

Targeting fusion protein/corepressor contact restores differentiation response in leukemia cells

Serena Racanicchi¹, Chiara Maccherani¹,
Concetta Liberatore¹, Monia Billi¹,
Vania Gelmetti^{2,3}, Maddalena Panigada¹,
Giovanni Rizzo¹, Clara Nervi^{2,3}
and Francesco Grignani^{1,*}

¹Patologia Generale and Medicina Interna e Scienze Oncologiche, Dipartimento di Medicina Clinica e Sperimentale, Perugia University, Policlinico Monteluce, Perugia, Italy, ²Dipartimento di Istologia ed Embriologia Medica, Università di Roma 'La Sapienza', Roma, Italy and ³Parco Bio-Medico Scientifico San Raffaele di Roma, Roma, Italy

The AML1/ETO and PML/RAR α leukemia fusion proteins induce acute myeloid leukemia by acting as transcriptional repressors. They interact with corepressors, such as N-CoR and SMRT, that recruit a multiprotein complex containing histone deacetylases on crucial myeloid differentiation genes. This leads to gene repression contributing to generate a differentiation block. We expressed in leukemia cells containing PML/RAR α and AML1/ETO N-CoR protein fragments derived from fusion protein/corepressor interaction surfaces. This blocks N-CoR/SMRT binding by these fusion proteins, and disrupts the repressor protein complex. In consequence, the expression of genes repressed by these fusion proteins increases and differentiation response to vitamin D3 and retinoic acid is restored in previously resistant cells. The alteration of PML/RAR α -N-CoR/SMRT connections triggers proteasomal degradation of the fusion protein. The N-CoR fragments are biologically effective also when directly transduced by virtue of a protein transduction domain. Our data indicate that fusion protein activity is permanently required to maintain the leukemia phenotype and show the route to developing a novel therapeutic approach for leukemia, based on its molecular pathogenesis.

The EMBO Journal (2005) 24, 1232–1242. doi:10.1038/sj.emboj.7600593; Published online 24 February 2005

Subject Categories: chromatin & transcription; molecular biology of disease

Keywords: corepressors; fusion proteins; histone deacetylase; leukemia; retinoic acid

Introduction

About 40% of acute myeloid leukemias (AML) are caused by the activity of chimeric proteins encoded by fusion genes generated by chromosomal translocations (Grignani *et al.*, 1993; Look, 1997; Tenen, 2003). Fusion proteins contribute

to a combination of genetic lesions that produce differentiation block and altered growth of leukemia cells. A number of fusion proteins function as transcriptional repressors (Melnick and Licht, 1999; Tenen, 2003), due to their ability to firmly bind transcriptional corepressor molecules. Retinoic acid receptor α (RAR α) fusion proteins in acute promyelocytic leukemia (APL) and AML1/ETO in AML M2–M4 aberrantly bind the corepressors N-CoR or SMRT. These molecules engage to the promoter of RAR α or AML1 target genes a multiprotein complex including Sin3A and histone deacetylases (HDACs) (Gelmetti *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998; Lutterbach *et al.*, 1998; Grignani *et al.*, 1998a; Wang *et al.*, 1998; Melnick and Licht, 1999). HDACs deacetylate histones and can secondarily enroll DNA methyltransferases (Baylin, 2002; Di Croce *et al.*, 2002). This pathologic sequence is caused by the ability of AML1/ETO, PML/RAR α or other RAR α fusion proteins to generate oligomeric structures that produce an abnormally stable corepressor binding (Kastner *et al.*, 1992; Nervi *et al.*, 1992; Grignani *et al.*, 1996; Lin and Evans, 2000; Minucci *et al.*, 2000). The overall effect is hypoacetylation of histones and DNA methylation that rearrange chromatin structure hampering transcription of crucial myeloid differentiation genes (Ferrara *et al.*, 2001; Di Croce *et al.*, 2002; Alcalay *et al.*, 2003; Tenen, 2003). Murine bone marrow transduction/transplantation models and cell line studies have shown that the activity of these leukemia fusion proteins can block cell differentiation (Grignani *et al.*, 1993, 1998a, 2000; Gelmetti *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998; Lutterbach *et al.*, 1998; Wang *et al.*, 1998; Melnick and Licht, 1999; Minucci *et al.*, 2002; Schwieger *et al.*, 2002; Tenen, 2003). However, in transgenic mouse models, PML/RAR α and AML1/ETO are not sufficient to induce differentiation block, which appears to require further molecular damages (reviewed in Melnick and Licht, 1999; Bernardi *et al.*, 2002). In this context, a crucial question is whether fusion protein functions are required to maintain a fully malignant leukemia phenotype, including the block of differentiation. In such a case, targeting the primary fusion protein/corepressor interaction could reverse gene silencing and restore the differentiation potential of leukemia cells. This would represent a therapeutic goal, specifically directed to leukemia cells, where abnormal corepressor recruitment occurs. Actually, differentiation treatment of APL with retinoic acid (RA) is based on the ability of this drug to detach corepressors from the PML/RAR α protein, inducing coactivators recruitment on both the fusion protein and the normal RAR α (Grignani *et al.*, 1998a; He *et al.*, 1998; Lin *et al.*, 1998). However, most acute leukemia blast cells are not induced to differentiate by RA. Moreover, APL themselves include RA-resistant cases, due to the expression of the PLZF/RAR α fusion protein or of mutant PML/RAR α proteins (Warrell, 1993; Shao *et al.*, 1997; Melnick and Licht, 1999). Thus, a general strategy to modify fusion protein/corepressor interactions and induce leukemia cell differentiation is currently unavailable.

*Corresponding author. Medicina Interna e Scienze Oncologiche, Dipartimento di Medicina Clinica e Sperimentale, Perugia University, Policlinico Monteluce, 06100 Perugia, Italy. Tel.: +39 075 572 6264; Fax: +39 075 578 3444; E-mail: fragrig@unipg.it

Received: 30 June 2004; accepted: 1 February 2005; published online: 24 February 2005

Targeting fusion protein/corepressor contact can be achieved by interfering with the protein interaction interfaces. The corepressor interaction surfaces of the PML/RAR α and AML1/ETO proteins have been mapped and contact well-defined domains of the corepressors (Gelmetti *et al*, 1998; Lutterbach *et al*, 1998; Wang *et al*, 1998; Glass and Rosenfeld, 2000). Peptides representing the corepressor core interaction domains inhibit the RAR/N-CoR association, and are released by RA (Hu and Lazar, 1999; Nagy *et al*, 1999; Perissi *et al*, 1999). Therefore, expression in leukemia cells of protein sequences that are representative of the interaction surfaces could block fusion protein/corepressor interactions.

In this work, we investigated whether interrupting the contact between fusion proteins and corepressors could modify the leukemia phenotype. We took advantage of two well-studied models: the leukemias derived from the expression of the PML/RAR α or the AML1/ETO fusion proteins. We show that expression of short sequences representing the fusion protein interaction domains of N-CoR efficiently blocks these interactions and restores the differentiation response of leukemia cells. These effects were also obtained by direct protein transduction. Our data indicate that fusion protein activity is a permanent requirement for the leukemia phenotype and that it is necessary for the leukemia differentiation block. The block of pathogenetic protein-protein interaction could represent a novel strategy for cancer treatment.

Results

Expression of interaction domain peptides restores differentiation response in leukemia cells

To block fusion protein/N-CoR interactions, we expressed in PML/RAR α or the AML1/ETO positive leukemia cell lines protein fragments representing the interaction sequences: the IDN and IDC domains of N-CoR that include the nuclear receptors corepressor binding sites (Hu and Lazar, 1999; Nagy *et al*, 1999; Perissi *et al*, 1999), and the N-CoR RD3 domain that interacts with ETO (Gelmetti *et al*, 1998; Lutterbach *et al*, 1998; Wang *et al*, 1998) (Figure 1A).

Using bicistronic retroviral vectors, we coexpressed green fluorescence protein (GFP) and the IDC or IDN domains of N-CoR in the APL cell lines NB4, containing PML/RAR α , and NB4R4, a derivative of NB4 made RA-resistant by a mutation in the RA binding region of PML/RAR α (Lanotte *et al*, 1991; Shao *et al*, 1997). In these cell lines, we also expressed a mutated IDC (M10) whose ability to bind nuclear receptors is greatly reduced (Nagy *et al*, 1999) and a RAR α fragment spanning the region of the corepressor binding surface (D403). We then expressed these protein fragments in U937 and HL60 cells, which do not contain fusion proteins. As a model of AML1/ETO leukemia, we utilized the SKNO1 cell line that has high and stable expression of the fusion protein (Matozaki *et al*, 1995). In SKNO1 cells, we expressed the RD3 and IDC domains of N-CoR, with the latter as a negative control. We added an HA tag to IDN, D403 and RD3 domains to make them recognizable by antibodies. GFP-expressing cells were purified by fluorescence-activated cell sorting (FACS). These cell lines expressed the different fragments (Figure 1B).

