A sumoylation site in PML/RARA is essential for leukemic transformation

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Summary

Pathogenesis of acute promyelocytic leukemia (APL) has been proposed to involve transcriptional repression through enhanced corepressors binding onto RARA moieties of PML/RARA homodimers. Unexpectedly, we show that the K160 sumoylation site in the PML moiety of PML/RARA is required for efficient immortalization/differentiation arrest ex vivo, implying that RARA homodimerization is insufficient to fully immortalize primary hematopoietic progenitor cells. Similarly, PML/RARA*K160R* transgenic mice develop myeloproliferative syndromes, but never APL. The Daxx repressor no longer binds PML/RARA*K160R*, but fusion of these two proteins restores the differentiation block ex vivo. Thus, transcriptional repression dependent on a specific sumoylation site in PML is critical for the APL phenotype, while forced RARA dimerization could control expansion of the myeloid compartment.

Introduction

Acute promyelocytic leukemia (APL) is initiated by the formation of a PML/RARA fusion protein (reviewed in Melnick and Licht, 1999). Briefly, PML/RARA is a potent repressor, at least in part through the ability of PML/RARA homodimers to recruit and tightly bind nuclear receptor corepressors (Grignani et al., 1998; He et al., 1998; Lin and Evans, 2000; Lin et al., 1998; Minucci et al., 2000). The fusion protein also binds a much larger set of response elements than the normal RARA/RXR heterodimers, thus extending the transcriptional network normally controlled by this complex (Jansen et al., 1995; Kamashev et al., 2004; Perez et al., 1993). Both of these properties rely on the presence of a strong dimerization/oligomerization domain in PML (Kastner et al., 1992; Perez et al., 1993). Yet, why PML, rather than any self-dimerizing protein, is the recurrent fusion partner of RARA in APL is not understood. One possible clue stems from the localization of PML to specific nuclear domains, PML nuclear bodies (NBs), which are disrupted upon expression of PML/RARA. PML and NBs play a role in the control of apoptosis or senescence, possibly through modulation of P53 activation (Gottifredi and Prives, 2001; Salomoni and Pandolfi, 2002). Indeed, PML/RARA expression induces a sharp resistance to apoptosis (Grignani et al., 1993; Wang et al., 1998). Nuclear bodies are structures where PML recruits a variety of proteins, including the Daxx repressor (Li et al., 2000). PML-triggered recruitment/sequestration of Daxx away from the chromatin could account for the fact that PML modulates transcription (Lehembre et al., 2001; Li et al., 2000; Zhong et al., 2000b). Conversely, when PML is artificially tethered to DNA, its interaction with Daxx may account for repression (Vallian et al., 1997; Wu et al., 2001).

Sumoylation is a posttranslational modification that tags a ubiquitin-like peptide, SUMO, onto specific lysine residues. Sumoylation appears to modulate tertiary structure, protein/protein interactions, and subcellular localization. Despite the large number of proteins that may be sumoylated, there are only a few examples where loss of a specific sumoylation site has yielded clear functional changes ex vivo, except for transcription factors whose sumoylation is often associated with the acquisition of transcriptional repression (Verger et al., 2003). PML sumoylation is required for the recruitment of partner proteins such as Daxx or Sp100, but not for NB formation

SIGNIFICANCE

The PML/RARA oncogene initiates acute promyelocytic leukemia (APL) by repressing myeloid differentiation programs. Homodimerization of PML/RARA triggers a tighter binding of RARA corepressors, which was proposed to account for transcriptional repression. Sumoylation is a posttranslational modification of lysines often associated with acquisition of repression. We show that a specific sumoylation site in PML is absolutely required for transformation ex vivo. In transgenic mice, expression of a PML/RARA mutant that cannot undergo sumoylation induces myeloid hyperplasia, but never APL. This PML or PML/RARA sumoylation site allows the recruitment of a potent repressor, Daxx. These results identify a repression domain in PML that controls the APL-specific differentiation block, which could explain why PML is the most common fusion partner of RARA in APL.

or nuclear matrix targeting (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001; Zhong et al., 2000a). There are three sumoylation sites within PML (Kamitani et al., 1998), but only K160 is required for arsenic-triggered PML or PML/RARA degradation, most likely through its ability to recruit the 11S proteasome (Lallemand-Breitenbach et al., 2001; Zhu et al., 1997). This specific lysine was also implicated in PML degradation by viral proteins (Boutell et al., 2003).

Transcriptional repression is central to the pathogenesis of APL, as shown by the sensitivity of the disease to HDAC inhibitors (He et al., 2001; Warrell et al., 1998). In the current view of APL pathogenesis, PML/RARA homodimers repress the expression of target genes. Indeed, a variety of RARA-fusion proteins are associated with rare or exceptional forms of human APL, and all of these RARA partners (PLZF, NPM/B23, STAT5b, NuMA) harbor strong homodimerization domains (Piazza et al., 2001). Any direct contribution of PML to PML/RARA-induced transcriptional repression is unclear, and why PML is the most common RARA partner remains unsettled. Here, we have addressed the role of PML sumoylation in PML/RARA transformation both in vivo and ex vivo.

Results

To examine the role of PML/RARA sumoylation in APL pathogenesis, we expressed PML/RARA or the PML/RARA*K160R* mutant in mouse primary hematopoietic precursor cells. After transduction and growth/selection in methylcellulose, PML/RARA-expressing cells are highly immature by immunophenotyping (Du et al., 1999). In contrast, PML/RARA*K160R* expression reproducibly yields differentiation profiles that are intermediate between those induced by expression of PML/RARA and empty vector (Figures 1A and 1B). Granulocytes were found on cytological preparations of vector-transduced cells, blasts, and promyelocytes in those expressing PML/RARA, but PML/RARA*K160R*-transduced cells displayed features of activated macrophages. PML/RARA-transduced cells can be indefinitely replated in methylcellulose. In contrast, PML/RARA*K160R*-expressing cells could only be replated twice, demonstrating that defective sumoylation impairs PML/RARA-dependent immortalization (Figure 1C). Similarly, PML/RARA-transformed cells consistently grew faster in liquid culture than those transduced with PML/RARA*K160R* (Figure 1D). Transduced cells expressed similar levels of PML/RARA or PML/RARA*K160R*, although, as previously shown (Lallemand-Breitenbach et al., 2001), several sumoylated forms of PML/RARA were absent from the mutant (Figure 1E). Note that, in these primary cells (including human APL cells), up to half of the total amount of the fusion protein is SUMO-conjugated. Thus, a critical sumoylation site in the PML moiety of PML/RARA is required for complete differentiation block and immortalization of primary hematopoietic precursor cells.

