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Letters to the Editor

ΔN-p73 is a transcriptional target of the PML/RARα oncogene in myeloid differentiation

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Dear Editor,

The p53 paralog TPp73 gene gives rise to a variety of functionally distinct polypeptides involved in the control of growth arrest, apoptosis and differentiation. Multiple TA (transactivation competent, pro-apoptotic and anti-proliferative) p73 carboxy-terminal splicing isoforms (α, β, γ, δ, ε, ζ, η and η_1) are expressed from the P1p73 promoter (reviewed by Giombini *et al*[1] and references therein). A second intragenic promoter, P2p73, controls the expression of dominant-negative (ΔN) variants that lack the amino-terminal transactivation domain and act as dominant-negative repressors of p53- and p73-dependent apoptosis (reviewed by Giombini *et al*[1] and references therein). Changes in TAp73 and ΔN-p73 expression rather than inactivating mutations within the TP73 gene, have been described in many human tumors.[2] Unlike acute lymphoid leukemias, which mainly display promoter CpG islands hypermethylation, acute myeloid leukemia is characterized by a relative enrichment of shorter TAp73 isoforms, and in the case of acute promyelocytic leukemia (APL), a peculiar lack of ΔN-p73 expression has been reported by our group.[3–5]

In this study, we have investigated the molecular mechanisms underlying the transcriptional repression of the P2p73 promoter that leads to the severe reduction of ΔN-p73 expression in APL blasts. We identified the promyelocytic leukemia (PML)/RARα fusion protein as a direct regulator of the P2p73 transcriptional activity and showed that retinoic acid (RA) treatment relieves P2p73 repression *in vitro* and restores ΔN-p73 expression in APL patients *in vivo*. Notably, we found that ΔN-p73 expression acts as a pro-differentiation factor in APL cells.

Sequence analysis and binding-site scanning of the P2p73 promoter reveals, in addition to the well-characterized p53 and AP1 sites, the presence of five conserved putative RA-responsive element (RARE) sites (positions −5242 −5209; −4413 −4372; −3843 −3826; −3081 −3043; −2871 −2824) and several RARE half-sites (Figure 1a). By generating deletion mutants of the P2p73 promoter, we found that deletion of a large genomic fragment containing either the putative RARE elements (P2-2500) or several RARE half sites (P2-1500) results in an increase of the basal transcriptional activity of the P2p73 promoter (Figure 1b, left panel). The transcriptional activity of the P2–55 promoter deletion mutant returns to levels comparable to those of the full-length P2–5800 promoter, thus suggesting the presence of positive regulatory elements between positions –5800 and –1500 (Figure 1b, left panel). To analyze the transcriptional effects of the PML/RARα fusion protein on the P2p73 promoter, we performed transactivation assays using the deletion mutants described above (Figure 1a). We found that exogenously expressed PML/RARα severely downregulates the P2–5800, P2–2500 and P2–1500 promoter constructs, whereas it has no effect on the P2–55 promoter mutant (Figure 1b, right panel). By using crosslinked chromatin derived either from PR9 cells, a U937-derived cell line in which exogenous PML/RARα expression is Zinc-inducible, or from NB4 promyelocytic leukemia cells, we found that PML/RARα is recruited *in vivo* onto multiple sites in the P2p73 promoter (Figure 1a, right panel; Figure 1c). Altogether, these findings indicate that PML/RARα fusion protein binds to and inhibits the transcriptional activity of the P2p73 promoter.

Several *in vitro* and *in vivo* studies have shown that RA treatment blocks PML/RARα activity either by inducing changes in the composition of chromatin-bound PML/RARα complexes or by directing PML/RARα to degradation, thus relieving its repressive effects on RA target genes transcription.[6] Indeed, we found that RA treatment of PR9 cells determined a significant reduction of the PML/RARα fusion protein bound to the P2p73 promoter (Figure 1c, upper panel; Supplementary Figure 1). A similar pattern of PML *in vivo* binding to the ΔN-p73 promoter was also found in NB4 cells (Figure 1c, lower panel). It has been shown previously that the binding of PML/RARα to the promoter of its transcriptional target gene RARβ2 is not affected upon RA treatment.[7] The switch from repression to transcriptional activation has been related to the substitution of HDAC1-containing complexes with transcriptionally active PML/RARα-p300 protein complexes.[7] Although we cannot exclude that the small amount of PML/RARα still bound to P2p73 promoter (Figure 1c) may be engaged in transcriptionally active complexes, our results strongly suggest that the actual amount of PML/RARα recruited *in vivo* is critical for the transcriptional repression of the P2p73 promoter. Our findings could also be explained by the existence of different subsets of PML/RARα target genes whose transcriptional control is exerted through distinct molecular mechanisms. Furthermore, transactivation assays confirmed that the inhibitory effect of PML/RARα on the P2p73 promoter is reversed in the presence of RA (Figure 1d). Conversely, Zinc-inducible expression of PML/RARα in PR9 cells (Figure 1e, left panel) leads to a decrease of ΔN-p73 transcripts (Figure 1e, right panel), whereas RA treatment strongly induces ΔN-p73 mRNA expression

