is also involved in the activation of caspase 8, a caspase involved in TGF-β mediated apoptosis. The colocalization of caspase 8 with PML within PML NBs upon TGF-β stimulation suggests a novel nuclear pathway for caspase 8 activation.

Collectively, our findings lead to the following conclusions presented in the model illustrated in Fig. 7 (i) IFNα increases the expression of caspase 8 and PML isoforms in the RIPA-soluble fraction, (ii) addition of TGF-β induces the shift of PML and caspase 8 to the nuclear matrix where SUMOylated PML, SUMOylated p75 caspase 8 and activated caspase 8 are found, (iii) TGF-β induces PML conjugation to SUMO, (iv) PML and caspase 8 are found colocalizing in PML NBs in response to TGF-β (v) The specific C-terminal region of PMLIV is essential for the interaction of PMLIV with caspase 8 and the recruitment of caspase 8 into PML NBs. This process results in enhanced induction of apoptosis, caspase 8-dependent nuclear PML degradation and PML NB disruption arguing for a role of PML NBs as regulators of apoptosis in response to TGF-β.

Author contributions

F.E-A., B.E-M. and M.A.M. performed experiments. F.E-A. and L.D. analyzed data and participated in the preparation of the manuscript. M.K.C-A developed the concept, managed the project and wrote the manuscript.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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