The expression of HDAC1, HDAC3 SMRT and N-CoR protein (Figure 1C) and the basal differentiation stage of all these

lines were not significantly affected by the expression of any of these fragments (Figure 2). We next measured by real-time PCR the mRNA expression of selected fusion protein target genes (Zhu *et al*, 2001; Linggi *et al*, 2002; Alcalay *et al*, 2003) (Figure 1D). IDC and IDN expression in both NB4 and NB4R4 cells increased the PML/RAR α target genes RAR α and granulocyte colony-stimulating factor receptor (G-CSF-R) mRNA, whereas RD3 expression in SKNO1 cells increased the AML1/ETO target genes G-CSF-R and p14^{ARF} mRNA. Since these genes participate in the differentiation process, we studied the cell response to differentiation inducers, vitamin D3 (D3) or RA. As expected, D3-induced differentiation is impaired in NB4, NB4R4 and SKNO1 cell lines. In contrast, in NB4-IDC/IDN, NB4R4-IDC/IDN and SKNO1-RD3 cells expressing the interaction peptides, we observed a strong D3-mediated differentiation induction as measured by the expression of the myeloid differentiation surface markers CD11b and CD14 (Figure 2A and B and Supplementary Figure 1). Confirming that the N-CoR fragments exerted their activity on the PML/RAR α -RAR α pathway, the expression of the RAR α D403 fragment restored D3-induced differentiation in NB4 D403 and NB4R4D403 cells (Figure 2A and B). Differentiation-unblocking effects were specific since they were not detectable in NB4 and R4 cells expressing the IDC M10 mutant, or in SKNO1 cells expressing IDC.

When treated with RA, both control NB4 and NB4-IDC/IDN cells differentiated efficiently, while NB4R4 cells were unresponsive. However, NB4R4-IDC/IDN cells displayed a strong induction of myeloid differentiation, as measured by morphology, NBT reduction assay and expression of surface differentiation markers (Figure 3A, C and E). In NB4R4D403, RA-induced differentiation was also clear. U937 and HL60 cells expressing IDC did not show any impairment of RAR α expression or RA-induced differentiation, indicating that IDC expression does not decrease the activity of wild-type RAR α (not shown). SKNO1 cells are unresponsive to RA. However, when these cells expressed the RD3 N-CoR fragment, RA treatment induced their differentiation, although it was less complete than in NB4, as shown by growth arrest, CD11b expression and NBT assay (Figure 3B, D and F). The cell growth of NB4 and NB4R4 cells was not significantly affected by the expression of the N-CoR fragments (not shown). SKNO1-RD3 cells grew significantly less rapidly than control SKNO1 cells (Figure 3F).

Taken together, these data suggest that the expression of specific fusion protein/corepressor interaction domains restores differentiation response in leukemia cells by derepression of fusion protein targets.

Expression of interaction domain peptides triggers specific PML/RAR α degradation

We next asked whether the altered interactions with N-CoR could affect stability and expression of the fusion proteins. Indeed, in both NB4IDC or IDN and NB4R4IDC or IDN cells, the PML/RAR α protein expression was markedly reduced with respect to control cells (Figure 4A). Notably, the RAR α protein was not affected. In RT-PCR assays, however, the expression of the PML/RAR α fusion mRNA was still abundant (Figure 4B), suggesting that protein degradation was occurring. The phenomenon is ligand independent since it occurred in the absence of RA and was maintained in the absence of serum (Supplementary Figure 2). To test whether

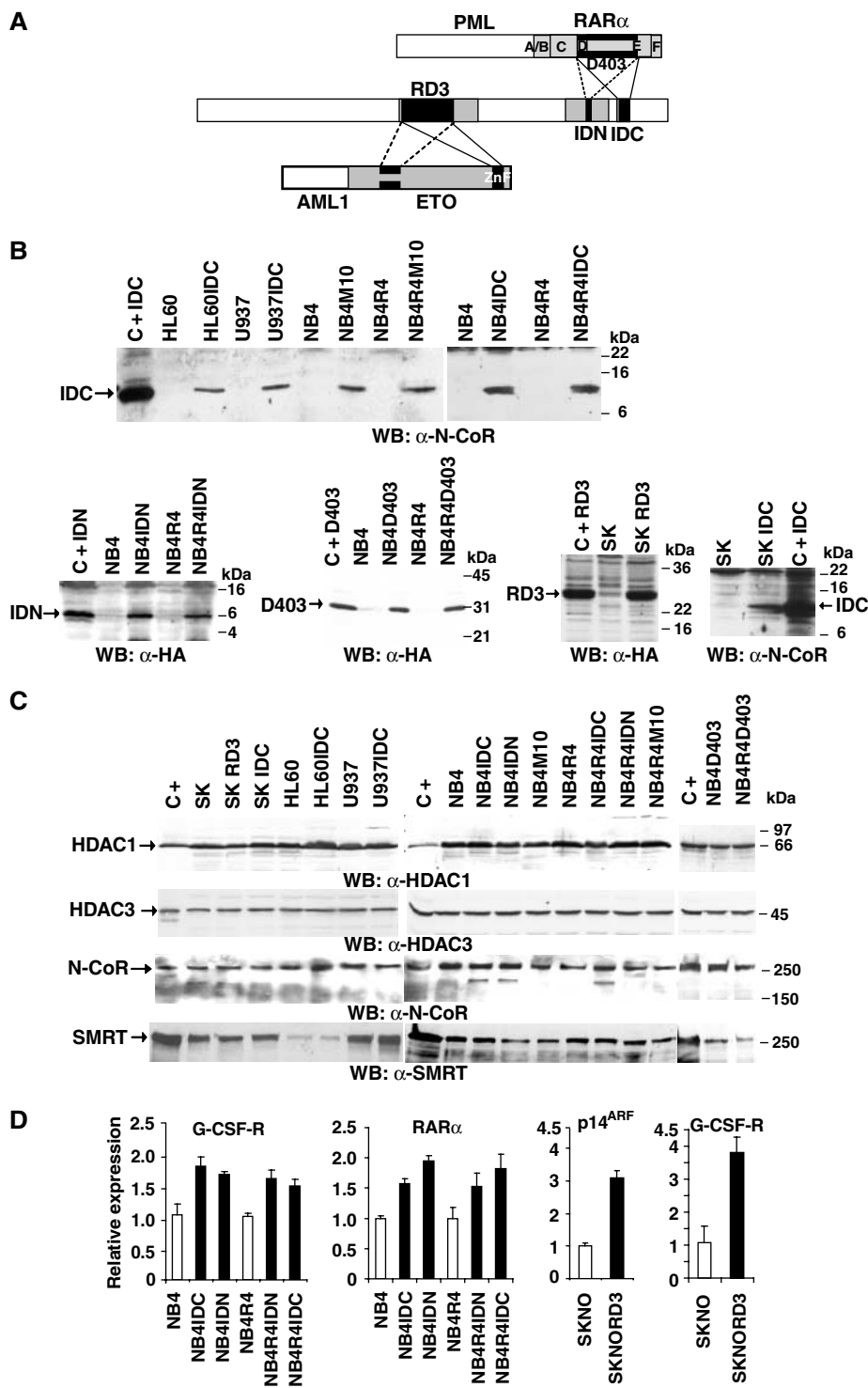


Figure 1 Effects of the different interaction domain protein fragments on the expression of corepressor and fusion protein target genes. (A) Interaction regions of the N-CoR protein and the PML/RAR α and AML1/ETO fusion proteins; gray areas within N-CoR represent the previously mapped RD3, IDN and IDC domains; black bars are the fragments used in this work. Black bars on fusion protein schemes indicate the sites of interaction with N-CoR. (B) Western blotting on lysates from the indicated cells, with the indicated antibody, showing the expression of IDC, IDN, D403 and RD3 fragments. M10 is an IDC mutant unable to bind RAR α . SK represents for SKNO1. C + : lysates from 293T cells transfected with the indicated expression vector, as positive controls. (C) Western blotting on the same lysates as in (B) and lysates of the indicated cell lines showing the expression levels of HDAC-1, HDAC3, N-CoR and SMRT proteins. C + : nuclear HeLa extract. (D) Real-time quantitative RT-PCR showing the effect of the interaction domains on mRNA expression of RAR α and G-CSF-R and p14^{ARF} in NB4, NB4R4 and SKNO cells as indicated. The expression of cells carrying an empty vector (NB4, NB4R4 and SKNO1) is given as 1.

proteasomal degradation could be responsible for the reduced PML/RAR α protein expression, we treated IDC-expressing cells with the proteasome inhibitors MG132 and lactacystin

(Zhu *et al*, 1999). Fusion protein expression was restored (Figure 4C). Since elastases and caspases can proteolyse PML/RAR α *in vivo* (Nervi *et al*, 1998; Lane and Ley, 2003),