(Figure 1e, right panel). We next investigated the impact of exogenously expressed ΔN-p73 on NB4 cells survival and differentiation. We found that ΔN-p73-expressing cells acquire the membrane differentiation markers CD11b, CD11c and CD15 in the absence of RA exposure (Figure 1f, right panel; Supplementary Figure 2) and respond to RA both with rapid (i.e. after 24 hours), morphological changes (i.e., size reduction, decreased nucleo/cytoplasmic ratio and more condensed chromatin) (Figure 1f, left panel) and with higher levels of CD11b, CD11c, CD15 and CD54 antigens expression (Figure 1f, right panel; Supplementary Figure 2). In contrast to these marked effects on cell differentiation, ΔN-p73 expression did not modify NB4 cells proliferation and survival (data not shown).

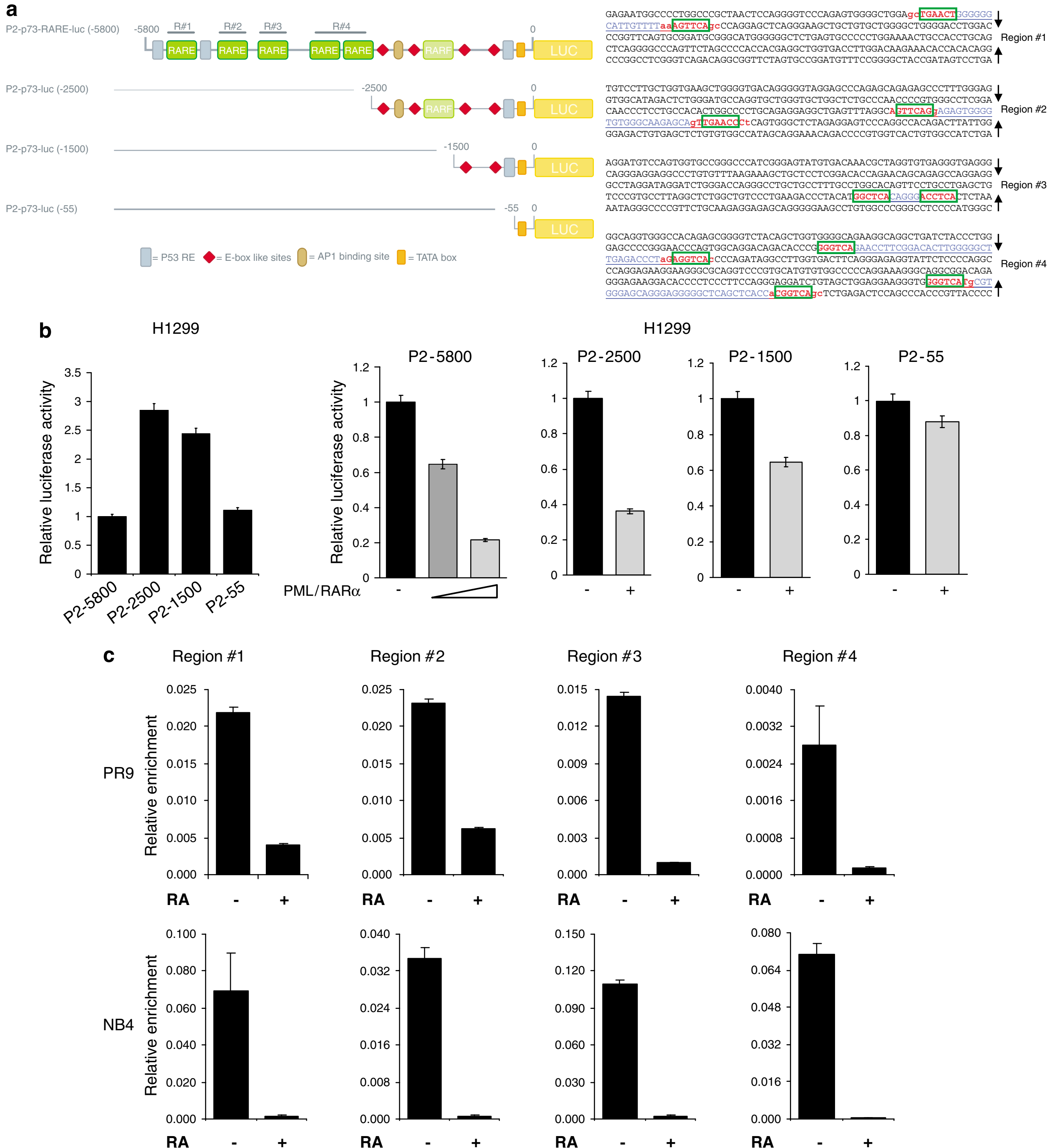
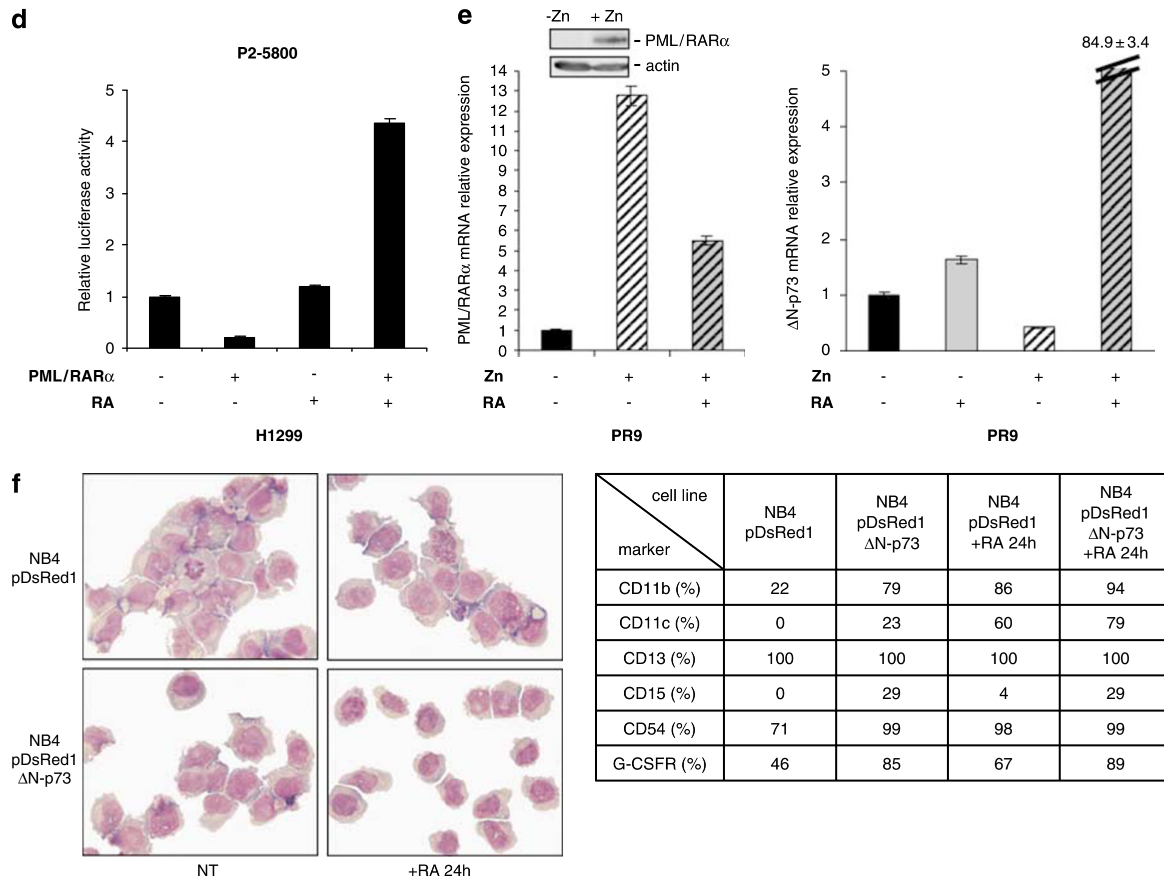