Transcriptional repression induced by forced RARA dimerization was proposed to underlie the pathogenesis of APL (Lin and Evans, 2000; Minucci et al., 2000). We thus examined whether the K160 sumoylation site would alter PML/RARA dimerization, DNA binding, or enhanced corepressor association. PML/RARA*K160R* retains the ability to impair retinoic acid response in transient transfection assays in SaOS2 cells (Figure 2A), although a modest, but consistent, decrease in basal PML/RARA-dependent transcriptional repression was observed. Similar results were obtained in Cos or 293T cells (data not shown). The ability of PML/RARA*K160R* to bind PML or RXR (Figures 2B and 3A) or to disrupt PML nuclear bodies was unaffected by this mutation (Figure 2C). Finally, using the DR5 response element from the *RARB* gene, we found that PML/RARA*K160R* behaved exactly as PML/RARA with respect to its ability to bind DNA as a homodimer or to display an enhanced affinity for the SMRT corepressor (Figure 2D). Altogether, these results show that PML/RARA*K160R* exerts very similar properties as PML/RARA as regards dimerization, corepressor binding, repression of RA-target genes, PML binding, and nuclear body disruption, but fails to significantly impair differentiation of primary hematopoietic progenitors. These observations therefore imply that the APL differentiation block is not solely due to enhanced binding of corepressors onto PML/RARA homodimers and is not properly reflected by transcriptional repression of retinoic acid target genes in transient transfections.

We have recently shown that in APL cells, PML/RARA is mostly complexed to RXR and hence can bind DNA in a variety of manners (e.g., as PML/RARA homodimers, [PML/RARA-RXR]^2 tetramers, or PML/RARA-RXR heterodimers [Kamashev et al., 2004]). In vivo, the K160R mutation might favor the formation of PML/RARA-RXR heterodimers, which would not exhibit enhanced SMRT binding. To directly demonstrate that RARA homodimers are insufficient to trigger differentiation arrest, we introduced in the RARA moiety of PML/RARA*K160R* two mutations previously shown to abolish RXR binding (Zhu et al., 1999), which hence force PML/RARA to bind DNA as a homodimer (Figure 3A). The double mutant PML/RARA*K160R,M883R,T886R* protein homodimerizes on DNA, binds the SMRT corepressor tightly, and still represses transcription from retinoic acid-sensitive reporters (Figures 3A and 3B). Yet, its expression in primary hematopoietic progenitor cells only induces a minor differentiation arrest and fails to immortalize cells, behaving essentially as PML/RARA*K160R* (Figures 3C and 3D). The PML/RARA*K160R,M883R,T886R* protein is more stable than PML/RARA (data not shown) and remains monosumoylated, but otherwise displays the classical microspeckled nuclear distribution characteristic for the fusion protein (Daniel et al., 1993) (Figure 3E). Note that the *M883R,T886R* mutation alone enhances, rather than impairs, the differentiation block induced by PML/RARA (Figures 3C and 3D). This experiment directly demonstrates that RARA homodimerization, as seen in PML/RARA*K160R* homodimers, is not sufficient to trigger full differentiation arrest and the immortalization of primary mouse hematopoietic progenitor cells.

To explore the significance of these observations in an in vivo setting, we generated transgenic mice expressing PML/RARA or nls-PML/RARA*K160R* under the control of the MRP8 promoter (Brown et al., 1997). Development of APL in MPR8-PML/RARA transgenic mice has been extensively described previously and shown to occur with an incomplete penetrance (Brown et al., 1997, Kogan et al., 2001). Transgenic mice with high PML/RARA expression develop a skin disease (Hansen et al., 2003), which precludes their reproduction. Mouse APL cells are transplantable to unirradiated syngenic recipients, and transplantation of prediseased transgenic bone marrow suffices to recapitulate leukemia development in the host, demonstrating that the disease is cell-autonomous. Similar ranges of expression of these two constructs were obtained (data not

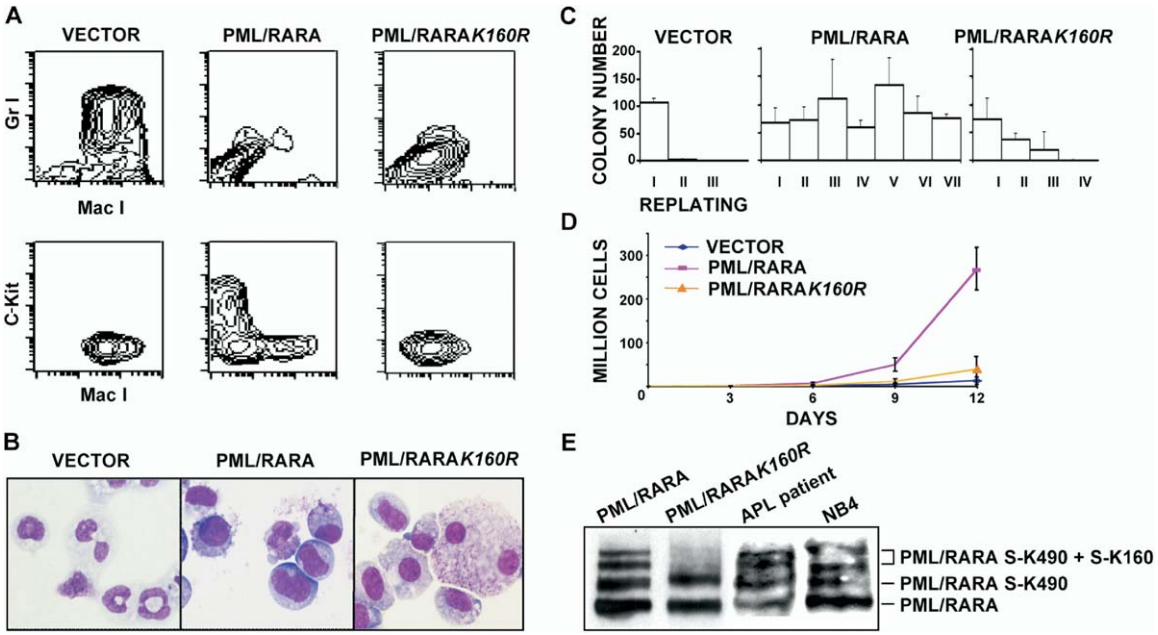


Figure 1. Comparison of primary hematopoietic precursors transduced by PML/RARA or PML/RARA*K160R*

A: Flow cytometry analysis of c-Kit, Gr1, and Mac1 in vector-, PML/RARA-, or PML/RARA*K160R*-transduced cells.
B: MGG stained cytospins of cells analyzed in **A**.
C: Serial replating in methylcellulose.
D: Comparison of the growth rates in liquid culture. Mean of 3 different experiments.
E: Western blot analysis of PML/RARA and PML/RARA*K160R* in transduced cells. The position of SUMO-modified PML/RARA proteins is indicated. As previously shown (Lallemand-Breitenbach et al., 2001), the K160 site is required for multiple sumoylation events.

shown) and only lines that express the fusion protein were considered for the study. A full account of the animal data is presented in Supplemental Table S1. We first examined the F1 progeny: in 2 of 3 PML/RARA lines, offspring developed splenomegaly at 22 and 23 months. Similarly, all of the 7 nls-PML/RARA*K160R* founders with progeny had at least 1 offspring (but up to 5) with splenomegaly, with a similar mean latency of 21 months (Supplemental Table S1, Figure 4A). In both PML/RARA and nls-PML/RARA*K160R* transgenics with splenomegaly, an increase in blood and marrow neutrophils, with a corresponding decrease in lymphocytes, was consistently found (data not shown), as previously reported in transgenic mice expressing other APL-associated fusion proteins (Sukhai et al., 2004). While splenomegaly due to splenic hematopoiesis was observed (Supplemental Table S1), extramedullar hematopoiesis was not observed in the livers, which remained of normal aspect on macroscopic examination. Thus, the K160R mutation does not abolish the ability of the PML/RARA transgene to initiate myeloid expansion.