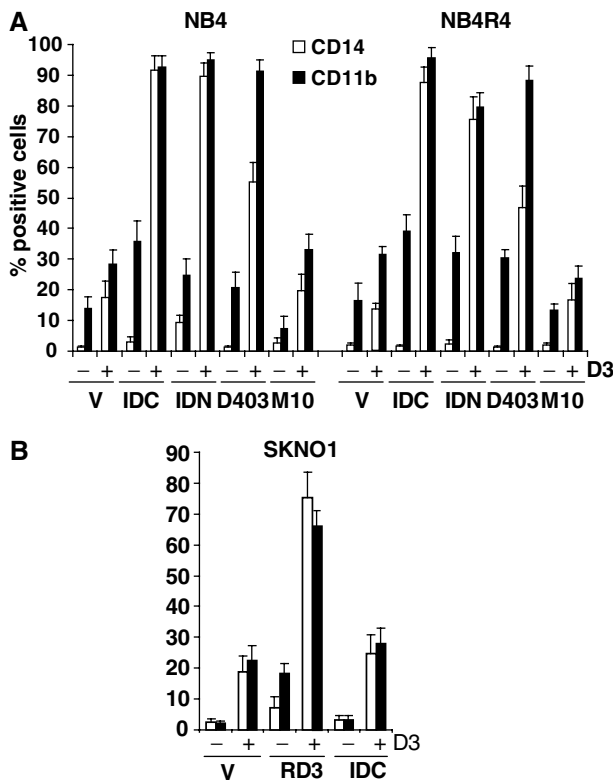


Figure 2 Expression of N-CoR interaction domain fragments restores D3-induced differentiation of leukemia cells. (A) D3-induced differentiation of NB4 or NB4R4 cells, carrying an empty GFP vector (V) or expressing IDC, IDN, D403, the IDC mutant M10 or RD3. (B) D3-induced differentiation of SKNO1 cells, carrying an empty GFP vector (V) or expressing RD3 or IDC. Percentage of cells expressing the differentiation surface markers CD11b and CD14 as assessed by cytofluorimetry is shown. Differentiation markers were measured 72 h after treatment. Error bars are the mean \pm standard deviation of four independent experiments.

we treated these cells with the serine protease inhibitor PMSF, and the caspase inhibitors Z-VAD and DEVD. Caspase inhibitors had no effect (not shown), in agreement with the PML/RAR α -dependent caspase activation by RA (Nervi *et al*, 1998). PMSF treatment increased the expression of PML/RAR α in NB4 and NB4R4 cells, but failed to restore PML/RAR α expression in IDC or IDN cells (Figure 4C and not shown). We concluded that IDC/IDN expression triggers degradation of the PML/RAR α fusion protein by proteasomal enzymes. Proteolysis was PML/RAR α specific, since SKNO1 cells expressing the N-CoR RD3 fragment (Figure 4A) displayed only a marginally reduced amount of AML1/ETO protein.

We then asked whether the restored differentiation responsiveness obtained in NB4 and NB4R4 cells was due to fusion protein degradation. We restored PML/RAR α protein expression in NB4 and NB4R4IDC or IDN cells by proteasomal inhibitors and induced cell differentiation with D3. Despite the re-established expression of PML/RAR α , both these cell lines were efficiently differentiated by treatment with D3 (Figure 4C and D), indicating that the disturbance of fusion protein association with N-CoR rather than fusion protein degradation was responsible for the restored differentiation potential of the leukemia cells.

Block of fusion protein/corepressor interactions is responsible for the restored differentiation potential of leukemia cells

To verify whether expression of the N-CoR fragments can block N-CoR /fusion protein association in live cells, we used the IDC, IDN or the RD3 vector to infect U937 cells with Zn-inducible expression of PML/RAR α (U937 PR9 cells) (Grignani *et al*, 1993) or AML1/ETO (U937 A/E), respectively (Gelmetti *et al*, 1998). Infected cells expressed the different N-CoR domains (Figure 5A). Notably, fusion protein expression was maintained at 6 h after Zn induction (Figure 5B and C), allowing the analysis of the fusion protein/corepressor complex formation. Thus, we performed co-immunoprecipitation experiments using anti-N-CoR antibodies, followed by Western blotting with anti-RAR α or -AML1 antibodies. Reciprocally, we immunoprecipitated the fusion proteins by anti-PML or -ETO antibodies and Western blotted the immunoprecipitated proteins with anti-Sin3A antibodies. The expression of the N-CoR fragments abolished co-precipitation of the PML/RAR α or AML1/ETO proteins with the N-CoR/Sin3A complex (Figure 5B and C). Since the same domains of the fusion proteins that bind N-CoR also recruit the SMRT corepressor, we tested whether the N-CoR fragments could impair SMRT interaction with the fusion proteins. Co-immunoprecipitation of SMRT with both PML/RAR α and AML1/ETO was markedly reduced by the expression of IDC or RD3 N-CoR fragments in PML/RAR α - or AML1/ETO-expressing cells, respectively (Figure 5D). To further prove that the disruption of the corepressor complex was occurring *in vivo*, we performed chromatin immunoprecipitation (ChIP) with anti-N-CoR and anti-SMRT antibodies and searched for specific sites in the promoter of fusion protein target genes. In PML/RAR α cells, we searched for the RA-responsive element on RAR α 2, and in AML1/ETO cells for an AML1 site on p14^{ARF}. Again, expression of the N-CoR interaction fragments markedly reduced the amount of N-CoR and SMRT on the promoter region of these genes (Figure 5E). Consistently, mRNA expression of RAR α , which is reduced by Zn-induced PML/RAR α expression, is increased in cells expressing IDC. Similarly, p14^{ARF} expression is repressed by Zn-induced AML1/ETO, but is increased by the presence of the RD3 fragment (Figure 5F). We concluded that fusion protein interaction with corepressors can be effectively competed *in vivo* by peptides representing specific interaction domains of N-CoR.

Block of fusion protein/N-CoR interactions can be obtained by protein transduction strategies

We next explored protein transduction as a tool for delivering therapeutic molecules into the cells (Schwarze *et al*, 1999, 2000). We produced in bacteria the N-CoR IDC, IDN, M10 and RD3 proteins fused to an HIV TAT protein transduction domain (PTD) and to an SV40-derived nuclear localization signal (NLS). We repeatedly exposed NB4, NB4R4 and SKNO1 cells to the TAT fusion peptides. Western blotting analysis after trypsin treatment of the cells (see Materials and methods) showed the presence of protein fragments in the cells (Figure 6A). The transduced TAT-IDC induced PML/RAR α degradation after 3 days of treatment, although partial degradation products, reacting with an anti-RAR α antibody, were still visible (Figure 6B). To verify whether the protein fragments were biologically active, we performed D3 and RA

differentiation experiments as described above (Figure 6C and D). NB4 and NB4R4 cells transduced with TAT-IDC and TAT-IDN cells displayed a high level of D3-induced differentiation (Figure 6C) as measured by CD11b and CD14 surface expression. Again, NB4R4 cells became highly sensitive to the differentiation effect of RA (Figure 6D). The effects of transducing SKNO1 cells by the TAT-RD3 protein were qualitatively similar to those obtained by expressing the N-CoR RD3 fragment by retroviral vectors. However, in SKNO1 cells, differentiation induction by D3 and RA was less effective, suggesting that the amount of TAT-RD3 protein entering the cells was not sufficient to warrant a complete release of the leukemia differentiation block (Figure 6E). Overall, these

results indicate that protein transduction of interaction domain peptides can be envisaged as an effective strategy to unblock differentiation in leukemia cells.

Discussion

In this study, we restored leukemia cell response to RA and D3, two physiological inducers of myeloid differentiation, by expressing short protein sequences representative of the surfaces used by N-CoR to bind the PML/RAR α and AML1/ETO proteins. Probably, these protein fragments saturate the N-CoR binding sites on the RAR α and ETO moiety of the fusion proteins, impairing N-CoR interaction. Importantly,

