2 Molecular Targets of Treatment in Acute Promyelocytic Leukemia

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Introduction

Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemia (AML), characterized at the cellular level by a differentiation block of the granulocytic lineage at the promyelocytic stage. On the molecular level, more than 98% of APL cases are caused by the chromosomal translocation t(15;17) which implicates the two genes promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARA), leading to the expression of the *PML/RARA* fusion oncoprotein [1]. In the RARA gene, the breakdown always occurs in the intron 2, whereas three breakdown regions were described occurring in the PML gene and resulting in the expression of two long isoforms (bcr1 and bcr2 transcripts) and one short isoform (bcr3 transcript) (Fig. 2.1) [1–3]. Other translocations were reported in APL always involving the RARA gene with various gene partners, of which PLZF is the most common [4]. This chapter will discuss the role of *PML/RARA* in the development of APL while focusing on the importance of treatment-triggered *PML/RARA* degradation and PML/P53-driven senescence in the pathophysiology of cure.

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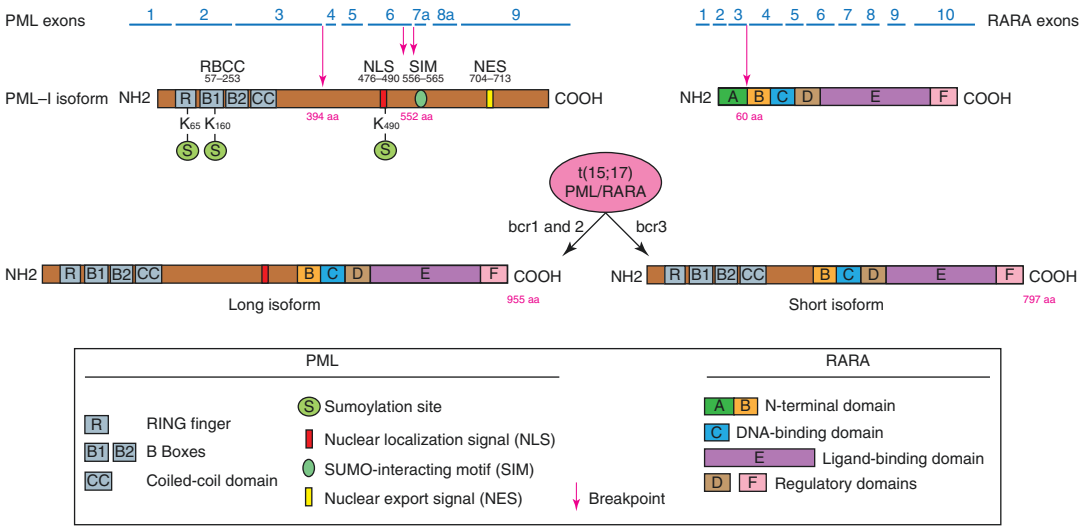

Fig. 2.1 The t(15;17) translocation partners. The t(15;17) translocation involves two genes, PML and RARA, leading to the expression of the PML/RARA fusion protein. The breakpoints always occur in the intron 2 of the RARA gene, whereas three breakdown regions were described in the PML gene: in the intron and exon 6 for bcr1 and bcr2 transcripts, respectively, and in the intron 3 for bcr3 transcript. This results in the expression of two long PML/RARA isoforms (bcr1 and 2) and one short (bcr3). PML/RARA retains all the functional domains of RARA (notably the DNA- and ligand-binding domains) and PML (in particular the RING finger and coiled-coil domains). *bcr* breakpoint cluster region

