C/EBP β : a major PML–RARA-responsive gene in retinoic acid-induced differentiation of APL cells

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In acute promyelocytic leukemia (APL), the translocation t(15;17) induces a block at the promyelocytic stage of differentiation in an all-*trans*-retinoic acid (ATRA)-responsive manner. Here we report that upon treatment with ATRA, t(15;17) cells (NB4) reveal a very rapid increase in protein level and binding activity of C/EBP β , a C/EBP family member, which was not observed in an ATRA-resistant NB4 cell line. We further provide evidence that ATRA mediates a direct increase of C/EBP β , only in PML–RARA (promyelocytic leukemia–retinoic acid receptor α)-expressing cells. In addition, transactivation experiments indicate that the PML–RARA fusion protein, but not PML–RARA mutants defective in transactivation, strongly transactivates the C/EBP β promoter. These results suggest that PML–RARA mediates ATRA-induced C/EBP β expression. Finally, we demonstrate the importance of C/EBP β in granulocytic differentiation. We show that not only does C/EBP β induce granulocytic differentiation of non-APL myeloid cell lines independent of addition of ATRA or other cytokines, but also that C/EBP β induction is required during ATRA-induced differentiation of APL cells. Taken together, C/EBP β is an ATRA-dependent PML–RARA target gene involved in ATRA-induced differentiation of APL cells.

Keywords: APL/C/EBP/granulopoiesis/PML–RARA/
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Introduction

Acute myeloid leukemias are often associated with specific chromosomal translocations that rearrange genes important for the commitment and the differentiation of hematopoietic cells. The t(15;17) translocation found in acute promyelocytic leukemia (APL) juxtaposes the promyelocytic (PML) gene to the retinoic acid receptor α (RARA) gene (Borrow *et al.*, 1990; de The *et al.*, 1990). It is believed that the aberrant PML–RARA fusion protein resulting from the translocation plays a role in the accumulation of the blasts arrested at the promyelocytic stage. This hypothesis is supported by studies of the expression of PML–RARA in transgenic mice, resulting in the development of leukemia with striking APL features

(Brown *et al.*, 1997; Grisolan *et al.*, 1997; He *et al.*, 1997). The interesting feature of the PML–RARA-bearing blasts is that they are acutely sensitive to therapeutic doses of all-*trans*-retinoic acid (ATRA) which is used effectively for the treatment of t(15;17) patients (Castaigne *et al.*, 1990; for a review see Mistry *et al.*, 2003). However, the precise mechanism by which the PML–RARA fusion protein blocks the cells at the promyelocytic stage, and how retinoic acid (RA) reverses this block is still unclear.

It is believed that PML–RARA acts as a dominant-negative factor on both the RARA and PML pathways (de The *et al.*, 1991; Kakizuka *et al.*, 1991). It has been shown that PML–RARA retains most functional domains of the RARA protein and behaves as an abnormal RA receptor with altered transactivation properties, suggesting a defect in the RA pathway to be responsible for the promyelocytic block. This has been strongly supported by experiments showing that PML–RARA recruits co-repressors and histone deacetylase that cannot be dissociated with physiological doses of RA, thus inhibiting RA-induced transcriptional activation (Grignani *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998). However, recently it has become evident that disruption in the RA signaling pathway does not afford an explanation as to how the cells become specifically blocked at the promyelocytic stage since granulocyte lineage commitment is not impaired in RARA and retinoic acid receptor γ (RARG)-deficient mice (Kastner *et al.*, 2001).

The sensitivity of promyelocytes in APL to ATRA remains an enigma. One explanation is that PML–RARA not only participates in the block of cell differentiation, but is also involved in the response of cells to ATRA. PML–RARA has been shown to homodimerize and act as a potent transcriptional repressor, acquiring an oncogenic potential which is reversible upon ATRA treatment (Lin and Evans, 2000; Minucci *et al.*, 2000). Moreover, PML–RARA has been involved in the regulation of genes during APL differentiation (Park *et al.*, 1999; Moog-Lutz *et al.*, 2001). In addition, ATRA resistance, a major problem in ATRA treatment, is mainly associated with defects in the RARA E-domain of PML–RARA (Imaizumi *et al.*, 1998; Duprez *et al.*, 2000). These findings highlight the role of the fusion protein in mediating the sensitivity of APL cells to ATRA.

In this work, we tested the hypothesis that ATRA could target members of the leucine zipper transcription factor family, the CCAAT/enhancer-binding protein (C/EBP) family, during APL differentiation. These transcription factors are implicated in the growth or the differentiation of several tissues or cell lineages including the myeloid lineage (for a review see Lekstrom-Himes and Xanthopoulos, 1998). In myeloid development, the effect of the C/EBP family on cell proliferation and differentiation is mediated by their ability to bind DNA (often

containing the sequence CCAAT) and control gene expression (reviewed in Tenen *et al.*, 1997), although recent studies provide evidence that these factors also regulate cell fate by protein–protein interaction, independent of their transcriptional activity (reviewed in McKnight, 2001). Three members of this family, C/EBP α , C/EBP β and C/EBP ϵ have been shown to be involved at different stages of myeloid development. C/EBP α plays a critical role for the engagement of the cells to the granulocytic lineage (Zhang *et al.*, 1997; Radomska *et al.*, 1998), while C/EBP ϵ is important late in the granulocytic differentiation process (Yamanaka *et al.*, 1997). However, the role of C/EBP β is less clear. Although C/EBP β knockout mice do not show any defects in myeloid differentiation, recent reports show that C/EBP β can drive immature cells into granulocytes (Popernack *et al.*, 2001; Iwama *et al.*, 2002). The apparent lack of any myeloid defects in C/EBP β mice may suggest that overlapping expression of C/EBP members provides functional redundancy among the different members (Hu *et al.*, 1998).

Here, we report that ATRA targets C/EBP β during APL differentiation. We show that ATRA increases C/EBP β activity and we demonstrate that this activation is due to an increase in C/EBP β RNA and protein levels and is one of the earliest events following ATRA treatment. We show that PML–RARA mediates ATRA-induced upregulation of C/EBP β through the C/EBP β proximal promoter and that ATRA resistance of APL cells correlates with the loss of C/EBP β induction. ATRA-independent expression of C/EBP β can drive multipotent hematopoietic cells to granulocytic differentiation. Inhibition of C/EBP β , obtained after using a dominant-negative C/EBP or using small interfering RNA (siRNA) against C/EBP β , reduces ATRA-induced maturation of APL cells. Therefore, we propose that C/EBP β is a major target of PML–RARA during APL differentiation.