Bone marrows from all F0 founders were engrafted into irradiated syngenic recipients, allowing the analysis of all transgenic lines, including those that had failed to reproduce. Similar to the F1 offspring, grafts from 5 of 8 PML/RARA founders developed splenomegaly with a mean latency of 13 months (including the time from donor birth to transplantation). Grafts from 4 of 10 nls-PML/RARA*K160R* founders also developed splenomegaly (mean latency 20 months), demonstrating that bone marrows expressing nls-PML/RARA*K160R* retain the ability to promote myeloid expansion upon engraftment (Figure 4B and Supplemental Table S1). Importantly, cytological analysis of the bone marrow demonstrated variable degrees of block in granulocytic differentiation in PML/RARA mice, in contrast to those derived from nls-PML/RARA*K160R* mice (data not shown). Similarly, the preleukemic marrow from PML/RARA mouse 26 could be replated at least 6 times, while 4 different marrows from PML/RARA*K160R* mice with splenomegaly never replated more than twice (data not shown), consistent with the ex vivo transduction data. To confirm these differences in myeloid differentiation, the hyperplastic marrows from F0 grafts were retransplanted into irradiated syngenic hosts. Transplantation greatly enhanced the differentiation block in PML/RARA mice, since 4 of 5 lines rapidly developed morphologically typical APL, on average 4 months after retransplantation (Supplemental Table S1, Figure 4B). Histologic examination reveals multiorgan invasion of blast cells (Figure 4C and data not shown). Very high numbers of APL blasts were present in the blood (Figure 4D). The bone marrow blasts are highly homogenous, have typical promyelocytic morphology, and express both immature markers (c-Kit and Scal) and, at intermediate levels, some myeloid markers (Mac and GR) (Figures 4C and 4E), reflecting the proliferation of immature myeloid cells. Hemoglobin levels were normal and platelet counts low (data not shown). These APL are very aggressive malignancies that kill all recipients, irradiated or not, with very short latencies (Figure 4B) and, as expected, were retinoic acid- and arsenic-sensitive (data not shown). In sharp contrast, none of the hy-

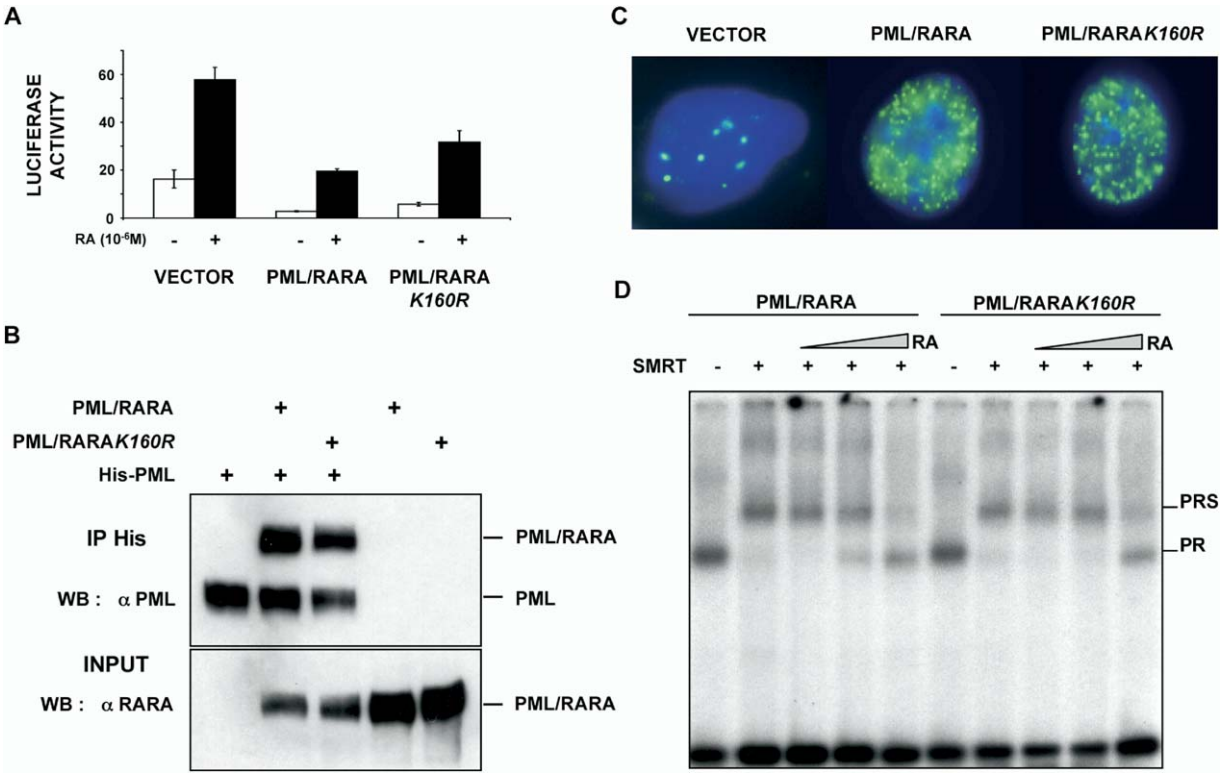


Figure 2. PML/RARA*K160R* shares all known features of PML/RARA

A: Transcriptional response to RA of vector-, PML/RARA-, or PML/RARA*K160R*-transfected SaOS2 cells.

B: PML similarly binds PML/RARA and PML/RARA*K160R*. PR, PML/RARA homodimers; PRS, PML/RARA-SMRT complex.

C: Immunofluorescence analysis (PML antibody) of primary hematopoietic cells, demonstrating that the *K160R* mutation does not change the microspeckled pattern of the fusion protein (Daniel et al., 1993).