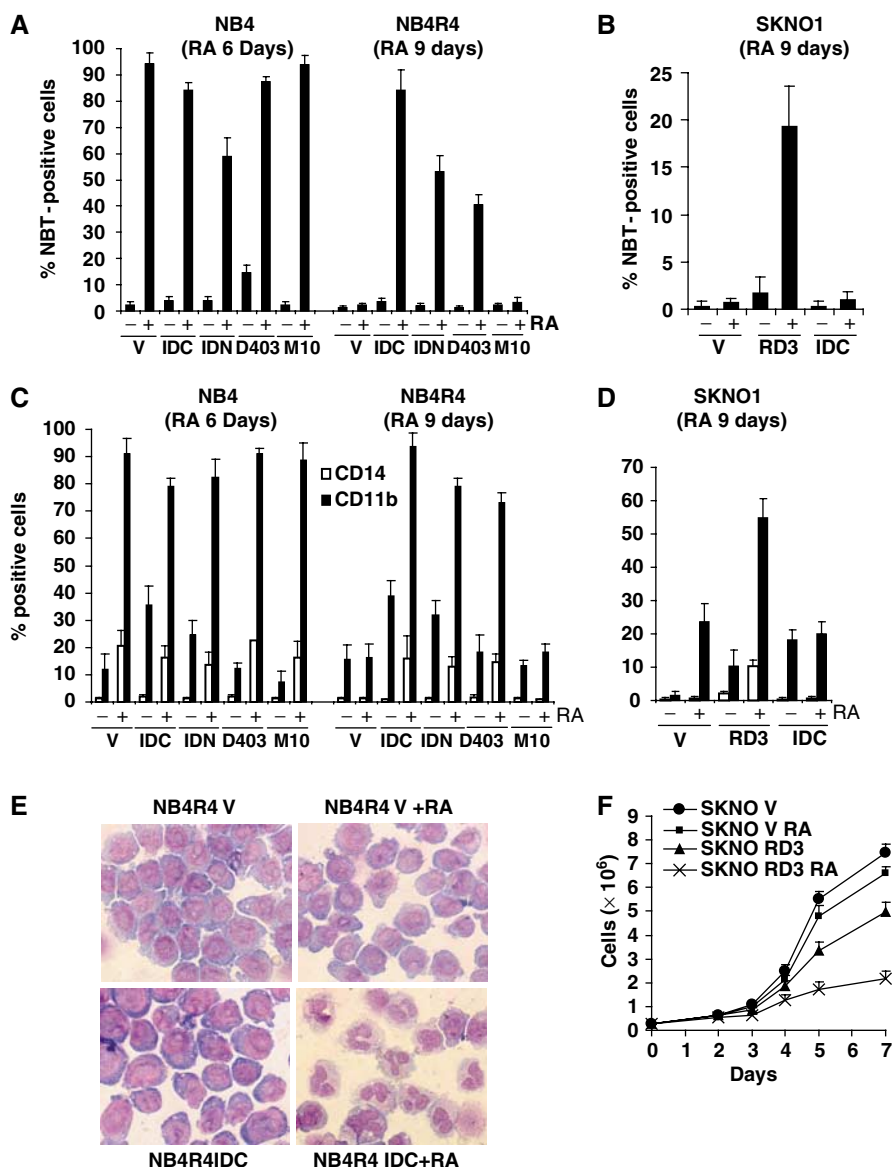


Figure 3 Expression of N-CoR interaction domain fragments restores RA-induced differentiation in RA-resistant leukemia cells. (A) RA-induced differentiation (+) of the indicated cell lines, carrying an empty GFP vector (V) or expressing IDC, IDN, D403, the IDC mutant M10 or RD3. NB4 cells were induced for 6 days and NB4R4 cells for 9 days. Differentiation measured as a percentage of cells reducing NBT. (B) RA-induced differentiation (+) of SKNO1 cells, carrying an empty GFP vector (V) or expressing RD3 or IDC. Cells were induced for 9 days. Differentiation was measured as in (A). (C) RA-induced differentiation (+) as in (A), measured as a percentage of cells expressing CD11b or CD14. (D) RA-induced differentiation (+) as in (B), measured as a percentage of cells expressing CD11b or CD14. (E) May-Grunwald-Giemsa staining of cytopsin slides from NB4R4 carrying an empty GFP vector (V) and NB4R4IDC cells untreated or treated with 10^{-6} M RA for 9 days. (F) Growth curve of SKNO1 cells carrying an empty GFP vector (V) or SKNO1 cells expressing RD3 in the presence and absence of 10^{-6} M RA. In (A–D, F), values represent mean and standard deviation of four experiments.

saturation of fusion protein–corepressor binding sites also displaces SMRT, increasing the effectiveness of this approach. Our co-immunoprecipitation and CHIP data indicate that N-CoR, SMRT and Sin3A may all contribute to the repressor activity of AML1/ETO and PML/RAR α . N-CoR fragments dislocate all these members of the repressor complex from the fusion proteins. This occurs *in vivo* at specific sites on the promoter of fusion protein target genes involved in the regulation of cell differentiation, such as RAR α , G-CSF-R and p14^{ARF}, resulting in their derepression. Although the AML1 moiety of AML1/ETO binds Sin3A (Lutterbach *et al*, 2000), the ETO-directed N-CoR fragment reduces the amount

of fusion protein-bound Sin3A below the sensitivity of our assay. Overall, the disruption of the repressor complex on direct fusion protein targets is likely to be a major contribution in the increased differentiation potential of the cells. The differentiation-unblocking effects of a segment of RAR α spanning the region that binds N-CoR further confirm that PML/RAR α activity is the major target of IDC/IDN.

Modification of other molecular pathways might contribute to the overall phenotype. Recruitment of N-CoR by vitamin D receptor may be altered, contributing to unblock differentiation. We observed an increased expression of the osteocalcin gene, a target of vitamin D receptor, in U937 cells

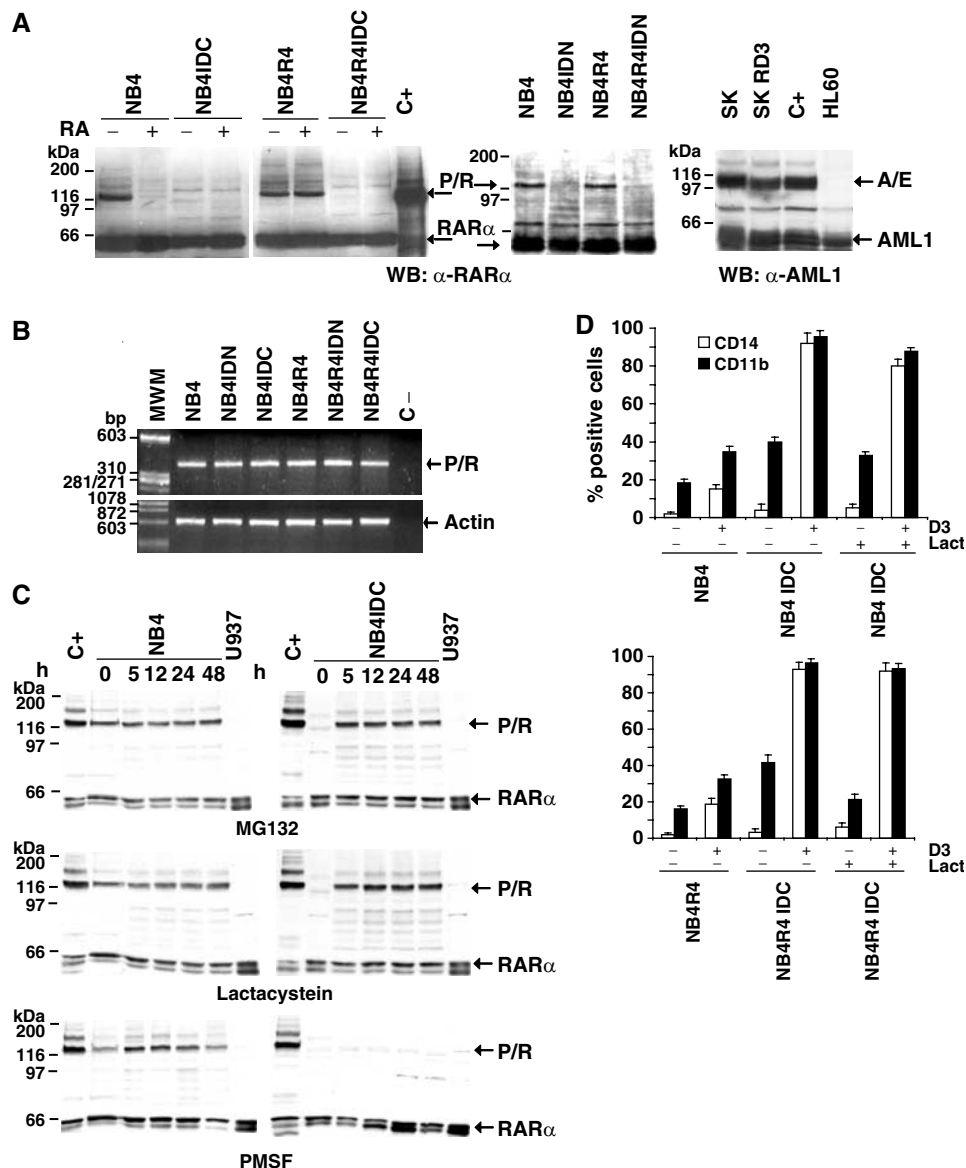


Figure 4 Expression of interaction domain protein fragments induces proteosomal degradation of the PML/RAR α fusion protein. (A) Western blotting with anti-RAR α -F or anti-AML1. P/R and A/E stand for the PML/RAR α protein and the AML1/ETO protein, respectively. The mutated PML/RAR α expressed in NB4R4 is resistant to RA-induced degradation (Raelson *et al*, 1996). (B) RT-PCR with PML/RAR α or actin oligonucleotides for detection of the PML/RAR α fusion transcript (P/R) or actin transcript as a control in the indicated cells. In (A, B), protein and RNA extraction was performed 2 weeks after retroviral transduction. (C) Western blotting showing PML/RAR α and RAR α expression in lysates from the indicated cells before and after the indicated treatment times with the proteasome inhibitors MG132 or lactacystin and the serine protease inhibitor PMSF. C+ is a lysate from 293T cells transiently transfected with a PML/RAR α expression vector, as positive control. U937 lysate: negative control. (D) D3-induced differentiation in NB4 and NB4R4 carrying an empty GFP vector or expressing IDC untreated (-) or pretreated for 6 h with lactacystin (+) to restore PML/RAR α expression and then with 250 ng/ml D3 (+) to induce differentiation. Expression of CD11b and CD14 was measured after 48 h. Values represent mean and standard deviation of three experiments.