The Translocation Partners: RARA and PML

RARA, the retinoic acid (RA) receptor alpha, is a nuclear transcription factor activated by retinoids—such as all-trans retinoic acid (ATRA). Upon heterodimerization with its cofactor the retinoic X receptor (RXR) [5], the RARA/RXR complex binds to specific DNA RA response elements (RARE) composed typically of two direct repeats of a core hexameric motif, PuG [G/T] TCA; the classical RARE is a 5 bp-spaced direct repeat [6]. In the absence of the ligand, RARA/RXR interacts with nuclear receptor corepressors such as N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor). This interaction leads to the recruitment of Sin3A and histone deacetylase (HDAC) complexes which maintain chromatin in a compacted and repressed state [7, 8]. The binding of retinoids induces conformational changes in the ligand-binding domain (LBD) of RARA, the most striking one being the repositioning of helix 12. This structural modification causes corepressor release and recruitment of coregulator complexes, some members of which exhibit enzymatic activities such as CBP/p300, then allowing transcription of target genes [9, 10]. Some of these target genes accelerate myeloid differentiation toward granulopoiesis. Accordingly, in vivo granulopoiesis is delayed in the presence of RARA, reflecting the basal repressive activity of unliganded RARA, while it is accelerated by RA solely in the presence of RARA [11].

The PML gene was originally identified in APL [3, 12] and is encoded by nine exons. Seven isoforms are generated by alternative splicing: six are nuclear isoforms designated PML-I to PML-VI, and one is cytoplasmic, PML-VII. PML belongs to the TRIM family,

many of which are ubiquitin ligases [13, 14]. Several members of this family are oncogenes: few of them were shown to promote malignant transformation as partners of fusion genes [15]. PML protein contains several regions: a RBCC/TRIM motif (amino acids 57–253 in exons 1–3) which harbors a C_3HC_4 RING finger, two B boxes (B1 and B2), and an α-helical coiled-coil homodimerization domain [14, 16, 17], a nuclear localization signal (NLS) (amino acids 476–490 in exon 6), a SUMO-interacting motif (SIM) (amino acids 556–565 in exon 7a) present only in PML-I to PML-V, and a nuclear export signal (NES) (amino acids 704–713 in exon 9) found only in PML-I and consistent with its nuclear and cytoplasmic distribution [17]. Some domains were described in isoform-specific sequences, like the interaction domain between PML-IV and ARF, a positive regulator of p53 [18], and the exonuclease-like domain in PML-I [19].

PML proteins aggregate in the nucleus and form speckles known as PML nuclear bodies (NBs). These domains are tightly associated with senescence and control of p53 activation, as recently reviewed [20]. Indeed, PML is required for senescence induction, as demonstrated upon stress, DNA damage, oncogene activation, or simply during replicative senescence [21]. At least, some of these functions are mediated through PML NBs which have been implicated in partner sequestration and/or posttranslational modifications, notably phosphorylation, sumoylation, and ubiquitinylation [22, 23]. PML NBs are dynamic structures which harbor a few constitutive, and numerous transiently, client proteins depending on different conditions (i.e., stress, interferon (IFN) treatment, viral infections) like the death domain-associated protein Daxx [22], p53, and many of its regulators [24–26]. Indeed, PML NBs regulate the subcellular localization of Daxx, thereby controlling its proapoptotic activity, and appear to be important for activation of p53-mediated senescence, most likely through posttranslational modifications [27, 28]. In addition, PML-controlled senescence can be initiated and furthermore reinforced at the transcriptional level: PML promoter contains IFN and p53 response elements, creating a positive feedback loop during senescence induction [27, 29].

The Oncoprotein PML/RARA

The expression of *PML/RARA* is sufficient to drive leukemogenesis by deregulating RA-dependent cell differentiation pathways and enhancing the self-renewal of myeloid progenitors [30]. In murine transgenic models, *PML/RARA* expression yields typical APL, although at variable penetrance [31]. From a structural point of view, the *PML/RARA* fusion protein retains all the functional domains of RARA (notably the DNA- and the ligand-binding domains) and PML (in particular the RING finger and coiled-coil domains). On one hand, PML/RARA binds DNA via its RARA domain and acts in a dominant-negative manner to repress the transcription of RARA target genes by strengthening the recruitment of corepressors (N-CoR and SMRT) and HDACs, enforcing DNA methylation and gene silencing. PML/RARA oligomers are complexed to RXR which greatly enhances PML/RARA ability to bind DNA and recognize highly degenerate sites [32, 33]. As RARA signaling regulates myeloid differentiation, its inhibition could explain the block in differentiation that is observed in APL cells. On the other hand, PML/RARA also heterodimerizes with PML via its coiled-coil domain leading to the disruption of NBs: in APL cells, PML is redistributed in a microspeckled pattern (Fig. 2.2). This could abrogate the PML-controlled senescence pathways and contribute to APL pathogenesis. Accordingly, PML/RARA expression was conclusively linked to defective p53 activation [26, 28], thus leading to senescence deregulation, as well as increased self-renewal.