Results

An increase in C/EBP binding activity is an early event in APL cells in response to ATRA

Because of the known role of C/EBP proteins in mediating granulocytic differentiation, we investigated C/EBP activity during ATRA-induced APL differentiation. Electrophoretic mobility shift assays (EMSAs) were performed using a C/EBP-binding site probe from the human granulocyte colony-stimulating factor (G-CSF) receptor promoter (Smith *et al.*, 1996) with nuclear extract from APL ATRA-sensitive NB4 cells. Consistent with previous findings (Lutz *et al.*, 2001), C/EBP binding was detected in NB4 cells (Figure 1A, lane 3). Addition of specific antibody against either C/EBP α , C/EBP β or C/EBP ϵ resulted for each family member tested in a partial supershift of the protein–oligonucleotide complex (Figure 1A, lanes 4–6), indicating that these three CEBP proteins were part of the DNA–protein complex initially observed. EMSA of nuclear extract from NB4 cells after ATRA stimulation revealed substantial modification in the DNA binding profile of C/EBP proteins. Levels of C/EBP α binding did not change within 24 h of ATRA treatment (Figure 1A, lane 8), and disappeared after 48 h (Figure 1A, lanes 12 and 16). In contrast, we observed an increase in C/EBP β binding after 24 h of ATRA treatment

Fig. 1. ATRA induces changes in the C/EBP DNA binding profile during promyelocytic differentiation. EMSA analysis of nuclear extracts from NB4 (A) and NB4-R2 cells (B) treated with ATRA. A double-stranded oligonucleotide including the C/EBP-binding site from the G-CSF receptor was radiolabeled with ^{32}P and incubated in the absence (free probe; lane 1) or presence of 10 μg of nuclear extracts from cells which were untreated (lanes 3–6), or treated for 24 (lanes 7–10), 48 (lanes 11–14) and 72 h (lanes 15–18) with ATRA. For competition analysis, a 50-fold molar excess of unlabeled oligonucleotide was used (lane 2). Antisera against C/EBP α (α), C/EBP β (β) or C/EBP ϵ (ϵ) were added to the binding reaction. Supershifted complexes are indicated with an arrow. Free probe was present in excess in each lane (shown for A only, but similar results apply to the EMSA shown in B and Figure 2).

(Figure 1A, lanes 9 and 13) as well as an increase of C/EBP ϵ binding (Figure 1A, lanes 10, 14 and 18). The C/EBP DNA binding profile was not modified upon ATRA treatment of NB4-R2 cells, an NB4 subclone which is resistant to ATRA treatment (Duprez *et al.*, 2000) (Figure 1B, lanes 3–18). These data demonstrate that the activity of different C/EBP proteins is modulated upon ATRA treatment and suggest that there is a correlation between ATRA-induced C/EBP binding modification and ATRA-induced APL differentiation.

C/EBP ϵ has been described as a target gene responsible for ATRA-induced differentiation (Park *et al.*, 1999). We found an increase in the binding activity of both C/EBP β and C/EBP ϵ upon ATRA-mediated differentiation. Thus, we next sought to determine the temporal sequence of the two events by studying the binding activity of C/EBPs following a short exposure of NB4 cells to ATRA. We found that C/EBP α binding activity was not modified during 8 h of ATRA treatment, whereas C/EBP β activity was stimulated as early as 2 h following ATRA treatment (supershifted bands, Figure 2A) and C/EBP ϵ required at least 8 h to display an increase in binding activity (Figure 2A). Thus, C/EBP β binding activity preceded that of C/EBP ϵ . Bone marrow cells from an APL patient were subjected to EMSA, and we demonstrated that *in vitro* treatment with ATRA for 15 h increased C/EBP β binding

Fig. 2. C/EBP β DNA binding activity is rapidly enhanced during ATRA-mediated differentiation of APL cells. **(A)** NB4 cells were treated with 1 μ M ATRA for 2 (lanes 4–6), 4 (lanes 7–9) and 8 h (lanes 10–12). EMSAs were performed using the same conditions as in Figure 1. DNA–C/EBP complexes were characterized by supershift experiments using antisera against C/EBP α (α), C/EBP β (β) or C/EBP ϵ (ϵ). **(B)** C/EBP α and C/EBP β binding profile in primary APL cells. Untreated cells and cells treated for 15 h with 1 μ M ATRA were subjected to EMSA analysis using the same conditions as in (A).

Fig. 3. The increase in C/EBP β protein after ATRA treatment reflects the increase in DNA binding activity. Nuclear extracts from ATRA-sensitive (NB4) and ATRA-resistant (NB4-R2) cells were tested for C/EBP β and C/EBP ϵ expression by western blotting following ATRA treatment. The membranes were incubated with antiserum against C/EBP β (upper panel) and then stripped and incubated with an antiserum against C/EBP ϵ (lower panel). The arrows designate the bands corresponding to the 43 kDa C/EBP β isoform, as well as the 32 and 27 kDa C/EBP ϵ isoforms. On the left of the figure, specificity of the C/EBP β antibody is shown using extracts from untransfected CV1 cells and from CV1 cells transfected with a human C/EBP β cDNA.

in the APL sample, resembling that seen in the NB4 cell line (Figure 2B). Our results thus implicate C/EBP β as a new RA target in ATRA-induced APL differentiation.

ATRA regulates C/EBP β expression through PML–RARA

Post-transcriptional modification of C/EBP β can enhance C/EBP β transcriptional activity by enhanced DNA binding (Poli *et al.*, 1990). Thus, we were interested in investigating the mechanism by which ATRA enhances C/EBP β binding. We used a C/EBP β -specific antibody to analyze the relative protein level in nuclear extracts from cells treated for varying lengths of time with ATRA. In untreated NB4 cells, we were unable to detect the C/EBP β protein by immunoblotting (Figure 3, upper panel), although the more sensitive EMSA revealed the presence of the protein (Figure 1). Nonetheless, expression of C/EBP β protein was markedly increased following ATRA treatment. The augmentation in relative abundance of C/EBP β protein following ATRA treatment (Figure 3,

upper panel) correlated with the kinetics observed for the increase in DNA binding activity (Figure 1). Moreover, no change in C/EBP β protein level was observed in the resistant cell line NB4-R2 (Figure 3, upper panel). However, analyses of the same extracts using a C/EBP ϵ -specific antibody failed to detect any significant changes in expression level of C/EBP ϵ isoforms (32 and 27 kDa) after a short exposure of the cells to ATRA. In agreement with previously published data (Chih *et al.*, 1997), the smaller, 27 kDa isoform increased in abundance after longer exposure of ATRA (Figure 3, lower panel). Therefore, we conclude that ATRA activates C/EBP β activity by increasing the nuclear level of the protein and that this effect precedes C/EBP ϵ upregulation.