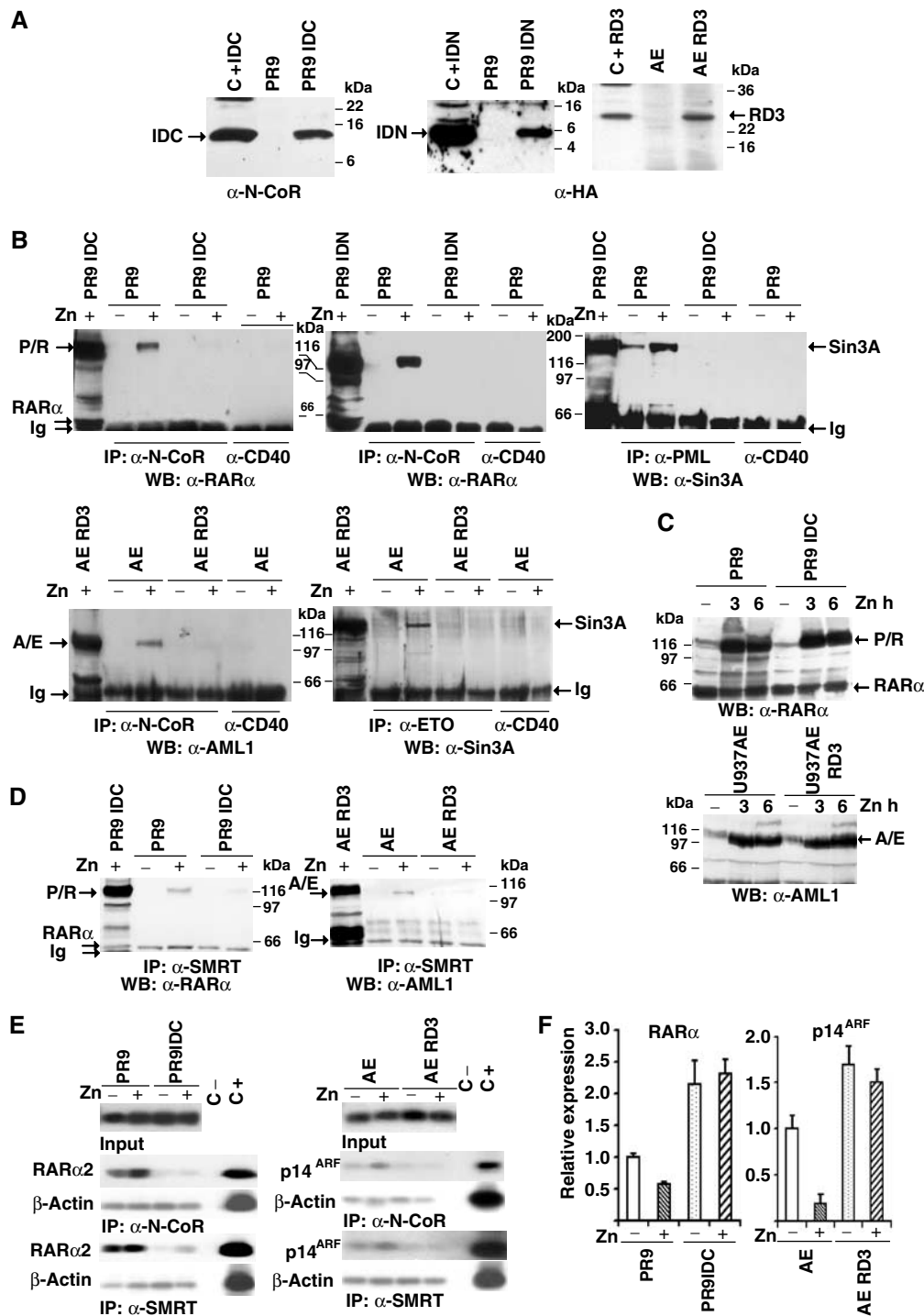


Figure 5 Expression of the N-CoR fragments disrupts the fusion protein/corepressor complexes on fusion protein target genes. (A) Western blotting of cell lysates stably expressing the indicated N-CoR fragments. The anti-N-CoR antibody recognizes the IDC domain. An anti-HA tag antibody visualizes the HA tag fused to the IDN and RD3 N-CoR fragments. C+ is a positive control as in Figure 1. PR9 and AE: U937 cells with Zn-inducible expression of PML/RAR α or AML1/ETO, respectively. (B) Co-immunoprecipitation and Western blotting experiments from PR9 and AE cells (as in (A)) stably expressing IDC or IDN or RD3, before (–) and after (+) Zn-induced expression of the PML/RAR α or AML1/ETO fusion proteins. IP indicates the immunoprecipitating antibody. CD40 was used as a negative control. Immunoprecipitates were analyzed by Western blotting (WB) with the indicated antibody. The first lane of each panel is a whole-cell lysate showing the expression of PML/RAR α (PR9 IDC or IDN), AML1/ETO (AE RD3) or Sin3A proteins in the indicated cells. Ig indicates the position of immunoglobulin-derived bands. (C) Western blotting with anti-RAR α or anti-AML1 antibodies showing short-term (3 and 6 h) Zn-inducible (+) expression of the PML/RAR α and AML1/ETO proteins in the indicated cell lines. (D) Co-immunoprecipitation with anti-SMRT antibodies and Western blotting from PR9 and AE cells as in (B). (E) ChIP with the indicated antibodies from the indicated cells before and after 5 h Zn induction (Zn+) of fusion protein expression. The RAR α 2 promoter or the p14^{ARF} promoter was PCR amplified from the immunoprecipitated chromatin as indicated. Input shows amplification from sonicated chromatin. Actin: amplification of actin DNA as a control of nonspecific precipitated sequences. C–: PCR without DNA; C+: PCR on genomic DNA. (F) Real-time quantitative RT-PCR showing the expression of RAR α or p14^{ARF} in the indicated cells before and after Zn induction (Zn+) of fusion protein expression. The expression in uninduced cells is taken as 1. Due to promoter leakage (C), uninduced cells have low expression of the fusion proteins.