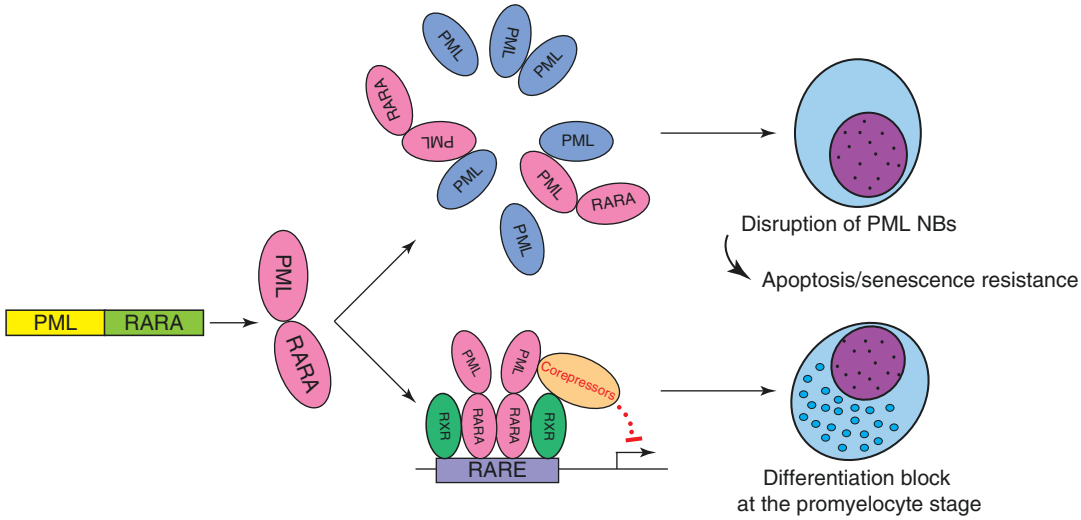

Fig. 2.2 Dual action of PML/RARA oncoproteins. The PML/RARA fusion protein interacts with PML via the coiled-coil domain of its PML moiety, resulting in the disruption of PML NBs. PML/RARA forms a heterodimer with RXR via its RARA moiety and binds DNA with poor selectivity to repress transcription of many genes, including RARA targets, by recruiting corepressors. This collectively causes the characteristic differentiation block of APL cells at the promyelocytic stage

Therapeutic Effects of Retinoic Acid in APL

In the 1980s, APL patients were treated with chemotherapy alone and had poor prognosis despite a complete remission rate of 50–90%. This was explained by a high rate of relapse. The addition of RA to anthracycline-based chemotherapy marked a major advance in the treatment of APL by increasing rates of clinical remission and cure. With these optimized historical regimens, the 5-year overall relapse-free survival is up to 75% [34]:

Uncoupling Differentiation and Loss of Clonogenicity Under RA Treatment

RA treatment of APL constitutes the first example of differentiation therapy in patients [35–38]. RA binds to the ligand-binding domain on the RARA moiety of PML/RARA, triggering a conformational change that releases corepressors and recruits transcriptional coactivators. This allows the activation of RARA target gene transcription and differentiation of leukemic cells (Fig. 2.3). It was first believed that the therapeutic effect of RA stems solely from its ability to reverse repression of myeloid differentiation. Nevertheless, experiments from APL transgenic mice have demonstrated that blast differentiation can be uncoupled from loss of leukemia-initiating cells (LIC) [39]. As reviewed below, multiple settings were described in which full differentiation was not accompanied by significant APL regression or prolongation of survival [40]. For example, in PML/RARA mice, treatment with various RA doses (low, intermediate, and high) or synthetic retinoids similarly yielded terminal granulocytic differentiation; however, survival of treated mice sharply differed by dose and retinoid type. In fact, loss of LIC was dose dependent with only intermediate and high all-trans RA was able to impede clonogenicity in secondary recipients [39–41]. In PLZF-RARA transgenic mice (PLZF-RARA APL is RA resistant in patients), cell differentiation levels upon RA treatment