D: Retinoic acid similarly regulates SMRT//PML/RARA interactions in gel-shift analysis.

perplastic bone marrows derived from nls-PML/RARA*K160R* ever evolved toward APL, despite an extended followup (Supplemental Table S1 and Figure 4B). Rather, the recipients of secondary grafts from nls-PML/RARA*K160R* bone marrows developed splenomegaly at low penetrance after a long latency, in irradiated animals only (Figure 4B and data not shown). Bone marrows from these animals were hyperplastic (Figure 4C), and contained more myeloid cells than marrows derived from control animals (Figures 4D and 4E), but exhibited neither a morphological differentiation block nor the appearance of immature cells on the immunophenotype. Multiorgan invasion was not observed (Figure 4C). Similarly, in the two PML/RARA lines whose offspring (but not F0 grafts) developed splenomegaly, F1 bone marrows also rapidly yielded APL upon engraftment (Supplemental Table S1), while, again, none of the bone marrows grafted using nls-PML/RARA*K160R* F1 offspring as donors ever yielded APL (Supplemental Table S1). Altogether, of the 8 PML/RARA founder lines, 6 eventually yielded APL from either F0 or F1 grafts, while APL was never detected in any of the grafts from hyperplastic bone marrows derived from the 10 nls-PML/RARA*K160R* founder lines (Supplemental Table S1 and Figure 4F).

The nls tag does not alter any of the known properties of PML/RARA, and transduction of PML/RARA*K160R* or nls-PML/RARA*K160R* in primary mouse hematopoietic cells induced similar incomplete differentiation block. Nevertheless, to confirm our results, we generated a second set of PML/RARA*K160R* transgenics. To date, 8 of 9 transgenic mouse lines developed a dense hyperplastic bone marrow, including two lines with splenomegaly without myeloid differentiation block in secondary grafts (Supplemental Table S1). All other findings (skin disease, shift toward myeloid markers in bone marrow immunophenotyping) in this second group of PML/RARA*K160R* mice were identical to the first set (Supplemental Table S1 and data not shown). We thus conclude that the K160R mutation does not block PML/RARA-induced myeloproliferation, but that this sumoylation site contributes to the APL-specific differentiation block and disease aggressiveness, fully consistent with the ex vivo data.

Upon sumoylation of its 3 conjugation sites, PML recruits a number of proteins, including Sp100, Daxx, and the 11S proteasome (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001; Zhong et al., 2000a). As Daxx is a potent and promiscuous repressor involved in PML-dependent modulation of transcription, we focused on the role of K160 in PML/Daxx interactions. Mutation of PML K160 completely abolished recruitment of Daxx onto PML bodies and also precludes Daxx recruitment toward the APL-specific microspeckles upon PML/RARA*K160R* expression (Figure 5A), further establishing the critical role of this specific sumoylation site in the recruitment of PML-associ-

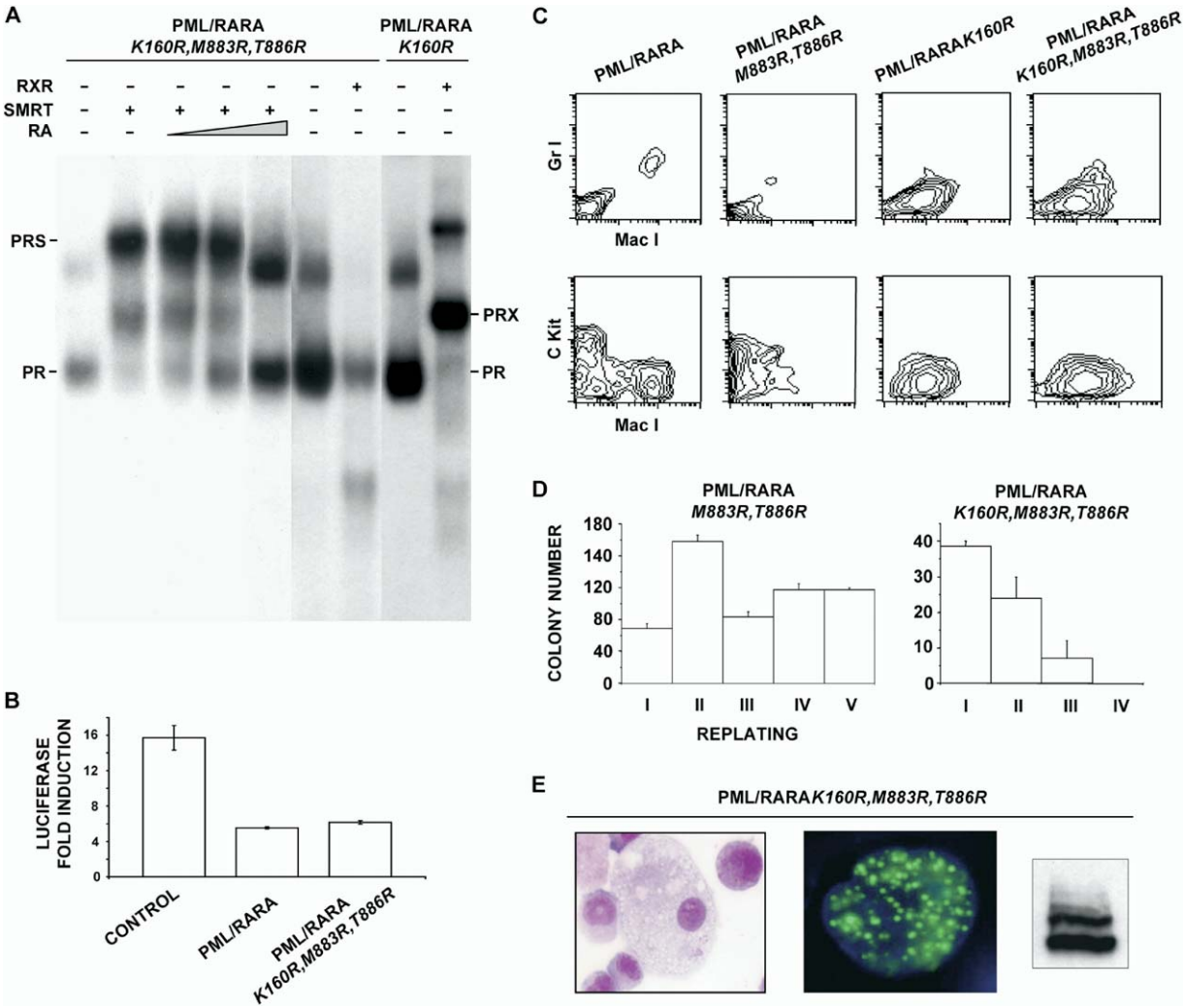
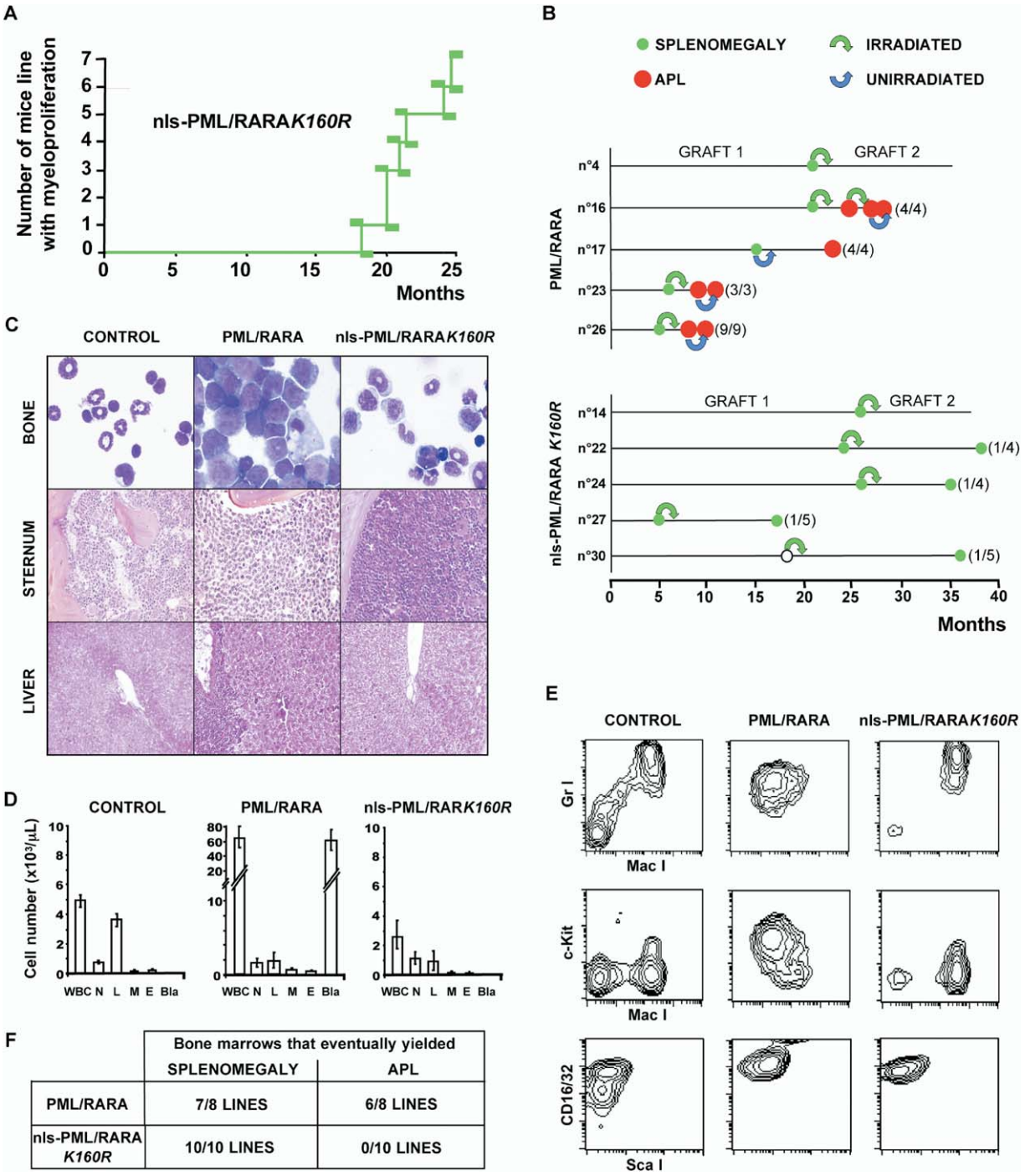