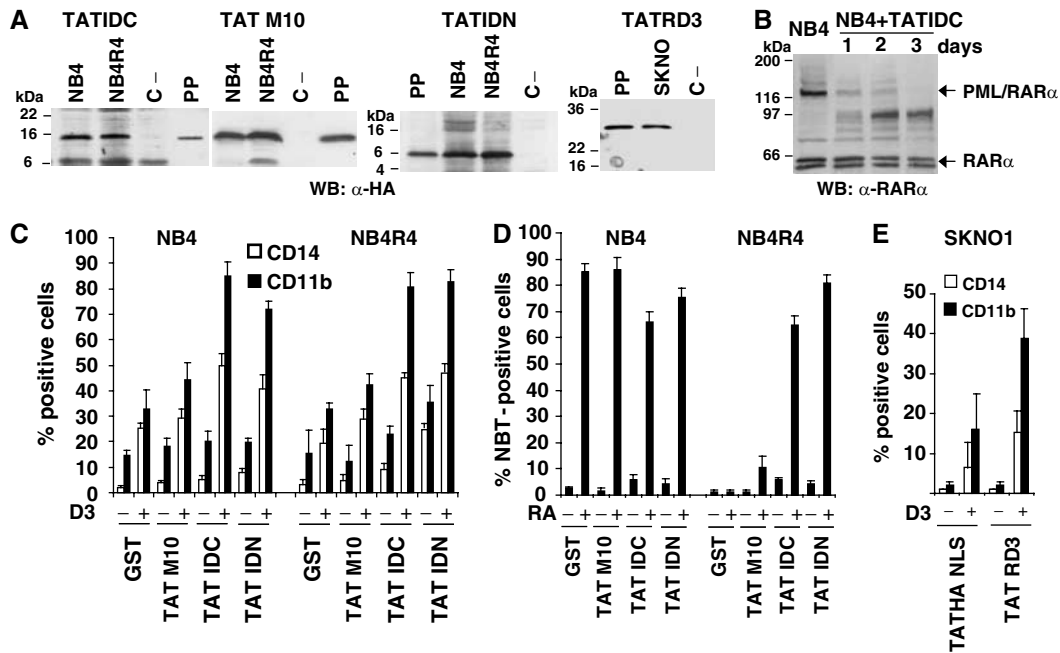


Figure 6 TAT PTD-mediated transduction of IDC, IDN or RD3 restores differentiation of leukemia cells. (A) Western blotting from lysates of the indicated cells transduced with TAT-IDC, TAT-IDN, TAT-M10 or TAT-RD3 (see Materials and methods). PP: purified TAT fusion protein as a positive control; C-: untransduced NB4 or SKNO1 cells. We used an antibody recognizing the HA tag included in the TAT fusions. (B) Anti-RAR α -F Western blotting on lysates from NB4 cells transduced with TAT-IDC for different times. (C) D3-induced differentiation of NB4 and NB4R4 cells transduced with the TAT-IDC or -IDN proteins. TAT-M10 and GST proteins were used as a negative control. Differentiation was measured as in Figure 2. (D) RA-induced differentiation of NB4 and NB4R4 cells transduced with the TAT-IDC or -IDN proteins. TAT-M10 and GST proteins were used as a negative control. Differentiation was measured as a percentage of cells reducing NBT after 6 days (NB4) or 9 days (NB4R4) of RA treatment. (E) D3-induced differentiation of SKNO1 cells transduced with the TAT-RD3 protein. A TAT-HA-NLS protein was used as a negative control. Differentiation was measured as in Figure 2. Values represent mean and standard deviation of four separate experiments.

overexpressing IDC (not shown). Activation of cAMP pathway can restore RA-induced differentiation in RA-resistant APL-derived cells (Kamashev *et al*, 2004). We cannot exclude that N-CoR fragments may activate the cAMP pathway, but our data suggest that their major effects are actually exerted through modulation of fusion protein target genes.

Expression of the N-CoR fragments specifically induces degradation of the PML/RAR α protein. HDAC1, HDAC3, N-CoR and SMRT, important components of the repressor complex, are unmodified. Degradation is ligand independent, since it occurs in serum-free medium, in the absence of retinoids. In fact, inhibitors of caspases, which participate in RA-induced degradation, do not restore PML/RAR α expression. Conversely, proteasomal inhibitors abolish this phenomenon, suggesting that the fusion protein is degraded in the proteasome. Serine protease inhibitors should prevent PML/RAR α degradation by elastases, recently implicated in the pathogenesis of APL (Lane & Ley, 2003). Actually, these agents appear to increase the expression of PML/RAR α and its SUMO-modified forms in NB4 cells, but do not restore PML/RAR α expression in IDC/IDN cells, indicating that the pathogenetic degradation is different from the proteolysis seen in this study. Here, PML/RAR α degradation is triggered by the loss of corepressor interaction. Likely, interaction with corepressors maintains the fusion protein in a steric conformation that makes it inaccessible to proteasomal enzymes. Likewise, release of corepressors from the fusion protein may also contribute to RA- and arsenic trioxide-induced PML/RAR α degradation (Yoshida *et al*, 1996; Zhu *et al*, 2001; Hong *et al*, 2003).

The AML1/ETO protein is only modestly degraded when separated from N-CoR, indicating that the degradation is PML/RAR α specific. The RD3 fragment may induce the same conformational changes as the entire corepressor molecule. Alternatively, a fraction of Sin3A protein below the sensitivity of our assays may remain bound to the AML1 moiety of the fusion protein and may be sufficient to stabilize it (Lutterbach *et al*, 2000; Imai *et al*, 2004).

Receptor proteolysis plays a role in ligand-dependent transcriptional activation by nuclear receptors (Zhu *et al*, 2001; Seeler and Dejean, 2003; Perissi *et al*, 2004). In NB4-IDC/IDN cells, PML/RAR α is proteolysed and the cells respond to RA. Although we cannot exclude a contribution of partially degraded forms of the fusion protein, RA response probably derives from the activity of the RAR α protein. Its expression is not modified in IDC/IDN cells although its mRNA is increased, possibly implying limited proteolysis that may contribute to ligand-dependent activation of target genes. Overall, in the absence of PML/RAR α , NB4-IDC/IDN cells may simply behave like other RA-responsive myeloid cells, since RA binding releases from RAR α peptides representing the N-CoR interaction regions, allowing coactivator recruitment (Hu and Lazar, 1999; Nagy *et al*, 1999; Perissi *et al*, 1999). Thus, the N-CoR fragments, which specifically target the abnormal protein interactions underlying leukemia transformation, may not affect normal cells. In agreement, IDC expression does not impair RA-induced differentiation of cells that do not express the PML/RAR α fusion protein.

Expression of the N-CoR fragments can convert leukemia cells from RA-resistant to RA-responsive. In NB4R4IDC or

IDN cells, the dominant-negative effect on RAR α of a mutant PML/RAR α is abolished. The kinetics of RA-induced differentiation in NB4-IDC/IDN and NB4R4-IDC/IDN cells was somewhat slower than in NB4 cells (Figure 3), possibly due to the loss of PML/RAR α contribution to differentiation (Grignani *et al*, 1993; Kogan *et al*, 2000) and to stable molecular alterations due to the long-term block of the RAR α pathway in NB4R4 cells. Also SKNO1/RD3 cells, which express AML1/ETO, became RA-responsive. These data are in agreement with our previous findings, suggesting that the AML1/ETO fusion protein is able to block the RAR α pathway (Ferrara *et al*, 2001), and show that this is the direct consequence of the AML1/ETO protein interaction with corepressors.

Overall, loss of N-CoR/SMRT interactions, rather than fusion protein degradation, is primarily responsible for restored differentiation response in cells expressing N-CoR fragments. Treatment of NB4IDC and NB4R4IDC cells with proteasome inhibitors re-establishes PML/RAR α expression, but the fusion protein cannot recruit N-CoR, due to the overexpression of interaction peptides, and, as a result, cannot block differentiation. Moreover, the SKNO1-RD3 cells differentiate efficiently despite the fact that AML1/ETO protein is only slightly degraded.

Our data have implications regarding the role of fusion proteins in the construction of the leukemia phenotype, a critical issue in the selection of targets for molecular therapy. Transgenic animal models indicate that fusion protein activity is not sufficient to cause differentiation block (reviewed in Melnick and Licht, 1999; Bernardi *et al*, 2002). However, fusion proteins block differentiation more effectively in murine bone marrow transduction-transplantation models and in cell lines (Melnick and Licht, 1999; Tenen, 2003). We show that fusion protein function is necessary to block leukemia cell response to physiologic myeloid differentiation inducers. Ligand-induced receptor stimulation is still required to trigger maturation of the cells. Overall, full malignant features in leukemia require fusion protein activity. This phenomenon has been referred to as 'addiction' to oncogenes and has been shown for myc-dependent cancers (Weinstein, 2002; Jain *et al*, 2003). In our model system, it implies that the removal of fusion protein function may restore leukemia cell differentiation response. Thus, fusion proteins are important targets for molecular therapy of leukemia.

Searching for a method to interfere with leukemia transformation *in vivo*, we obtained direct transduction of ID fragments by fusing them with HIV TAT PTD. The transduced fragments had effects that were qualitatively similar to those obtained by retroviral transduction including PML/RAR α protein degradation, although partial degradation products were still visible after 3 days of treatment. SKNO1 cells appear to be less accessible to TAT PTD-mediated transduction than APL-derived cell lines. Nevertheless, we show that this strategy is feasible and can be applied to diverse protein-protein interactions. Protein transfer has already proven to be therapeutically effective in live animals (Asoh *et al*, 2002; Kilic *et al*, 2003). Our data establish the foundation for a targeted treatment approach to leukemia, based on its molecular pathogenesis (Rabbits and Stocks, 2003). As a further support to the relevance of this approach, while this paper was in preparation, it has been published that TAT-mediated transfer of SMRT fragments inactivates the repressor activity

of Bcl-6 *in vitro* and *in vivo*, leading to growth arrest and apoptosis of lymphoma cells (Polo *et al*, 2004). Future improvements of protein transfer efficiency or the development of small interfering molecules that act on protein interactions may render this strategy applicable in human therapy.

Materials and methods

Cell culture

The amphotropic packaging cell line Phoenix, the APL cell lines NB4 and NB4R4, the myeloid cell lines HL60 and U937 and its derivatives were cultured in RPMI medium with 10% FBS. For SKNO1 cells, 10 ng/ml GM-CSF was added to the medium.