To analyze further the mechanisms involved in ATRA-induced C/EBP β upregulation, we studied the expression of C/EBP β RNA. In agreement with the increase in protein levels, C/EBP β RNA expression was increased following exposure of the NB4 cells to ATRA, while no induction was observed in the NB4-R2 resistant cells (Figure 4A). In

Fig. 4. The presence of PML-RARA is required to rapidly induce C/EBP β RNA. (A) Northern blot analysis of total RNA isolated from NB4, NB4-R2 and HT93 cells untreated or treated with 1 μ M ATRA for the indicated time. The 28S rRNA level is shown as a loading control. (B) Comparison by northern blot of C/EBP β induction after ATRA treatment between PML-RARA- and PLZF-RARA-expressing cells. PML-RARA and PLZF-RARA were induced respectively by pre-treating the U937-PR9 cells or the U937-B412 cells with zinc for 15 h. (C) Northern blot analysis of total RNA from NB4, U937-PR9 or zinc-pre-treated U937-PR9 cells untreated or treated with 1 μ M ATRA for 1, 2 or 4 h in the absence (-) or presence (+) of cycloheximide (CHX). The 28S rRNA level is shown as a loading control. (D) Level of PML-RARA protein after a short period of ATRA treatment is revealed by western blot analysis of NB4 nuclear extracts using an anti-RARA antibody. (E) Northern blot analysis of the half-life of C/EBP β mRNA in uninduced and ATRA-induced NB4 cells. NB4 cells were pre-treated with 1 μ M ATRA for 4 h and then actinomycin D (10 μ g/ml) was added for the indicated times to the cells prior to extraction of RNA. Results are presented as RNA level normalized against actin with respect to time.

addition, we also observed the same pattern of ATRA-induced C/EBP β induction in another RA-sensitive APL cell line, HT93 (Figure 4A), indicating that this effect is not an artifact of the NB4 cell system. To study the APL-specific nature of C/EBP β induction by ATRA, we took advantage of the U937-derived cell lines, U937-PR9 and U937-B412 which contain respectively a zinc-inducible

PML-RARA and PLZF-RARA (Ruthardt *et al.*, 1997). Using this system, we demonstrated that the level of C/EBP β mRNA expression was strongly enhanced with ATRA in PML-RARA-expressing cells (Figure 4B). This enhanced expression required the presence of the PML-RARA fusion protein, since C/EBP β remained only a little responsive to ATRA in U937-PR9 cells that were not pre-

treated with zinc. More interestingly, induction of the fusion protein PLZF-RARA that is associated with an ATRA-resistant form of APL has no effect on C/EBP β expression after ATRA treatment (Figure 4B). Treatment of NB4 cells for short periods of time revealed that the increase in C/EBP β RNA occurred after only 1 h. The expression profile of C/EBP β RNA following ATRA treatment was not perturbed by pre-treating the cells with cycloheximide, indicating that *de novo* protein synthesis is not required for ATRA to exert its effect (Figure 4C, left panel). The same short ATRA treatment did not modify C/EBP β RNA in myeloid U937-PR9 cells in which PML-RARA was not expressed (Figure 4C, middle panel) but strongly enhanced C/EBP β RNA in the same cells pre-treated with zinc, similar to that observed in NB4 cells (Figure 4C, right panel). These observations indicate that the direct induction of C/EBP β mRNA by ATRA is PML-RARA specific. Given the literature showing that prolonged ATRA treatment results in PML-RARA degradation, the presence of PML-RARA was checked in the nuclear extract of NB4 cells after a short time of ATRA stimulation (Figure 4D). For the 4 h treatment time tested, we observe no degradation of the PML-RARA fusion protein. Taken together, our data demonstrate that ATRA directly regulates C/EBP β expression and that this regulation requires the presence of the PML-RARA fusion protein.

In order to investigate whether ATRA induced post-transcriptional stabilization of C/EBP β mRNA, the mRNA half-life was measured in uninduced and ATRA-induced NB4 cells by exposing the cells to actinomycin D for the times indicated in Figure 4E. The average half-life of the mRNA was 2 h in both uninduced and ATRA-induced NB4 cells (Figure 4E), suggesting a transcriptional effect of ATRA rather than post-transcriptional mRNA stabilization accounting for the upregulation of steady-state level of C/EBP β mRNA.

ATRA treatment activates the C/EBP β promoter in a PML-RARA-dependent manner

ATRA is known to exert its effects by transcriptional activation via the heterodimer RAR/RXR (Perez *et al.*, 1993), and transcriptional activation by ATRA has been shown to be mediated through an RA response element (RARE) motif composed of direct repeats of (A/G)G(G/T)TCA (Naar *et al.*, 1991; Umesono *et al.*, 1991). As our results suggested that ATRA was mediating PML-RARA activation of C/EBP β transcription, we assessed the efficiency of PML-RARA in activating the C/EBP β promoter in the absence or presence of ATRA. We subcloned 0.8 kb of the human C/EBP β promoter region (bp -600 to +200) which includes the putative TATA-binding protein-binding site and transcriptional initiation site, into a luciferase (LUC) reporter vector (Figure 5A). Transfection of the resulting BetaP0.8LUC reporter construct into U937-PR9 cells demonstrated that the C/EBP β promoter did not respond like a RA-responsive promoter. Treatment of the non-zinc-stimulated cells with ATRA failed to significantly transactivate the promoter, compared with the control RARE-LUC reporter plasmid (Figure 5B, compare lane 1 with lane 11). In contrast, the relative luciferase activity was enhanced in the presence of PML-RARA after ATRA treatment

(Figure 5B, lane 2), showing that the C/EBP β promoter region was activated only in the presence of the fusion protein after ATRA treatment (Figure 5B). This correlated well with our northern blot results (Figure 4), which demonstrated elevation of C/EBP β expression exclusively in PML-RARA-expressing cells. Deletion analyses of the C/EBP β promoter implicated the region between bp -112 and +56 as being important for this activation (Figure 5B).

To investigate whether the integrity of the fusion protein is necessary for the transactivation, C-terminal deletion mutants of PML-RARA were tested (Figure 5C). PML-RARA Δ 903, a mutant which lacks half of α -helix 12 of the ligand-binding domain, could repress basal transcriptional activity but, importantly, this repression could not be overcome with ATRA treatment (Figure 5C, lane 4). Deletion of the F domain did not affect the transcription activity of PML-RARA (Figure 5C). These results demonstrate that a functional ligand-binding domain is required for the transactivation by PML-RARA. It is noteworthy that this Δ 903 mutant has been shown to be expressed in the ATRA-resistant NB4 subclone NB4-R2 (Duprez *et al.*, 2000).