Figure 3. Forced homodimerization of PML/RARA does not induce differentiation arrest or immortalization

A mutation that abolishes RXR binding (*M883R, T886R*) was introduced in PML/RARA or PML/RARA*K160R* to enforce homodimer formation; SMRT binding (**A**), transcriptional activation (**B**), immunophenotyping (**C**), replating assays (**D**), and morphology, immunofluorescence, and Western blot (**E**) were performed as described above. In **A**, PR denotes the PML/RARA dimer; PRS, its complex with the SMRT corepressor; and PRX, the PML/RARA//RXR tetramer.

ated proteins (Lallemand-Breitenbach et al., 2001). In contrast, mutation of the other major sumoylation site, K490, does not affect the ability of PML to recruit Daxx onto PML bodies (data not shown). Similarly, yeast two-hybrid experiments demonstrated that Daxx could efficiently repress transcription of the *His* gene to which PML was tethered by a Gal4-PML fusion (Figure 5B). Note that the yeast strain used in these experiments grows on histidine-less media, as some basal transcription of the His gene occurs prior to Gal4-dependent transcriptional activation. PML/Daxx interaction results in growth arrest, presumably as a consequence of Daxx-induced *His* silencing, while Daxx expression does not block growth in the absence of Gal4-PML expression (Figure 5B). Strikingly, Daxx failed to block yeast growth in the presence of GAL4-PML*K160R* (Figures 5B and 5C). A similar role of K160 was found when PML/RARA was fused to the GAL4 DNA binding domain, except that some repression of *His* gene expression was already noted upon expression of PML/RARA. Similarly, mammalian two-hybrid also showed that tethering of PML or PML*K160R* to the promoter of a luciferase reporter gene impairs its activity (Li et al., 2000; Vallian et al., 1997; Wu et al., 2001), demonstrating that PML contains sequences implicated in transcriptional repression, apart from the K160 sumoylation site. Coexpression of Daxx further represses luciferase expression in the presence of PML, but not PML*K160R* (Figure 5D). Altogether, these results establish that K160 is the critical residue in PML implicated in the recruitment of Daxx, resulting in transcriptional silencing.

Recruitment of Daxx onto PML/RARA should trigger transcriptional repression of PML/RARA targets, but, in addition, PML/RARA may also titrate Daxx off its chromatin binding sites and could hence enhance expression of Daxx-responsive genes (Kim et al., 2003; Lehembre et al., 2001; Li et al., 2000; Lin et al., 2003). We undertook a set of experiments to clarify



F

	Bone marrows that eventually yielded	
	SPLENOMEGALY	APL
PML/RARA	7/8 LINES	6/8 LINES
nls-PML/RARA *K160R*	10/10 LINES	0/10 LINES

Figure 4. Characterization of PML/RARA and nls-PML/RARA*K160R* transgenics

A: Incidence of myeloproliferation/splenomegaly in the 7 lines of nls-PML/RARA*K160R* transgenics with offspring. Each founder line was scored positive on a date corresponding to the average latency required for splenomegaly development in the offspring.

B: Bone marrows of F0 founders were serially grafted in irradiated (green arrow) or unirradiated (blue arrow) syngenic recipient mice. All reported mice, except 30, showed splenomegaly at the first necropsy (green dot). Graft 2 for mouse 30 is shown by an open circle. Upon secondary grafts, bone marrows derived from PML/RARA mice rapidly developed APL (red dot), while those derived from nls-PML/RARA*K160R* mice only showed a late recurrence of myeloid hyperplasia in a fraction of the recipients. Note that APLs are aggressive acute leukemias that kill all recipient mice, irradiated or unirradiated.