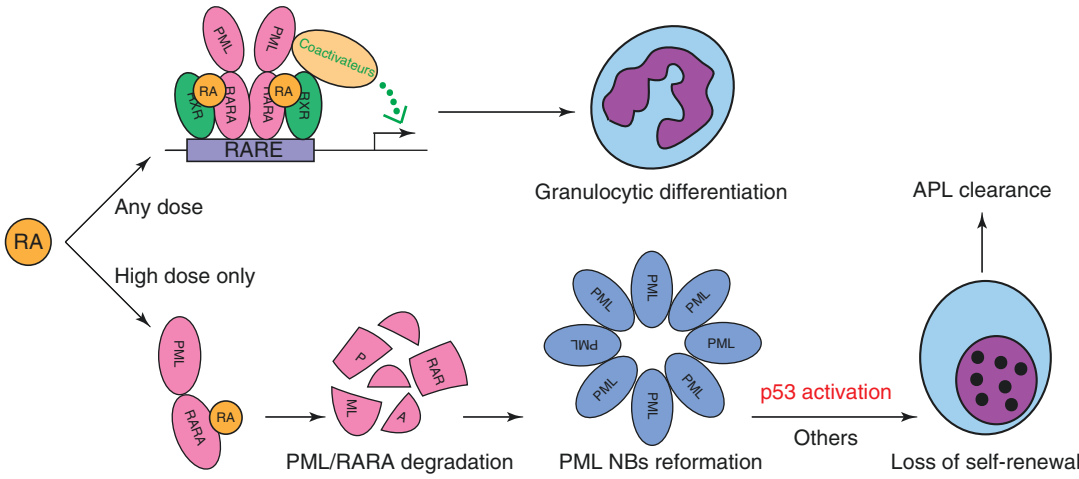

Fig. 2.3 Model of APL eradication with RA treatment. RA binds to RARA LBD of PML/RARA and elicits a conformational change releasing transcription corepressors and leading to the recruitment of coactivators. This allows transcription of RARA target genes and the terminal granulocytic differentiation of the blast. Moreover, RA treatment leads to PML/RARA proteasomal degradation that is followed by PML NB reformation and activation of p53. This latter effect results in loss of LIC

were comparable to that observed in PML/RARA mice but with a strong difference in survival of primary- and secondary-treated recipients [41]. Moreover, RA-resistant APL cells are highly sensitive to cAMP-induced differentiation, particularly in the presence of RA but fail to regress [39, 42]. Similarly, treatment of PML/RARA leukemic mice with histone deacetylase inhibitors (HDACi) led to tumor regression as well as a release in the differentiation block; however, HDACi failed to induce disease clearance [43]. Collectively, those results clearly establish the uncoupling of blast differentiation and tumor eradication in APL: significant transcriptional activation can indeed be obtained with small doses of RA, whereas clearance of LIC necessitates exposure to higher RA levels, an observation that was not yet fully transferred to clinical protocols. Indeed, a unique study has reported the use of single-agent liposomal RA in the treatment of APL patients and has found that some patients—mainly low-risk APL—can be cured without any additional chemotherapy [44], supporting the existence of dose-response in patients upon treatment with RA.