C/EBP β can induce granulocytic differentiation of a multipotential hematopoietic cell line

Our data strongly implicate an important role for C/EBP β in ATRA-induced promyelocytic differentiation of APL cells and suggested that C/EBP β directed differentiation. To determine whether C/EBP β can induce hematopoietic cells to undergo differentiation to granulocytes, we used the multipotent K562 cell line as a model (Sutherland *et al.*, 1986) that does not express C/EBP α , C/EBP β or C/EBP ϵ . Therefore, K562 cells were transduced with a pBabePuro retrovirus encoding either an estrogen receptor hormone-binding domain fused to C/EBP β (C/EBP β -ER) or the estrogen receptor hormone-binding domain alone (ER). When infected with the C/EBP β -ER or ER viruses, the expressed exogenous proteins were located in the cytoplasm, thus partitioning any nuclear activities they might have (Figure 6A). However, upon treatment of infected cells with β -estradiol for 48 h, the exogenous protein translocated to the nucleus (Figure 6A, i), thus allowing the direct study of any associated nuclear C/EBP β activity. Following β -estradiol treatment, the C/EBP β -ER-infected cells developed morphological changes characteristic of granulocytic differentiation (Figure 6A, ii), the cells stained positive for the nitroblue tetrazolium (NBT) reduction assay (Figure 6A, iii) and showed an increase in CD11b expression (Figure 6B). In order to characterize further granulocytic differentiation following the induction of C/EBP β , the expression of granulocytic-specific genes was analyzed by northern blot RNA prepared before and after varying times of β -estradiol treatment. C/EBP β nuclear translocation led to the specific induction of G-CSF receptor and C/EBP ϵ RNA (Figure 6C). Thus, in conclusion, the translocation of C/EBP β into the nucleus of K562 cells drives the cells to differentiate into mature granulocytes as assessed by morphological changes, NBT reduction assay, expression of a cell surface differentiation marker and granulocyte-specific gene expression.

Fig. 5. C/EBP β promoter is specifically activated by PML-RARA in the presence of ATRA. (A) Schematic representation of promoter constructs used in transient transfections shown in (B) and (C). The white rectangle labeled 'TATAA' represents the putative TATA box of the C/EBP β promoter. (B) U937-PR9 cells were transiently transfected with the different deletions of the C/EBP β reporter constructs. PML-RARA induction was obtained by pre-treating U937-PR9 cells with zinc for 15 h. Relative luciferase activity in the absence (white bar) or presence (black bar) of 2 μ M ATRA is presented. U937 cells (without PML-RARA) were treated with zinc for 15 h to control for the effect of zinc treatment. The RAREx3LUC reporter vector was used as a control for ATRA treatment. (C) C-terminal PML-RARA deletion mutants were tested for their ability to activate the C/EBP β promoter in U937-PR9 cells. The BetaP0.3LUC reporter was co-transfected in non-zinc-stimulated U937-PR9 cells with expression vector for PML-RARA wild-type and mutants (lanes 3–5). Relative luciferase activity in the absence (white bar) or presence (black bar) of 2 μ M ATRA is presented.

C/EBP β activity is required for ATRA induction of APL cell differentiation

To investigate further the role of C/EBP β in APL cell differentiation, the effect of overexpression of a dominant-negative form of C/EBP was examined in U937-PR9 and NB4 cells. The dominant-negative C/EBP (A-C/EBP) contains an acid amphipathic helix juxtaposed to a 49 amino acid leucine zipper domain of C/EBP. This combination allows formation of stable heterodimer with other C/EBP proteins and inhibits C/EBP gene activation (Olive *et al.*, 1996). By using a retrovirus-mediated gene transfer system (Iwama *et al.*, 2002), we expressed A-C/EBP in PML-RARA-expressing cells. Following infection, cells were treated with ATRA and expression of the cell surface differentiation marker CD11b was evaluated (Figure 7). Cells transfected with the control empty vector or the green fluorescent protein (GFP)-negative population of A-C/EBP-infected cells exhibited an increase in

expression of the CD11b marker upon ATRA treatment. The GFP-positive population from the A-C/EBP-infected cells did not show an increase of CD11b expression after ATRA treatment in either zinc-pre-treated U937-PR9 or NB4 cells (Figure 7). These data suggest that disruption of C/EBP function leads to a block in ATRA-induced APL cell differentiation. To look specifically for the involvement of C/EBP β in the ATRA-regulated maturation process, we used DNA vector-based RNAi technology to suppress C/EBP β expression in NB4 cells. NB4 cells were transfected with the empty vector MSCV-U6 or the MCV-U6-beta vector, and transfected cells were selected in media containing puromycin. One month after selection, cells were treated with ATRA. As shown as Figure 8A, cells that had been transfected with MSCV-U6-beta had a significantly reduced level of C/EBP β expression in response to ATRA. The maturation state of the cells was assessed by morphology and we demonstrated a

Fig. 6. C/EBP β directs differentiation of a multipotent hematopoietic cell line. (A) β -Estradiol induces nuclear localization of the C/EBP β -ER fusion protein followed by granulocytic differentiation. (i) Stably transfected K562 cells were subjected to immunofluorescence using an anti-C/EBP β antibody after treatment for 24 h with vehicle only (left panel) or β -estradiol (right panel). Cell maturation was evaluated by morphology (ii) and by NBT reduction assay (iii) following 48 h of treatment with vehicle only (left panel) or β -estradiol (right panel). (B) Cell surface expression of CD11b was determined by FACS. Cells transfected with empty ER vector or with C/EBP β -ER were treated with vehicle only or with β -estradiol for 72 h. (C) RNA expression of the G-CSF receptor (upper panel) or C/EBP ϵ (lower panel) was assessed using northern blot analysis. As indicated in the figure, cells infected with empty vector (ER) or C/EBP β -ER were treated for different times with β -estradiol.

Fig. 7. Inhibition of ATRA-induced granulocytic differentiation by a dominant-negative C/EBP. U937-PR9 (left panel) and NB4 (right panel) cells were infected with a virus expressing the dominant-negative form of C/EBP, A-C/EBP (bottom two panels) or mock virus (top panels). The infected cells were treated with ATRA to induce differentiation. CD11b expression in the GFP-negative cell population (bottom panels) or GFP-positive cell population (top two panels) was examined by flow cytometry 72 h after induction of differentiation.

correlation between the low level of C/EBP β expression (Figure 8A) and an immature shape of the cells (Figure 8B) in response to ATRA. Quantification of maturation was obtained by NBT reduction assay, and a poor response of

the low C/EBP β -expressing cells to ATRA treatment could be confirmed (Figure 8C).