C: Bone marrow cytology and marrow and liver histology of mice with APL (PML/RARA) or myeloproliferation (nls-PML/RARA*K160R*).

D: Blood counts of normal, PML/RARA, or nls-PML/RARA*K160R* grafts at the time they develop APL or splenomegaly with myeloproliferation. WBC, white blood cells; N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; Bla, blasts. Means and standard deviations are taken from at least 3 animals of different lines.

E: Immunophenotypes of bone marrow cells of normal mice (control), or mice with APL (PML/RARA) or myeloproliferation (nls-PML/RARA*K160R*).

F: Summary of the fate of the bone marrows derived from the different F0 or F1 transgenic upon serial transplantation.

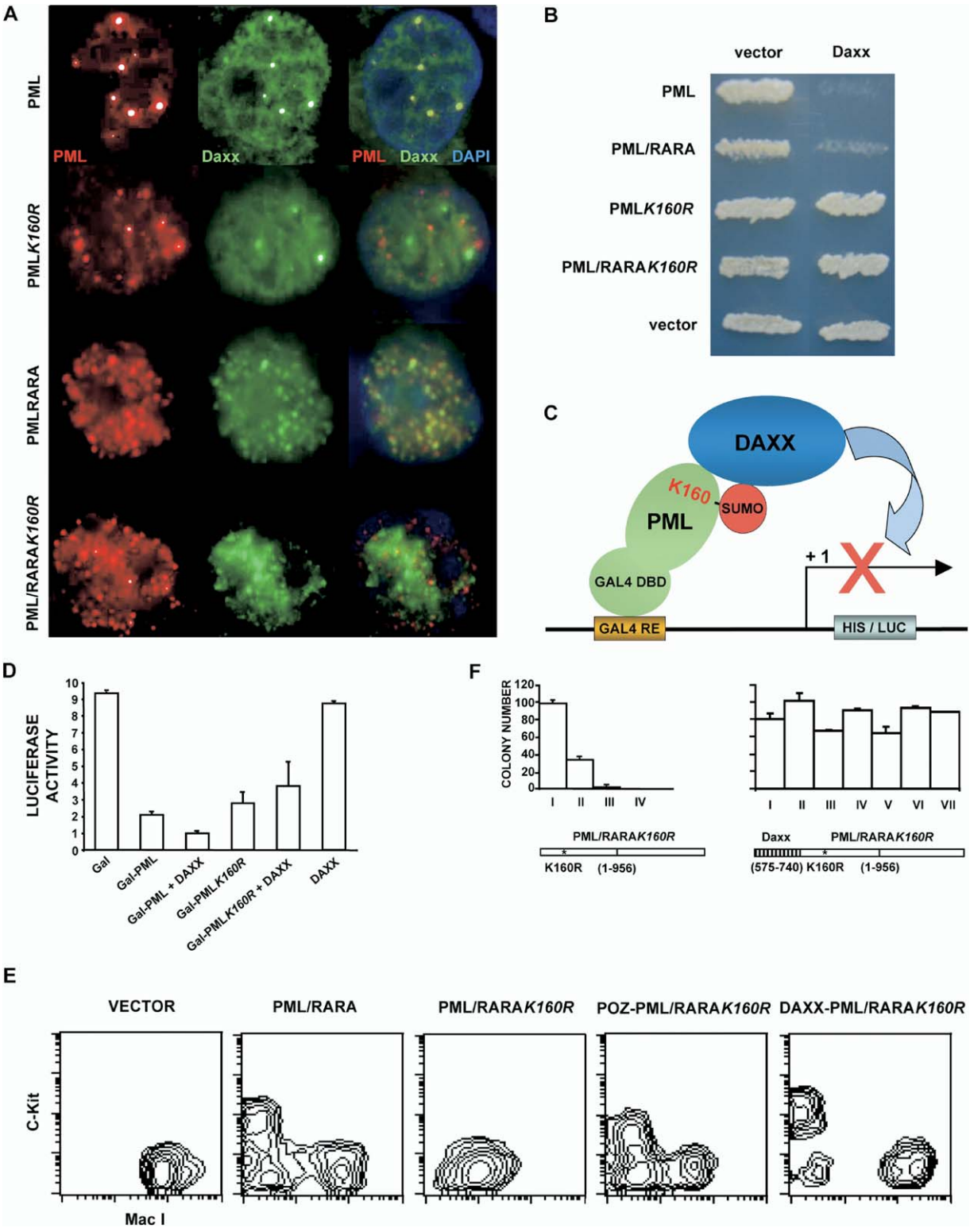


Figure 5. PML/RARA-triggered transformation is associated with recruitment of the Daxx repressor

A: Daxx localization in PML- or PML*K160R*-expressing fibroblasts (top) and in PML/RARA-or PML/RARA*K160R*-expressing hematopoietic precursors (bottom) derived from $PML^{-/-}$ mice.

B: Yeast two-hybrid experiments demonstrating that repression of the *His* locus requires the PML/Daxx interaction, which depends on K160 for both PML and PML/RARA.

C: Schematic representation of the two-hybrid repression experiments in **B**.

D: Mammalian double hybrid demonstrating that the PML/Daxx interaction requires K160.

E: Flow cytometry analysis of Mac1 or c-Kit expression in hematopoietic precursor cells transduced with vector, PML/RARA, PML/RARA*K160R*, POZ-PML/RARA*K160R*, or Daxx-PML/RARA*K160R*.

F: Serial replating assays of PML/RARA*K160R*- and Daxx-PML/RARA*K160R*-transduced cells.

the role of Daxx recruitment in cells transformation. As expected, transduction of PML/RARA*K490R* yields cells indistinguishable from PML/RARA-transformed ones (data not shown). Coexpression of PML/RARA and Daxx triggers essentially the same features as that of PML/RARA alone, which does not favor a role of PML/RARA to titrate Daxx off its other DNA binding sites (data not shown). In contrast, fusion to the N terminus of PML/RARA*K160R* of two distinct repression domains (either the C terminus of Daxx [Hollenbach et al., 1999] or the POZ domain of PLZF PLZF [Dong et al., 1996]) restores all the features of cells transformed ex vivo by PML/RARA. Notably, Kit expression (Figure 5E), promyelocyte-specific differentiation arrest, and continued replating up to the 7th passage (Figure 5F and not shown) were all restored, strongly suggesting that the function lost by the PML/RARA*K160R* mutant is its ability to fully repress transcription. In contrast, fusion of Daxx to RARA did not trigger differentiation arrest or immortalization ex vivo (data not shown), strongly suggesting that enforced homodimerization of RARA fusions contributes to these processes. Direct fusion of SUMO-1 or SUMO-2 to the N terminus of PML/RARA*K160R* allows one further round of replating, but yields cells with an immunophenotype similar to PML/RARA*K160R* (data not shown), pointing to the importance of position-specific sumoylation for the differentiation block. Altogether, these results very strongly suggest that the function lost in PML/RARA*K160R* is transcriptional repression, likely resulting from Daxx recruitment onto the PML/RARA-RXR complexes.