RA-Induced PML/RARA Degradation

Several studies have shown that RA triggers PML/RARA proteasomal degradation [40, 45, 46, 47]. Indeed, RA binding to PML/RARA allows direct recruitment of the proteasome to the ligand-activated transcriptional activation domain AF2 of RARA moiety, leading to PML/RARA degradation (Fig. 2.3) [46, 47]. This proteasome-mediated degradation is additionally modulated by a cAMP-triggered PML/RARA phosphorylation at serine 873 [39, 48]. A caspase-dependent cleavage was also reported [49]. Resistance to RA of some APL cell lines was associated with failure to degrade the fusion protein [46, 50]. In fact, most of these cell lines were mutated for PML/RARA [51, 52]. Thus, PML/RARA proteolysis seems to be linked to clearance of leukemic cells under RA treatment. Phosphorylation at serine 873 sharply enhances RA-induced LIC clearance [39], and the use of theophylline, an inhibitor of cAMP degradation, was beneficial in the treatment of a RA-resistant APL patient [42]. Ablain et al. further showed that treating

APL mice with retinoids other than RA did not affect PML/RARA degradation, although cell differentiation was induced. In secondary recipient experiments, loss of clonogenicity was only observed with RA [40] demonstrating that PML/RARA degradation by RA is followed by reformation of PML NBs [53]. Collectively, these data pharmacologically prove the uncoupling of differentiation and blast clearance and underscore the key role of PML/RARA in vivo degradation in APL eradication.

Role of PML and p53 in the Cure of APL Under RA Treatment

Loss of RA-treated PML/RARA leukemic cells was linked to cell cycle arrest and P53 activation. Examination of bone marrow transcriptome revealed that genes strongly associated with cell cycle arrest were activated only when APL mice were treated with high RA doses that also significantly affect LIC survival. Among the 30 most upregulated genes in this context, 10 were drivers of cell senescence directly linked to p53. For example, a massive induction of the master senescence gene Serpine1, also known as plasminogen activator inhibitor-1 (PAI-1), was observed. PML/RARA degradation was followed by PML NB reformation and triggered p53 stabilization, possibly through posttranslational modifications occurring on NBs (Fig. 2.3). This leads to a cell cycle arrest with senescence-like features resulting in elimination of leukemia-propagating cells [41]. The role of p53 in RA-induced APL elimination was demonstrated by in vivo survival experiments in $p53^{+/+}$ and $p53^{-/-}$ PML/RARA-driven APLs [41]. In addition, the importance of PML in inducing p53 activation and APL clearance was further established by mice survival experiments showing a much shorter survival of $Pml^{-/-}$ APL compared to that of $Pml^{+/+}$ APL. Definitely, these data demonstrate that functional PML NB reorganization upon RA treatment leading to p53 activation is a determining step in the cure of APL.

Therapeutic Effects of Arsenic Trioxide in APL

Arsenic trioxide (ATO) was first utilized in APL patients in the early 1990s and led to cure in 70% of patients [54, 55]. Thereby, APL is exquisitely sensitive to ATO which, in contrast to RA, may cure APL as a single agent. Moreover, the combination of RA and ATO in clinical trials appeared to be much superior to the conventional treatment with RA and chemotherapy [56]:

ATO-Induced PML NB Reformation and PML/RARA Degradation

Although RA and ATO are two unrelated therapeutic agents in APL, they share the biochemical property of inducing PML/RARA degradation. As described above, PML/RARA loss was directly linked to loss of self-renewal in leukemic cells and cure of APL [39]. Furthermore, ATO-induced PML/RARA loss could explain the differentiation observed in vivo in APL cells upon ATO exposure by a promoter clearance mechanism [57]. At the molecular level, while RA targets the RARA moiety of the fusion oncoprotein, ATO targets its PML moiety [50, 58] and induces its oxidation [59]. The same effect is observed on normal PML, and a specific ATO binding site was identified in the second B box. Arsenic sharply enhances the reformation of PML NBs by multimerization of PML and PML/RARA proteins. Then, through recruitment of UBC9 SUMO-E2 ligase, it favors the sumoylation of PML [23, 60]. Sumoylation of PML is followed by recruitment of the