In summary, C/EBP β is the first C/EBP activity induced following ATRA treatment of APL cells (Figures 2 and 3), and induction of C/EBP ϵ by C/EBP β is not dependent on ATRA (Figure 6). These results support a primary role for C/EBP β in the differentiation of APL cells.

Discussion

Is C/EBP β activation necessary for normal granulopoiesis?

In this study, we have shown the importance of the role of C/EBP β in inducing granulocytic differentiation. The role of C/EBP β in myeloid cells was first determined by the study of *C/ebpβ*^{-/-} mice which present a defect in macrophage activation (Screpanti *et al.*, 1995; Tanaka *et al.*, 1995). However, the exact role of C/EBP β in myeloid differentiation is unclear. Depending upon which system is used, gain-of-function experiments have shown a role for C/EBP β in either monocytic differentiation, eosinophil maturation (Nerlov *et al.*, 1998) or, recently, in granulocytic differentiation (Popernack *et al.*, 2001; Iwama *et al.*, 2002; this study). We show that expression of C/EBP β in the multipotent cell line K562 leads to its preferential differentiation towards granulocytes, as indicated by cell morphology and gene expression induction. Interestingly, in this cell line without any ATRA treatment, C/EBP β induces C/EBP ϵ , an important mediator of granulocytic differentiation (Park *et al.*, 1999). One possibility would be that C/EBP ϵ is a target of C/EBP β and participates in the differentiation of the cells. In

Fig. 8. Reduction of C/EBP β expression induces reduction of ATRA-induced differentiation of NB4 cells. (A) C/EBP β expression was evaluated by northern blot after 4 h of ATRA treatment in two different pools of cells transfected with the RNAi vector MSCV-U6-beta (pool1 and pool2) in comparison with cells transfected with an empty vector MSCV-U6 (ev). Cell maturation after 5 days of ATRA treatment was evaluated by morphology using MGG staining (B) and/or by NBT reduction assay (C). Results are presented as the percentage of NBT-positive cells and correspond to a count of 500 cells.

addition, we present evidence that C/EBP β is enhanced and required in ATRA-induced differentiation of the APL cells. We show that inhibiting C/EBP activity and more specifically C/EBP β expression dramatically reduces the APL response to ATRA. In addition, during ATRA-induced APL cell differentiation, C/EBP β modulates granulocytic-specific genes and binds to the G-CSF receptor promoter *in vivo* (data not shown). However, it remains to be determined whether C/EBP β is indispensable for normal granulopoiesis, or whether what we observed in APL cells is specific to this cell pathology. C/EBP activity is known to be necessary for granulocytic differentiation, since our previous work showed that the loss of C/EBP α impairs granulopoiesis (Zhang *et al.*, 1997). The role of C/EBP α versus C/EBP β in granulopoiesis has not yet been reported. However, a gene replacement strategy has shown that when C/EBP β is expressed from the *C/ebp α* gene locus, C/EBP β can functionally replace C/EBP α to restore granulopoiesis (Jones *et al.*, 2002). Moreover, immortalized fetal liver *C/ebp α* ^{-/-} cells can give rise to granulocytes under interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation, and this granulocytic differenti-

ation is characterized by an increase in C/EBP β expression (Zhang *et al.*, 2002).

C/EBP β is targeted by PML-RARA

Downstream events leading to ATRA-induced differentiation of APL cells are still poorly understood. A list of potential ATRA target genes has been drawn from multiple studies (reviewed in Melnick and Licht, 1999), which have been complemented by reports using systematic analyses, leading to the identification of new potential ATRA target genes (Liu *et al.*, 2000). In spite of these efforts, the important target genes have yet to be identified. In this work, we provide evidence that a specific myeloid transcription factor, C/EBP β , is targeted by ATRA during APL differentiation. We provide evidence that C/EBP β is a very early response gene, directly regulated after 1 h of ATRA treatment. This induction is not dependent on a RARE in the C/EBP β promoter and does not require a normal RA transduction pathway. This probably explains why C/EBP β has not yet been described as an RA-responsive gene in normal cells, and that direct repeats of the RARE motif, (A/G)G(G/T)TCA, are not found in the C/EBP β proximal promoter. Our data reveal that ATRA induction of C/EBP β occurs only in the presence of the PML-RARA fusion protein, placing PML-RARA as the mediator of this effect. Thus, C/EBP β is a gene that is not regulated by a normal retinoic pathway but specifically targeted by the fusion protein PML-RARA. This novel finding supports the hypothesis that PML-RARA is an active member of the response of APL cells to ATRA and fits with the model stipulating that PML-RARA expression confers sensitivity to ATRA in myeloid cell lines (Ruthardt *et al.*, 1997).

One enigma posed by our data is how the PML-RARA fusion protein, which is involved in blocking cell differentiation by repressing transcription, could then become the stimulator of the differentiation by becoming a transcriptional activator. This process could be difficult to picture in the light of the extensive literature showing that PML-RARA is degraded upon ATRA treatment (Duprez *et al.*, 1996; Zhu *et al.*, 1999). However, we show that C/EBP β activation occurs as early as 1 h after ATRA stimulation and that within the first few hours of treatment, PML-RARA is still present in the cell and is not degraded. This confirms previous data showing that the degradation process is neither complete nor immediate after the addition of ATRA (Duprez *et al.*, 1996; Zhu *et al.*, 1999). PML-RARA has been shown to activate target promoters *in vitro* in response to ATRA, and this activation involves the formation of a stable multimeric complex involving the coiled-coil region of PML (Lin and Evans, 2000; Minucci *et al.*, 2000). More recently, evidence that PML-RARA binds directly to *in vivo* target promoters was reported (Di Croce *et al.*, 2002). These models support the hypothesis that PML-RARA can directly bind DNA sites via the RARA DNA-binding domain and repress transcription in an ATRA-reversible way. Our work illustrates the fact that PML-RARA activates a promoter in response to ATRA, and we were able to determine the region in the C/EBP β promoter that mediates PML-RARA activation. This activation is probably the result of an indirect effect of the fusion protein on this promoter as we could not demonstrate any RARE and

any direct binding of PML–RARA to the DNA element (data not shown). We hypothesize that this activation is achieved by PML–RARA-mediated modification of the activity of a transcription factor, which is important for the regulation of C/EBP β . This model is supported by previous observations showing that PML–RARA can modify transcription factor activity through direct interaction (e.g. Gata-2; Tsuzuki *et al.*, 2000) or by recruiting co-repressors and HDAC1 (e.g. Mad; Khan *et al.*, 2001). Additional studies aimed at identification of proteins binding to the C/EBP β promoter will be required in order to understand the mechanism of activation.