PCR and RT-PCR, interaction domain fragments, retroviral vector construction, cell infection and cell sorting

cDNA fragments encoding N-CoR interaction domain peptides IDC, IDN and RD3 were cloned by PCR on the N-CoR cDNA (NM_011308) with the following oligonucleotides: RD3 sequence (amino acids 1071–1309) 5'-GCCACCATGGTTCGGCTCCGACAACCGACCAAC-3' and 5'-TCACATCCCTTGCTTATATTCCTTCCAC-3'; IDN sequence (amino acids 2059–2085) 5'-CGCCACCATGGCCAGGACCCATCGACTG-3' and 5'-TCAATTTCTAGCAAAATCTTGTA-3', IDC sequence (amino acids 2217–2323) 5'-ACCGCGCCACCATGGTTAAATCAAAG-3' and 5'-ATCTCACCGTGCCTCGCTGCTCGTAC-3'. In the IDC mutant M10, the amino acids 2275, 2278 and 2279 were mutagenized to alanine by a Quick Change mutagenesis kit (Stratagene, La Jolla, CA). The RAR α D403 fragment was obtained by PCR on a RAR α cDNA (NM_000964) with the following oligonucleotides: 5'-GCCGCCACCATGGTGACCCGGAAC-3' and 5' CATGGATCAGCGGATCCTCCATCTT-3' (amino acids 133–403). An HA tag was subcloned in-frame in the IDN, RD3 and D403 vectors. The cDNAs were cloned in a PINCO vector (Grignani *et al*, 1998b) where the CMV promoter was substituted for by an encephalomyocarditis virus internal ribosomal entry site (IRES). The resulting bicistronic vector encoded both the interaction peptides and GFP. Retroviral vector production and usage have been described previously (Grignani *et al*, 1998b, 2000; Minucci *et al*, 2002). Cells infected with empty control vectors and vectors encoding the described protein fragments were purified by FACS as reported (Grignani *et al*, 1998b, 2000). RT-PCR for detection of PML/RAR α mRNA was performed as described (Biondi *et al*, 1992) with the following oligonucleotides: 5'-CAGTGACGCTTCTCCATCA-3' and 5'-AGAATGCTGCTCTGGGTCTCAAT-3'.

Antibodies, Western blotting, immunoprecipitation, caspase and protease inhibitors

Western blotting and co-immunoprecipitation experiments were performed as described (Grignani *et al*, 1996, 1998a) using the following antibodies: anti-RAR α -F (a gift of P Chambon), anti-Sin3A AK-11, anti-N-CoR for immunoprecipitation, N-19 and anti-PML PG-M3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HDAC-1 and anti-N-CoR for Western blotting rabbit polyclonal IgG 06-720 and 06-892 respectively (Upstate Biotechnology, Lake Placid, NY), anti-AML-1/RHD Ab-2 (Oncogene Science, Boston, MA), anti-ETO Ab-1 (Oncogene Science), anti-HA.11 (Babco, Richmond, CA), anti-HDAC-3 CHIP grade and anti-SMRT-1542 (Abcam Ltd, Cambridge, UK).

The proteasome inhibitors MG132 and lactacystin (Biomol Research Laboratories Butler Pike Plymouth Meeting, PA) were used at a concentration of 10⁻⁶ M. PMSF (Sigma-Aldrich, Milano, Italy) was used at 0.5 mM. DEVD and Z-VAD (Sigma-Aldrich, Milano, Italy) were used at 10⁻⁶ M.

Real-time PCR analysis

Quantitative real-time PCR was performed as published (Linggi *et al*, 2002) in ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using Taqman oligonucleotides for GAPDH, G-CSF-R and RAR α (Applied Biosystem) according to the manufacturer's instructions. p14^{ARF} primers have been previously described (Linggi *et al*, 2002). Gene expression, normalized for GAPDH expression serving as endogenous control, was calculated using the $\Delta\Delta CT$ method.

Growth and differentiation and immunophenotyping experiments

Differentiation of U937, HL60, NB4, NB4R4 and SKNO1 cells and derivatives was induced with 250 ng/ml D3 (a gift from Hoffmann-La Roche, Basel, Switzerland) for 3 days. RA (Sigma-Aldrich, Milano, Italy) was used at 10^{-6} M. Cells were seeded at 10^5 /ml. Immunophenotyping was performed as published (Grignani *et al*, 1993) using PE-conjugated Serotech antibodies (Serotech, Oxford, UK). Nitro blue tetrazolium (NBT) assay was performed as described (Grignani *et al*, 1993).

Chromatin immunoprecipitation

ChIP was performed using previously described oligonucleotides and methods (Linggi *et al*, 2002) (see the antibodies above) using the same number of cells for each sample. PCR amplification of the RAR α 2 promoter (sequence AF283809) containing the RA-responsive element was obtained with the following oligonucleotides: Fwd 5'-ACAATGACACAAGCCGGTGTCTCA-3'; Rev 5'-CTTACAGATCA GACGTC AAGCCC-3'. PCR on β -actin (NM_001101) was used to detect nonspecific DNA: Fwd 5'-CTTCTACAATGAGCTGCGTGTGG-3'; Rev 5'-CATGGATCACGGGATCTCCATCTT-3'. PCR products were run on an agarose gel, Southern blotted and probed with a cloned and sequenced DNA fragment amplified from genomic DNA with the same oligonucleotides.

TAT fusion protein production and usage

The N-CoR cDNA fragments were subcloned in a 6xHis-based bacterial expression vector, in-frame with the HIV TAT PTD

(Schwarze *et al*, 1999), an HA tag and an NLS from SV40 (Hodel *et al*, 2001), added by PCR. TAT fusion proteins were purified as described (Vocero-Albani *et al*, 2001) and added to cell culture in serum-free medium at a final concentration of 200 nM. FCS (10%) was added 30 min after protein addition. During differentiation experiments, addition of TAT fusion protein was repeated four times a day. To detect protein uptake by Western blotting, cells were washed three times in PBS, treated with 2.5 mg/ml trypsin at 37°C for 20 min, washed again as before and lysed in sample buffer. In all, 10^{-6} M RA or 250 ng/ml D3 was added to cell culture medium 5 h after the first TAT protein treatment.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr PG Pelicci for helpful advice and reagents, Dr M Cioccoloni and Dr S De Matteis for preliminary experiments, Professor P Chambon for the anti-RAR α antibody, Dr W Miller Jr for the NB4R4 cell line, Dr Y Honma and Dr J Licht for the SKNO1 cell line and Roche Pharmaceuticals for the supply of D3. This work was supported by grants from AIRC, Italian Ministry for Instruction University and Research (MIUR and FIRB) and Ministry of Health to FG and CN and Fondazione Cenci Bolognetti to CN. SR and MP are recipients of an FIRC fellowship.

References

- Alcalay M, Meani N, Gelmetti V, Fantozzi A, Fagioli M, Orleth A, Riganelli D, Sebastiani C, Cappelli E, Casciari C, Scirpi MT, Mariano AR, Minardi SP, Luzi L, Muller H, Di Fiore PP, Frosina G, Pelicci PG (2003) Acute myeloid leukemia fusion proteins deregulate genes involved in stem cell maintenance and DNA repair. *J Clin Invest* **112**: 1751–1761
- Asoh S, Ohsawa I, Mori T, Katsura K, Hiraide T, Katayama Y, Kimura Y, Ozaki D, Yamagata K, Ohta S (2002) Protection against ischemic brain injury by protein therapeutics. *Proc Natl Acad Sci USA* **99**: 17107–17112
- Baylin SB (2002) Mechanism underlying epigenetically mediated gene silencing. *Semin Cancer Biol* **12**: 331–337
- Bernardi R, Grisendi S, Pandolfi PP (2002) Modelling haematopoietic malignancies in the mouse and therapeutic implications. *Oncogene* **21**: 3445–3458
- Biondi A, Rambaldi A, Pandolfi PP, Rossi V, Giudici G, Alcalay M, Lo Coco F, Diverio D, Pogliani EM, Lanzi EM (1992) Molecular monitoring of the myl/retinoic acid receptor-alpha fusion gene in acute promyelocytic leukemia by polymerase chain reaction. *Blood* **80**: 492–497
- Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, Fuks F, Lo Coco F, Kouzarides T, Nervi C, Minucci S, Pelicci PG (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* **295**: 1079–1082
- Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, Mancini M, Pelicci PG, Lo Coco F, Nervi C (2001) Histone deacetylase targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. *Cancer Res* **61**: 2–7
- Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Mol Cell Biol* **18**: 7185–7192
- Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* **14**: 121–141
- Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Ciocce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Grignani Fa, Lazar MA, Minucci S, Pelicci PG (1998a) Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia. *Nature* **391**: 815–818
- Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Peschle C, Nicoletti I, Pelicci PG (1993) The acute promyelocytic leukaemia specific PML/RAR α fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* **74**: 423–429
- Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Grignani F, Lanfrancone L, Peschle C, Nolan GP, Pelicci PG (1998b) High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res* **58**: 14–19
- Grignani F, Testa U, Rogaia D, Ferrucci PF, Samoggia P, Pinto A, Aldinucci D, Gelmetti V, Fagioli M, Alcalay M, Seeler J, Grignani Fa, Nicoletti I, Peschle C, Pelicci PG (1996) Effects on differentiation by the promyelocytic leukemia PML/RAR α protein depend on the fusion of the PML protein dimerization and RAR α DNA binding domains. *EMBO J* **15**: 4949–4958
- Grignani F, Valtieri M, Gabbianelli M, Gelmetti V, Botta R, Luchetti L, Masella B, Morsilli O, Pelosi E, Samoggia P, Pelicci PG, Peschle C (2000) PML/RAR alpha fusion protein expression in normal human hematopoietic progenitors dictates myeloid commitment and the promyelocytic phenotype. *Blood* **96**: 1531–1537
- He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A, Pandolfi PP (1998) Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat Genet* **18**: 126–135
- Hodel MR, Corbett AH, Hodel AE (2001) Dissection of a nuclear localization signal. *J Biol Chem* **276**: 1317–1325
- Hong SH, Yang Z, Privalsky ML (2003) Arsenic trioxide is a potent inhibitor of the interaction of SMRT corepressor with its transcription factor partners, including the PML-retinoic acid receptor alpha oncoprotein found in acute promyelocytic leukemia. *Mol Cell Biol* **21**: 7172–7182
- Hu X, Lazar MA (1999) The CoRR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402**: 93–96
- Imai Y, Kurokawa M, Yamaguchi Y, Izutsu K, Nitta E, Mitani K, Satake M, Noda T, Ito Y, Hirai H (2004) The corepressor mSin3A regulates phosphorylation-induced activation, intranuclear location, and stability of AML1. *Mol Cell Biol* **24**: 1033–1043
- Jain M, Arvantitis C, Chu K, Dewey W, Leonhardt E, Trinh M, Sunderberg CD, Bishop JM, Felsner DW (2003) Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* **297**: 104
- Kamashev D, Vitoux D, De The H (2004) PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. *J Exp Med* **199**: 1163–1174