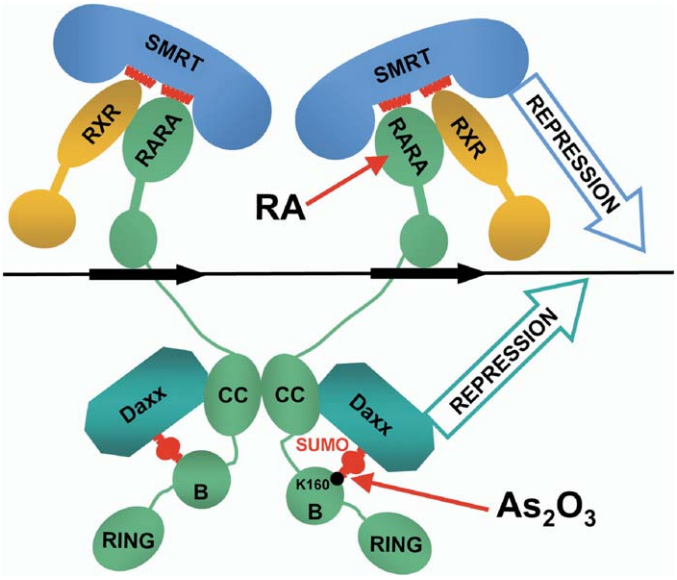


Figure 6. A model for APL pathogenesis and response to targeted therapies

PML/RARA, dimerized, but also bound to RXR (Kamashev et al., 2004), binds one or two SMRT molecules, resulting in transcriptional repression (blue arrow) that is released by RA (red arrow). In a symmetrical manner, the K160 sumoylation site recruits Daxx, resulting in strong repression (blue arrow). Arsenic trioxide (red arrow) modulates the sumoylation of lysine 160 in PML/RARA (Lallemand-Breitenbach et al., 2001), which could modulate Daxx binding prior to PML/RARA catabolism (Zhu et al., 1997). Protein interfaces critical for transformation and targeted by the therapies are shown in red.

Discussion

Combination of PML-mediated RARA homodimerization, enhanced corepressor binding, and repression of RA target genes was proposed to trigger the APL-specific differentiation block (Lin and Evans, 2000; Minucci et al., 2000). Probing PML/RARA function in primary hematopoietic progenitor cells demonstrates that RARA homodimerization is insufficient to initiate APL, as PML/RARA mutants that efficiently dimerize and tightly bind corepressors do not fully impair differentiation or immortalize these cells ex vivo and were incapable of initiating APL in mice. The PML/ RARA*M883R,T886R* mutant also directly demonstrates that titration of RXR (Kastner et al., 1992) does not make an important contribution to the APL phenotype, at least ex vivo. In contrast, the K160 sumoylation site appears to be critical for the differentiation block ex vivo and APL development in vivo, although it does not enhance the association of nuclear receptor corepressors. Restoration of the differentiation block through fusion of PML/RARA*K160R* to several distinct repression domains strongly suggests that the function lost upon mutation of K160 is the ability of the fusion to alter transcription of target genes. Surprisingly, such loss of K160-dependant transcriptional repression is not easily detected when probing PML/RARA function by transient transfections using RA-sensitive reporters. PML/RARA sumoylation could allow the fusion to permanently silence target genes, for example through histone and DNA methylation (Di Croce et al., 2002). Daxx was indeed proposed to bind the DNA-methylase-associated protein DMAP-1 (Muromoto et al., 2004), which might explain why the consequences of the K160 mutation were always more visible in stable expression systems than in transient assays (Figures 5B and 5D). Interestingly, experiments in *Xenopus* oocytes had shown that RARA-associated corepressors and histone deacetylases cannot fully account for PML/RARA-dependent repression and nucleosome reorganization (Segalla et al., 2003). RARA homodimerization alone (as observed in PML/RARA*K160R*) is insufficient to trigger a full APL phenotype. Yet, that the Daxx-RARA fusion did not alter differentiation or induce self-renewal of primary hematopoietic cells strongly suggests that enforced RARA dimerization nevertheless makes an important contribution to these processes. Dimerization could be important through enhanced SMRT binding (implying a dual, nonredundant, contribution of the two repression domains to transformation, Figure 6) and/or by extending the repertoire of DNA binding sites and hence the number of target genes (Kamashev et al., 2004).

To our knowledge, we have identified the first setting where SUMO-dependant repression controls transformation ex vivo and where any sumoylation mutant has an in vivo phenotype. We cannot formally rule out the possibility that K160 is the target of other posttranslational modifications (ubiquitination, isgylation, acetylation, methylation, etc.), but the fact that it is a well-characterized sumoylation site (Kamitani et al., 1998), previously implicated in arsenic-enhanced PML sumoylation by SUMO-1 and -2 (Lallemand-Breitenbach et al., 2001), as well as SUMO-dependent recruitment of partner proteins onto nuclear bodies (Lallemand-Breitenbach et al., 2001) (Figure 5), does not favor this hypothesis. For a large number of transcription factors, sumoylation was shown to induce repression through the recruitment of a variety of interacting proteins (Verger et al., 2003). For example, sumoylation enhances the ability of PLZF (the other recurrent RARA fusion partner) to re-

press expression of its target genes (Kang et al., 2003). Transcriptional repression could be due to Daxx recruitment, since this potent repressor interacts with PML/RARA in a strictly K160-dependent manner (Figure 5). Similarly, SUMO-dependant recruitment of Daxx onto Smad4 was very recently shown to account for transcriptional repression (Chang et al., 2005). Yet, in our situation, attempts to directly implicate Daxx in the differentiation arrest, using lentiviral expression of Daxx SiRNA, failed, because Daxx downregulation triggered apoptosis (Michaelson et al., 1999; Michaelson and Leder, 2003). Identification of a critical repression function in the PML moiety of PML/RARA, as previously seen with the POZ domain in the PLZF moiety of variant PLZF/RARA fusions (Grignani et al., 1998; He et al., 1998; Lin et al., 1998), points to t(15,17) APL as yet another example of myeloid leukemias where the translocation fuses a potent repression domain to a heterologous DNA binding domain (Rabbitts, 1994) (Figure 6). This likely explains why PML, rather that any self-dimerizing protein, is the most common RARA partner in APL.