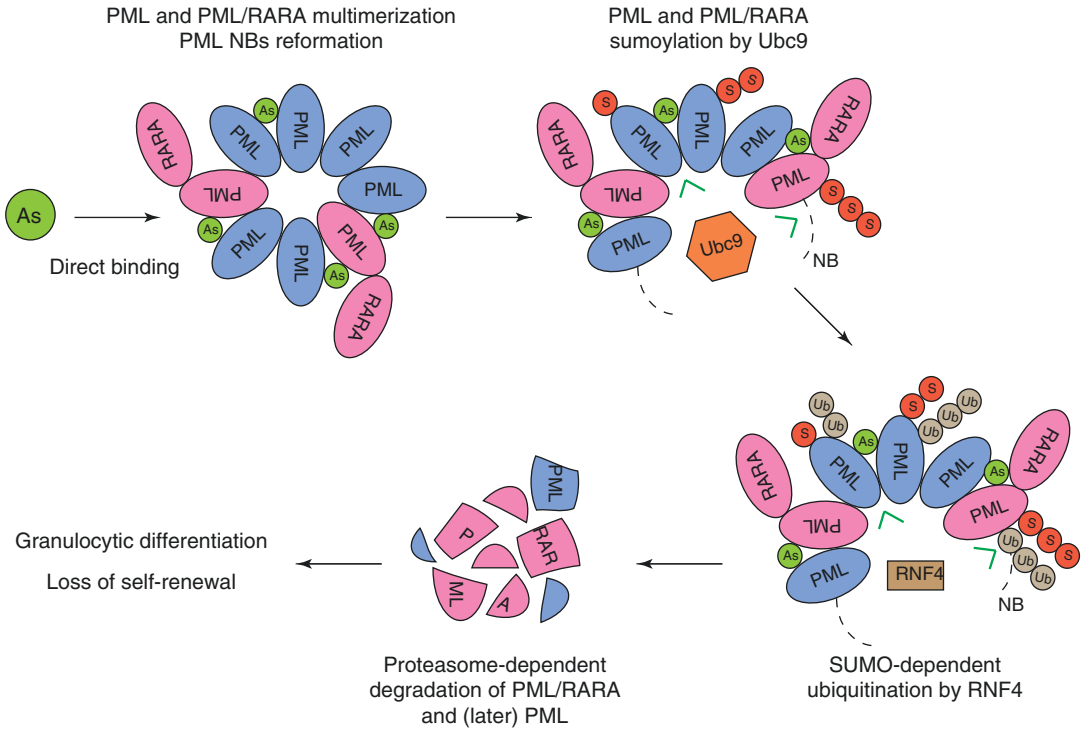

Fig. 2.4 ATO-induced degradation of PML/RARA. ATO prompts PML and PML/RARA multimerization that triggers PML NB reorganization. This is followed by recruitment of SUMO E2 ligase Ubc9 which sumoylates PML and PML/RARA on its PML part. Then, the sumoylated proteins are polyubiquitinated by the SUMO-dependent ubiquitin E3 ligase RNF4 resulting in their degradation. This likely explains the great efficacy of ATO in APL treatment. As is ATO

SUMO-dependent ubiquitin E3 ligase RNF4, which catalyzes polyubiquitination and subsequent proteasome-mediated proteolysis of PML and PML/RARA (Fig. 2.4) [61, 62]. In conclusion, degradation of PML/RARA and enhanced NB biogenesis are the two main effects of ATO which results in p53 activation and clearance of APL LICs. Note that the dual targeting of PML/RARA and PML likely explains the clinical superiority of this drug.

RA and ATO Synergy in APL Cure

In several mice models, combined RA and ATO treatment causes a rapid disappearance of APL cells and cures leukemia. Yet, those two therapeutic agents do not synergize (even antagonize) to induce cell differentiation [63–65], but they do cooperate to induce PML/RARA degradation by non-overlapping biochemical pathways [39, 50, 58]. Actually, NB reformation with RA-ATO treatment was much more complete in APL blasts than with RA alone, which can be explained both by the synergistic effect of both drugs on PML/RARA degradation but also by the direct PML targeting by ATO [41]. Accordingly, this treatment elicited enhanced activation of p53 target genes [41]. Hence, ATO cooperates with RA to cure APL by increasing RA-induced PML/RARA degradation and also by potentiating PML NB

reorganization yielding enhanced NB formation, p53 activation, and senescence (Fig. 2.5).