In summary, our data support a model in which C/EBP β can play an important role in granulocytic differentiation and, more specifically, at the promyelocytic stage of myeloid maturation. Our model suggests that in ATRA-stimulated APL cells, PML–RARA activates C/EBP β , providing a novel additional mechanism to explain the unique sensitivity of APL cells to ATRA. Identifying other genes activated by ATRA in association with PML–RARA and understanding how these genes are regulated may be especially useful for the treatment of relapsed APL patients.

Materials and methods

Constructs

An 800 bp DNA fragment from the C/EBP β promoter region encompassing bp –600 to +200 was amplified by PCR using genomic DNA from U937 cells as template with the following oligos: sense, 5'-TCCCGTCTGTGAAAATGGCAG-3'; antisense, 5'-ACCAGGCG-TTGCATGACG-3'. The cDNA product was cloned into a thymidine kinase minimal promoter luciferase reporter plasmid, PT81-LUC (Nordeen, 1988). BetaP0.3 and BetaP0.5 were generated by digesting the 800 bp promoter fragment using the internal restriction site *SacII*. P0.15 was generated by PCR using the oligos 5'-TTCACAAAT-AGCGGCCACC-3' and 5'-ACCAGGCGTTGCATGACG-3'. The unmodified ('empty') PT81-LUC was used as a negative control. A LUC reporter construct containing three copies of a RARE upstream of the tk promoter was used as a control for a RARE. PSG5-RARA, PSG5-PML–RARA and PML–RARA Δ 907 expression vectors have been described previously (Duprez *et al.*, 2000). PML–RARA Δ F was obtained by digesting full-length PSG5-PML–RARA with *KpnI* and *BamHI* (*KpnI* cuts in the PML part) and by replacing the digested fragment with a PCR-amplified PML–RARA which lacks the F domain. For retrovirus construction, a human C/EBP β cDNA fragment encoding the full-length protein was inserted in-frame with the murine estrogen receptor- α vector (Zhang *et al.*, 2002).

Cell culture and evaluation of cell differentiation

Human acute promyelocytic cell lines NB4, NB4-R2 and HT93 and human myelomonoblastic cell lines U937, U937-PR9 or U937-B412 were maintained at exponential growth in RPMI supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD). U937-PR9 and U937-B412 contain respectively PML–RARA cDNA and PLZF–RARA cDNA under the control of the zinc-inducible human metallothionein promoter (Ruthardt *et al.*, 1997). ATRA (Sigma Chemical Co., St Louis, MO) was used at final concentration of 1 μ M or as indicated in the figure legends, and cycloheximide (Sigma Chemical Co.) at a final concentration of 10 μ g/ml. When using ATRA and cycloheximide together, cycloheximide was added 30 min before ATRA treatment. For mRNA stability studies, actinomycin D was used at 10 μ g/ml and added for the times indicated in the figures. For the generation of the cell lines with conditional C/EBP β expression, K562 cells were electroporated with pBabePuro vector or pBabePuroC/EBP β -ER. The presence of the fusion protein C/EBP β -ER was screened in the different puromycin-resistant clones by western blot analysis using a C/EBP β -specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Three independent clones were analyzed. For virus infection, we used a producer cell line of the dominant-negative C/EBP virus, A-C/EBP (Iwama *et al.*, 2002). Between

2 and 4% of the cells were GFP positive 48 h after infection. Differentiation was evaluated by morphology using light microscopy of May–Grunwald–Giemsa staining and NBT staining or by flow cytometry analysis. We assessed the expression of the differentiation-related surface marker CD11b with a specific phycoerythrin (PE)-conjugated mouse monoclonal antibody (Pharmingen, San Diego, CA). Results were analyzed on a FACscan flow cytometer (Beckton Dickinson, Mountain View, CA) using Cellquest software.

Protein extracts, western blotting and EMSA

Nuclear proteins were extracted by an NP-40 detergent lysis procedure. For western blotting, nuclear extracts (20 μ g) were separated on 10% SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad). Proteins of interest were revealed with a C/EBP β monoclonal antibody or C/EBP ϵ polyclonal antibody (Santa Cruz Biotechnology) or with an antibody raised against the RARA F-region (Ab9 α -F, a gift from P. Chambon). To assess C/EBP binding activity, a double-stranded G-CSF receptor promoter oligonucleotide extending from bp –57 to –38 was used as a probe (Smith *et al.*, 1996). EMSA was performed by incubating 10 μ g of nuclear extract with 1 ng of the radiolabeled probe in binding buffer [10 mM HEPES pH 7.9, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol (DTT)] supplemented with 1 mg/ml bovine serum albumin (BSA), 10% glycerol and 0.5 μ g of poly(dI–dC) (Amersham, Piscataway, NJ) for 30 min at room temperature. For competition experiments, a 50-fold excess of unlabeled competitor oligonucleotide was added before addition of labeled oligonucleotide. For supershift experiments, 1.5 μ l of specific C/EBP α , C/EBP β or C/EBP ϵ antisera (Santa Cruz Biotechnology) were added before addition of the radiolabeled probe. Complexes were resolved in a 4% non-denaturing polyacrylamide gels in 1 $\times$ TBE.

Northern blot analysis

Northern blot analysis was performed as described previously (Duprez *et al.*, 1996). A fragment corresponding to bp 1427–1771 of the human C/EBP β cDNA was amplified by PCR and used as a probe. The C/EBP ϵ probe was a 0.5 kb *PstI* fragment of the *plurkat1* clone (Yamanaka *et al.*, 1997). The G-CSF receptor probe was a 0.7 kb *SacII*–*NdeI* fragment of the human G-CSF receptor cDNA.