PML/RARA*K160R* transgenic mice never developed APL, but rather myeloproliferations. In that respect, when expressed in mice, other APL-associated RARA-fusions (PLZF/RARA, NuMA/RARA, NPM/RARA) also trigger myeloproliferation without maturation defects, rather than typical APL (Cheng et al., 1999; He et al., 1997; Sukhai et al., 2004), stressing the importance of the PML moiety of the fusion to trigger the promyelocytic differentiation block (Bernardi et al., 2002). Enhanced corepressor binding on PML/RARA homodimers could underlie the myeloid hyperplasia observed in PML/RARA*K160R* transgenic mice. That PML/RARA sumoylation controls the differentiation block, but not the expansion of myelopoiesis in vivo, implies that distinct sets of target genes control these two functions. Interestingly, the normal *RARA* gene was previously implicated in both stem cell expansion (Purton et al., 2000) and granulocytic differentiation (Kastner et al., 2001). Molecular profiling of gene expression in progenitor cells expressing PML/RARA or its sumoylation mutant may allow the dissection of the pathways that control these two features of APL cells.

Our observations identify an unanticipated symmetry in the determinants for RA and arsenic response within PML/RARA (Figure 6). Either of the two agents triggers rapid degradation of PML/RARA through the respective targeting of the PML (arsenic) or RARA (RA) moieties of the fusion (Zhu et al., 1997, 1999, 2001). At the molecular level, the determinant of RA response is the AF2 activation domain in RARA, which is implicated in transcriptional repression, RA-dependent transactivation, and receptor degradation (Zhu et al., 1999). Accordingly, mutations in this region of PML/RARA have been associated with RA resistance, through both enhanced transcriptional repression and absence of RA-triggered PML/RARA degradation (Cote et al., 2000; Duprez et al., 2000; Shao et al., 1997; Zhou et al., 2002). Similarly, the K160 sumoylation site in the PML moiety of the fusion is required for transcriptional repression and transformation, but also for arsenic-modulated sumoylation and PML or PML/RARA degradation (Lallemand-Breitenbach et al., 2001; Zhu et al., 1997), which may raise new hypotheses as to the mechanism(s) of arsenic action in APL (Zhu et al., 2002).

Experimental procedures

Retroviral transduction and cell analyses

The cDNA encoding *PML/RARA* corresponds to the bcr1 breakpoint and includes the alternatively spliced exon 5 as well as the PML nls (de Thé et al., 1991). The encoded PML/RARA fusion protein hence contains all 3 PML sumoylation sites. The PML/RARA*K160R* mutant was described elsewhere (Lallemand-Breitenbach et al., 2001). The C terminus of Daxx (aa 575–740), containing the major repression domain (Hollenbach et al., 1999) or the N-terminal POZ domain of PLZF (Dong et al., 1996; Li et al., 1997), was fused in-frame to the N terminus of PML/RARA*K160R*. Similarly, SUMO-1 or -2 (kindly provided by R. Yeh) were fused in-frame to the N or C terminus of PML/RARA*K160R*. The two mutations that abolish RXR binding to RARA were transferred from RARA*M380R/T383R* into PML/RARA (Zhu et al., 1999).

Infection of lineage-depleted bone marrow from 5-fluorouracil-treated C57BL/6 mice and culture and G418 selection of the transduced progenitors cells in methylcellulose with stem cell factor, IL3, IL6, and GM-CSF were performed as described (Du et al., 1999). After a week, neomycin-selected cells were recovered from methylcellulose and either analyzed (FACS, MGG staining, immunofluorescence, and Western blot) or replated at a density of 10,000 cells/dish. Cells were serially replated until they stopped growing. Affinity-purified anti-PML rabbit or hen sera were used for immunofluorescence and Western blotting. Cell growth was assessed in RPMI 1640 medium supplemented with 10% fetal calf serum and the same cytokines, except GM-CSF.

Transgenic mice

PML/RARA was expressed using the MRP8 promoter (Brown et al., 1997) in B6/CBA F1 hybrids. Hematological disorders such as splenomegaly were not observed in nontransgenic littermates. The K160 mutation was introduced into the transgene with an SV40 nls tag (11 transgenics, 39-month followup) or without (9 transgenics, 24-month followup). Only transgenics lines expressing the fusion proteins by both Western blot and immunofluorescence are reported here (8/8 for PML/RARA, 10/11 nls-PML/RARA*K160R*, 9/9 PML/RARA*K160R*). For the sake of simplicity, mice that had "acute myeloid leukemia with features of human acute promyelocytic leukemia" are described as having "APL" (Kogan et al., 2002). Myeloproliferation was defined by the association of splenomegaly (spleen larger than 160 mg) with highly hyperplastic marrow, in the absence of significant differentiation abnormalities. Several founders developed a severe skin disease precluding their reproduction (Hansen et al., 2003), and some founders gave no progeny. F0 founders were sacrificed and phenotypes were observed in offspring (when available) or primary grafts in irradiated syngenic recipients. The second graft was used to demonstrate (or not) rapid progression from preleukemia to full-blown APL. Founder lines were scored positive for APL or myeloproliferation on the average time required to develop a phenotype, taking in account the time spent since birth of the donor bone marrow in the case of grafts. Blood cell counts were performed with a Celldyn 3700 counter from Abbot diagnosis. Bone marrow and pathology analyses were performed as before (Lallemand-Breitenbach et al., 1999).

Reporter assays and yeast double hybrid

SaOS2, COS, or 293T cells were transiently cotransfected with indicated constructs (20 ng expression vector, 100 ng RARE3-tk-Luc reporter [de Thé et al., 1990], and 100 ng of tk-renilla control vector). Cells were treated with RA (10^{-6} M) or vehicle, overnight. For yeast two-hybrid assays, we used the strain HF7c (Clontech) carrying His as reporter gene. PML, PML*K160R*, PML/RARA, or PML/RARA*K160R* were cloned in the yeast expression vector pBridge (Clontech) carrying the GAL4 DNA binding domain (DBD) and the selective Trp gene. A cDNA encoding the complete *hDaxx* gene was cloned in the yeast vector pGADGH (Clontech) carrying, or not, the GAL4 activator domain (AD) and the selective Leu gene. Transfections were performed using the lithium acetate method, and yeast strains were grown in appropriate selective medium as drop-out Trp/Leu or drop-out Trp/Leu/His media. Mammalian two-hybrid was performed as described using vectors kindly provided by A. Zelent (Chester Beaty Laboratories, London), where PML was expressed as a fusion protein with the GAL4 DNA binding domain and a luciferase expression vector under the control of GAL4 binding sites.

Analysis of the properties of transduced proteins

Electrophoretic shift analyses were performed as previously described (Zhu et al., 1999), using extracts from Cos-transfected cells and a bacterially expressed SMRT fragment (He et al., 1998). Immortalized $PML^{-/-}$ mouse embryo fibroblasts (Quignon et al., 1998) were infected by retroviruses ex-

pressing various PML derivatives (Lallemand-Breitenbach et al., 2001). The subnuclear localization of endogenous mouse Daxx protein was analyzed using an anti-Daxx antibody (Hollenbach et al., 1999).

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/2/143/DC1/.

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