This model for PML NB-based APL eradication was strongly supported by the discovery of a mutation in normal PML gene in a therapy-resistant patient [66–68]. Strikingly, this mutation (A216V), located immediately next to the ATO binding site on PML, is the predominant one observed within PML/RARA in ATO-resistant patients (Fig. 2.6) [69–72]. Finally, mutations in the p53 gene have been reported in rare, fully therapy-resistant patients [51, 52].

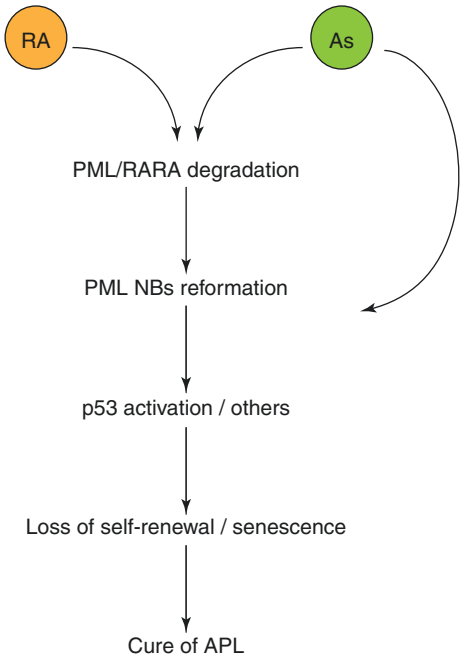

Fig. 2.5 Molecular effects of treatment combination in APL cure. PML/RARA degradation is strongly enhanced by the two therapeutic agents as well as PML NB reformation. This effect drives a greater NB reformation and p53 activation than with RA alone. Hence, ATO cooperates with RA to cure APL

Conclusions

PML/RARA degradation by RA and/or ATO appears to be the driving force underlying the cure of APL patients. Triggering the degradation of oncoproteins in other leukemias and sarcomas caused by fusion proteins could be a promising therapeutic approach as in APL. Downstream of PML/RARA degradation and PML NB reformation drives P53 activation and is required for loss of self-renewal by a senescence-like program. Importantly, targeting PML by ATO could drive cancer cell senescence in other diseases. Indeed, there are some indications that PML may be important in other hematological malignancies, like adult T-cell leukemia/lymphoma (ATL). Thus, this model of APL cure not only constitutes a success story of molecularly targeted therapy but may actually open new therapeutic avenues in other malignancies.

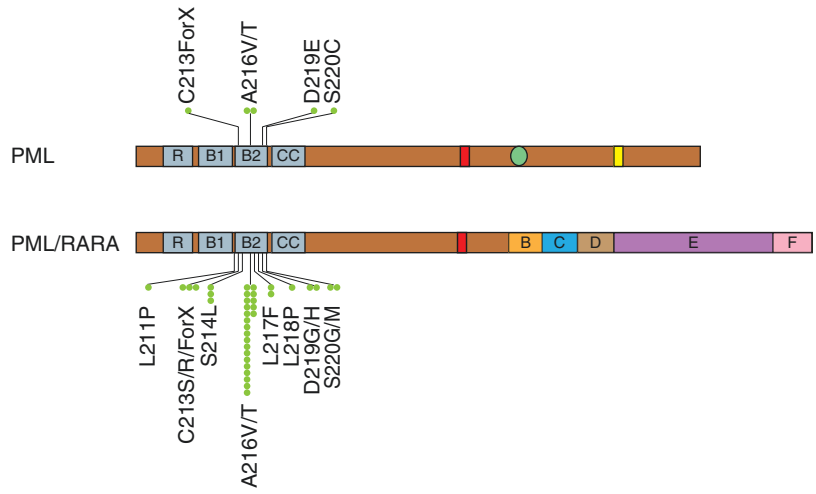

Fig. 2.6 Spectrum of mutations in PML and PML/RARA in ATO-resistant patients. Schematic representation of PML and PML/RARA proteins with observed mutations. *Circles* depict individual mutations

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