- Kastner P, Perez A, Lutz Y, Rochette-Egly C, Gaub MP, Durand B, Lanotte M, Berger R, Chambon P (1992) Structure, localization and transcriptional properties of two classes of retinoic acid receptor α fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. *EMBO J* **11**: 629–642
- Kilic U, Kilic E, Dietz GP, Bahr M (2003) Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice. *Stroke* **34**: 1304–1310
- Kogan SC, Hong SH, Shultz DB, Privalsky ML, Bishop JM (2000) Leukemia initiated by PMLRAR α : the PML domain plays a critical role while retinoic acid-mediated transactivation is dispensable. *Blood* **95**: 1541–1550
- Lane AA, Ley TJ (2003) Neutrophil elastase cleaves PML-RAR α and is important for the development of acute promyelocytic leukemia in mice. *Cell* **115**: 305–318
- Lanotte M, Martin-Thouvenin V, Najman S, Ballerini P, Valensi F, Berger R (1991) NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* **77**: 1080–1086
- Lin RJ, Evans RM (2000) Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol Cell* **5**: 821–830
- Lin RJ, Nagy L, Inoue S, Shao W, Miller WHJ, Evans RM (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* **391**: 811–814
- Linggi B, Muller-Tidow C, van de Locht L, Hu M, Nip J, Serve H, Berdel WE, van der Reijden B, Quelle DE, Rowley JD, Cleveland J, Jansen JH, Pandolfi PP, Hiebert SW (2002) The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(Arf) tumor suppressor in acute myeloid leukemia. *Nat Med* **8**: 743–750
- Look AT (1997) Oncogenic transcription factors in the human acute leukemias. *Science* **278**: 1059–1064
- Lutterbach B, Westendorf JJ, Linggi B, Isaac S, Seto E, Hiebert SW (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J Biol Chem* **275**: 651–656
- Lutterbach B, Westendorf JJ, Linggi B, Patten A, Moniwa M, Davie JR, Huynh KD, Bardwell VJ, Lavinsky RM, Rosenfeld MG, Glass C, Seto E, Hiebert SW (1998) ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol Cell Biol* **18**: 7176–7184
- Matozaki S, Nakagawa T, Kawaguchi R, Aozaki R, Tsutsumi M, Murayama T, Koizumi T, Nishimura R, Isobe T, Chihara K (1995) Establishment of a myeloid leukemia cell line (SKNO-1) from a patient with t(8;21) who acquired monosomy 17 during disease progression. *Br J Haematol* **89**: 805–811
- Melnick A, Licht JD (1999) Deconstructing a disease: RAR α , its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* **93**: 3167–3215
- Minucci S, Maccarana M, Ciocce M, De Luca P, Gelmetti V, Segalla S, Di Croce L, Giavara S, Matteucci C, Gobbi A, Bianchini A, Colombo E, Schiavoni I, Badaracco G, Hu X, Lazar MA, Landsberger N, Nervi C, Pelicci PG (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. *Mol Cell* **5**: 811–820
- Minucci S, Monestiroli S, Giavara S, Ronzoni S, Marchesi F, Insinga A, Diverio D, Gasparini P, Capillo M, Colombo E, Matteucci C, Contegno F, Lo Coco F, Scanziani E, Gobbi A, Pelicci PG (2002) PML-RAR induces promyelocytic leukemias with high efficiency following retroviral gene transfer into purified murine hematopoietic progenitors. *Blood* **100**: 2989–2995
- Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, Krishna V, Chatterjee K, Evans RM, Schwabe JW (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes Dev* **13**: 3209–3216
- Nervi C, Ferrara FF, Fanelli M, Rippon MR, Tomassini B, Ferrucci PF, Ruthardt M, Gelmetti V, Gambacorti-Passerini C, Diverio D, Grignani F, Pelicci PG, Testi R (1998) Caspases mediate retinoic acid induced degradation of the acute promyelocytic leukemia PML-RAR α fusion protein. *Blood* **92**: 2244–2251
- Nervi C, Poindexter EC, Grignani F, Pandolfi PP, Lo Coco F, Avvisati G, Pelicci PG, Jetten AM (1992) Characterization of the PML-RAR α chimeric product of the acute promyelocytic leukemia specific t(15;17) translocation. *Cancer Res* **52**: 3687–3692
- Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG (2004) A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* **116**: 511–526
- Perissi V, Staszewski LM, McInerney EM, Kurokawa R, Kronen A, Rose DW, Lambert MH, Milburn MV, Glass CK, Rosenfeld MG (1999) Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev* **13**: 3198–3208
- Polo JM, Dell'oso T, Ranuncolo SM, Cerchetti L, Beck D, Da Silva GF, Prive GG, Licht JD, Melnick A (2004) Specific peptide interference reveals BCL6 transcriptional and oncogenic mechanisms in B-cell lymphoma cells. *Nat Med* **10**: 1329–1335
- Rabbits TH, Stocks MR (2003) Chromosomal translocation products engender new intracellular therapeutics technologies. *Nat Med* **9**: 383–386
- Raelson JV, Nervi C, Rosenauer A, Benedetti L, Monczak Y, Pearson M, Pelicci PG, Miller WHJ (1996) The PML/RAR α oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. *Blood* **88**: 2826–2832
- Schwarze SR, Ho A, Vocero-Albani A, Dowdy SF (1999) *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**: 1569–1572
- Schwarze SR, Hruska KA, Dowdy SF (2000) Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol* **10**: 290–295
- Schwieger M, Löhler J, Friel J, Scheller M, Horak I (2002) AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSPB deficiency. *J Exp Med* **196**: 1227–1240
- Seeler JS, Dejean A (2003) Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol* **4**: 690–699
- Shao W, Benedetti L, Lamph WW, Nervi C, Miller WHJ (1997) A retinoid-resistant acute promyelocytic leukemia subclone expresses a dominant negative PML-RAR α mutation. *Blood* **89**: 4282–4289
- Tenen DG (2003) Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer* **3**: 89–101
- Vocero-Albani A, Chellaiah MA, Hruska KA, Dowdy SF (2001) Protein transduction: delivery of Tat-GTPase fusion proteins into mammalian cells. *Methods Enzymol* **332**: 36–49
- Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM (1998) ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. *Proc Natl Acad Sci USA* **95**: 10860–10865
- Warrell RPJ (1993) Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. *Blood* **82**: 1949–1953
- Weinstein IB (2002) Addiction to oncogenes—the Achilles heel of cancer. *Science* **297**: 63–64
- Yoshida H, Kitamura K, Tanaka K, Omura S, Miyazaki T, Hachiya T, Ohno R, Naoe T (1996) Accelerated degradation of PML-retinoic acid receptor α (PML-RAR α) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. *Cancer Res* **56**: 2945–2948
- Zhu J, Gianni M, Kopf E, Honore N, Chelbi-Alix MK, Koken M, Quignon F, Rochette-Egly C, de Thè H (1999) Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RAR α) and oncogenic RAR α fusion proteins. *Proc Natl Acad Sci USA* **96**: 14807–14812
- Zhu J, Lallemand-Breitenbach V, de Thè H (2001) Pathways of retinoic acid- or arsenic trioxide-induced PML/RAR α catabolism, role of oncogene degradation in disease remission. *Oncogene* **20**: 7257–7265