Transient transfection and luciferase assays

Transactivation studies of the C/EBP β promoter were performed in U937 or U937-PR9 cells. Transfections were carried out using Effecten reagent (Qiagen, Valencia, CA). Total cDNA transfected per assay was 0.5 μ g: 0.4 μ g of the reporter plasmid and 0.1 μ g of a reference plasmid (CMV β -gal). PML–RARA was induced by adding 100 μ M ZnSO₄ to the culture medium 15 h before transfection. When expression vectors were added, total cDNA transfected per assay was 1 μ g: 0.5 μ g of the reporter plasmid, 0.25 μ g of an expression plasmid and 0.25 μ g of a reference plasmid (CMV β -gal). After the addition of DNA, cells were incubated in the presence or absence of ATRA at 2 μ M final concentration for an additional 15 h. Cell lysates were assayed for luciferase activity (Promega, Madison, WI) and β -gal activity (Tropix, Bedford, MA). Luciferase activity values are standardized against β -galactosidase activity obtained with the reference plasmid.

siRNA

For knocking-down C/EBP β expression in the NB4 cell line, we used DNA-based vector-delivered siRNA technology. The targeted sequence of C/EBP β was empirically determined in the C/EBP β coding sequence but was verified by BLAST searches to ensure the non-homology with other C/EBP genes. Based on the strategy described in Devroe *et al.* (2002), two complementary oligos were designed corresponding to nucleotides 1280–1300 of the C/EBP β mRNA sequence (accession number AY193834): oligo1, GGCCAACTTCTACTACGAGGAagcttCC-TCTCGTAGTAGAAGTTGGCCCTTTTTC; and oligo2, ATTCAAAA-AGGGCCAACTTCTACTACGAGGAagcttCCTCGTAGTAGAAGTT-GGCC. The oligos were annealed and inserted in a modified MSCV-U6 siRNA retroviral expression vector digested with *ApaI* and *EcoRI* (Devroe *et al.*, 2002). The inserted sequence was verified by sequencing. NB4 cells were electroporated with MSCV-U6-beta vector or MSCV-U6 empty vector and selected with puromycin (1 μ g/ml). Pools of cells were treated with ATRA and subjected to northern blot analysis or differentiation assay.

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References

- Borrow,J., Goddard,A.D., Sheer,D. and Solomon,E. (1990) Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science*, **249**, 1577–1580.
- Brown,D., Kogan,S., Lagasse,E., Weissman,I., Alcalay,M., Pelicci,P.G., Atwater,S. and Bishop,J.M. (1997) A PML/RAR α transgene initiates murine acute promyelocytic leukemia. *Proc. Natl Acad. Sci. USA*, **94**, 2551–2556.
- Castaigne,S., Chomienne,C., Daniel,M.T., Berger,R., Miclea,J.M., Ballerini,P. and Degos,L. (1990) Retinoic acids in treatment of acute promyelocytic leukemia. *Nouv. Rev. Fr. Hematol.*, **32**, 36–38.
- Chih,D.Y., Chumakov,A.M., Park,D.J., Silla,A.G. and Koeffler,H.P. (1997) Modulation of mRNA expression of a novel human myeloid-selective CCAAT/enhancer binding protein gene (C/EBP ϵ). *Blood*, **90**, 2987–2994.
- de The,H., Chomienne,C., Lanotte,M., Degos,L. and Dejean,A. (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature*, **347**, 558–561.
- de The,H., Lavau,C., Marchio,A., Chomienne,C., Degos,L. and Dejean,A. (1991) The PML–RAR α fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell*, **66**, 675–684.
- Devroe,E. and Silver,P. (2002) Retrovirus-delivered siRNA. *BMC Biotechnol.*, **2**, 15.
- Di Croce,L. et al. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science*, **295**, 1079–1082.
- Duprez,E., Lillehaug,J.R., Gaub,M.P. and Lanotte,M. (1996) Differential changes of retinoid-X-receptor (RXR α) and its RAR α and PML–RAR α partners induced by retinoic acid and cAMP distinguish maturation sensitive and resistant t(15;17) promyelocytic leukemia NB4 cells. *Oncogene*, **12**, 2443–2450.
- Duprez,E., Benoit,G., Flexor,M., Lillehaug,J.R. and Lanotte,M. (2000) A mutated PML/RARA found in the retinoid maturation resistant NB4 subclone, NB4-R2, blocks RARA and wild-type PML/RARA transcriptional activities. *Leukemia*, **14**, 255–261.
- Grignani,F. et al. (1998) Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia. *Nature*, **391**, 815–818.
- Grisolano,J.L., Wesselschmidt,R.L., Pelicci,P.G. and Ley,T.J. (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML–RAR α under control of cathepsin G regulatory sequences. *Blood*, **89**, 376–387.
- He,L.Z., Tribioli,C., Rivi,R., Peruzzi,D., Pelicci,P.G., Soares,V., Cattoretti,G. and Pandolfi,P.P. (1997) Acute leukemia with promyelocytic features in PML/RAR α transgenic mice. *Proc. Natl Acad. Sci. USA*, **94**, 5302–5307.
- He,L.Z., Guidez,F., Tribioli,C.D., Ruthardt,M., Zelent,A. and Pandolfi,P.P. (1998) Distinct interactions of PML–RAR α and PLZF–RAR α with co-repressors determine differential responses to RA in APL. *Nat. Genet.*, **18**, 126–135.
- Hu,H.M., Baer,M., Williams,S.C., Johnson,P.F. and Schwartz,R.C. (1998) Redundancy of C/EBP α , - β and - δ in supporting the lipopolysaccharide-induced transcription of IL-6 and monocyte chemoattractant protein-1. *J. Immunol.*, **160**, 2334–2342.
- Imaizumi,M. et al. (1998) Mutations in the E-domain of RAR portion of the PML/RAR chimeric gene may confer clinical resistance to all-trans retinoic acid in acute promyelocytic leukemia. *Blood*, **92**, 374–382.
- Iwama,A. et al. (2002) Reciprocal roles for CCAAT/enhancer binding protein (C/EBP) and PU.1 transcription factors in Langerhans cell commitment. *J. Exp. Med.*, **195**, 547–558.
- Jones,L.C., Lin,M.L., Chen,S.S., Krug,U., Hofmann,W.K., Lee,S., Lee,Y.H. and Koeffler,H.P. (2002) Expression of C/EBP β from the C/ebp α gene locus is sufficient for normal hematopoiesis *in vivo*. *Blood*, **99**, 2032–2036.
- Kakizuka,A., Miller,W.H., Jr, Umesono,K., Warrell,R.P., Jr, Frankel,S.R., Murty,V.V., Dmitrovsky,E. and Evans,R.M. (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcription factor, PML. *Cell*, **66**, 663–674.
- Kastner,P., Lawrence,H.J., Waltzinger,C., Ghyselinck,N.B., Chambon,P. and Chan,S. (2001) Positive and negative regulation of granulopoiesis by endogenous RAR α . *Blood*, **97**, 1314–1320.
- Khan,M.M. et al. (2001) Role of PML and PML–RAR α in Mad-mediated transcriptional repression. *Mol. Cell*, **7**, 1233–1243.
- Lekstrom-Himes,J. and Xanthopoulos,K.G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.*, **273**, 28545–28548.
- Lin,R.J. and Evans,R.M. (2000) Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol. Cell*, **5**, 821–830.
- Lin,R.J., Nagy,L., Inoue,S., Shao,W., Miller,W.H., Jr and Evans,R.M. (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*, **391**, 811–814.
- Liu,T.X. et al. (2000) Gene expression networks underlying retinoic acid-induced differentiation of acute promyelocytic leukemia cells. *Blood*, **96**, 1496–14504.
- Lutz,P.G., Houzel-Charavel,A., Moog-Lutz,C. and Cayre,Y.E. (2001) Myeloblastin is an Myb target gene: mechanisms of regulation in myeloid leukemia cells growth-arrested by retinoic acid. *Blood*, **97**, 2449–2456.
- McKnight,S. (2001) McBindall: a better name for CCAAT/enhancer binding proteins. *Cell*, **107**, 259–261.
- Melnick,A. and Licht,J.D. (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. et al. (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. *Mol. Cell*, **5**, 811–820.
- Mistry,R.A., Pedersen,E.W., Solomon,E. and Grimwade,D. (2003) The molecular pathogenesis of acute promyelocytic leukemia: implications for the clinical management of the disease. *Blood Rev.*, **17**, 71–79.
- Moog-Lutz,C. et al. (2001) PRAM-1 is a novel adaptor protein regulated by retinoic acid (RA) and promyelocytic leukemia (PML)–RAR α receptor α in acute promyelocytic leukemia cells. *J. Biol. Chem.*, **276**, 22375–22381.
- Naar,A.M., Boutin,J.M., Lipkin,S.M., Yu,V.C., Holloway,J.M., Glass,C.K. and Rosenfeld,M.G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell*, **65**, 1267–1279.
- Nerlov,C., McNaghy,K.M., Doderlein,G., Kowenz-Leutz,E. and Graf,T. (1998) Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. *Genes Dev.*, **12**, 2413–2423.
- Nordeen,S.K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques*, **6**, 454–458.
- Olive,M., Williams,S.C., Dezan,C., Johnson,P.F. and Vinson,C. (1996) Design of a C/EBP-specific, dominant-negative bZIP protein with both inhibitory and gain-of-function properties. *J. Biol. Chem.*, **271**, 2040–2047.
- Park,D.J., Chumakov,A.M., Vuong,P.T., Chih,D.Y., Gombart,A.F., Miller,W.H., Jr and Koeffler,H.P. (1999) CCAAT/enhancer binding protein ϵ is a potential retinoid target gene in acute promyelocytic leukemia treatment. *J. Clin. Invest.*, **103**, 1399–1408.
- Perez,A., Kastner,P., Sethi,S., Lutz,Y., Reibel,C. and Chambon,P. (1993) PML/RAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. *EMBO J.*, **12**, 3171–3182.
- Poli,V., Mancini,F.P. and Cortese,R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell*, **63**, 643–653.
- Popernack,P.M., Truong,L.T., Kamphuis,M. and Henderson,A.J. (2001) Ectopic expression of CCAAT/enhancer binding protein β (C/EBP β) in long-term bone marrow cultures induces granulopoiesis and alters stromal cell function. *J. Hematother. Stem Cell Res.*, **10**, 631–642.
- Radomska,H.S., Huettner,C.S., Zhang,P., Cheng,T., Scadden,D.T. and Tenen,D.G. (1998) CCAAT/enhancer binding protein α is a regulatory

- switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol. Cell. Biol.*, **18**, 4301–4314.
- Ruthardt,M., Testa,U., Nervi,C., Ferrucci,P.F., Grignani,F., Puccetti,E., Peschle,C. and Pelicci,P.G. (1997) Opposite effects of the acute promyelocytic leukemia PML–retinoic acid receptor α (RAR α) and PLZF–RAR α fusion proteins on retinoic acid signalling. *Mol. Cell. Biol.*, **17**, 4859–4869.
- Screpanti,I. *et al.* (1995) Lymphoproliferative disorder and imbalanced T-helper response in C/EBP β -deficient mice. *EMBO J.*, **14**, 1932–1941.
- Smith,L.T., Hohaus,S., Gonzalez,D.A., Dziennis,S.E. and Tenen,D.G. (1996) PU.1 (Spi-1) and C/EBP α regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood*, **88**, 1234–1247.
- Sutherland,J.A., Turner,A.R., Mannoni,P., McGann,L.E. and Turc,J.M. (1986) Differentiation of K562 leukemia cells along erythroid, macrophage and megakaryocyte lineages. *J. Biol. Response Mod.*, **5**, 250–262.
- Tanaka,T. *et al.* (1995) Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*, **80**, 353–361.
- Tenen,D.G., Hromas,R., Licht,J.D. and Zhang,D.E. (1997) Transcription factors, normal myeloid development and leukemia. *Blood*, **90**, 489–519.
- Tsuzuki,S., Towatari,M., Saito,H. and Enver,T. (2000) Potentiation of GATA-2 activity through interactions with the promyelocytic leukemia protein (PML) and the t(15;17)-generated PML–retinoic acid receptor α oncoprotein. *Mol. Cell. Biol.*, **20**, 6276–6286.
- Umesono,K., Murakami,K.K., Thompson,C.C. and Evans,R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell*, **65**, 1255–1266.
- Yamanaka,R., Kim,G.D., Radomska,H.S., Lekstrom-Himes,J., Smith,L.T., Antonson,P., Tenen,D.G. and Xanthopoulos,K.G. (1997) CCAAT/enhancer binding protein ϵ is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. *Proc. Natl Acad. Sci. USA*, **94**, 6462–6467.
- Zhang,D.E., Zhang,P., Wang,N.D., Hetherington,C.J., Darlington,G.J. and Tenen,D.G. (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α -deficient mice. *Proc. Natl Acad. Sci. USA*, **94**, 569–574.
- Zhang,P., Nelson,E.A., Radomska,H.S., Iwasaki-Arai,J., Akashi,K., Friedman,A.D. and Tenen,D.G. (2002) Induction of granulocytic differentiation by two pathways. *Blood*, **99**, 4406–4412.
- Zhu,J., Gianni,M., Kopf,E., Honore,N., Chelbi-Alix,M., Koken,M., Quignon,F., Rochette-Egly,C. and de The,H. (1999) Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor α (RAR α) and oncogenic RAR α fusion proteins. *Proc. Natl Acad. Sci. USA*, **96**, 14807–14812.

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