Figure 1

Finally, to further evaluate whether RA-mediated release of ΔN-p73 expression from PML/RARα transcriptional repression is recapitulated in APL patients, we analyzed ΔN-p73 mRNA expression in blasts from 22 APL patients before and after conventional RA treatment. In agreement with our previous observation,[4] we found that ΔN-p73 expression is



marker \ cell line	NB4 pDsRed1	NB4 pDsRed1 ΔN-p73	NB4 pDsRed1 +RA 24h	NB4 pDsRed1 ΔN-p73 +RA 24h
CD11b (%)	22	79	86	94
CD11c (%)	0	23	60	79
CD13 (%)	100	100	100	100
CD15 (%)	0	29	4	29
CD54 (%)	71	99	98	99
G-CSFR (%)	46	85	67	89

Figure 1 Effects of PML/RARα fusion protein and RA on P2-p73promoter. (**a**) The left panel shows a schematic representation of the P2p73-promoter luciferase reporter plasmids used in transient transfection assays. Putative RA-responsive elements, the E-box-like sites, the AP1-binding site, the p53-responsive elements (RE) and the TATA box position are indicated. R1, R2, R3 and R4 identify the P2p73 promoter regions containing the five conserved RAREs. The P2-p73-RARE-luc (–5800) reporter construct was obtained by cloning into the luciferase reporter plasmid pGL3-basic (Promega Inc), a 5.8-kb PCR fragment of the genomic region located upstream the putative transcription initiation site of ΔN-p73 mRNA. Deletion mutants were obtained using standard cloning techniques. Sequence analysis confirmed the identity with the sequence present in Genebank. Right panel: location of the RARE sites in the P2p73 promoter sequence. Canonical RARE half sites are indicated by boxes. The arrows show the promoter regions amplified by specific primer pairs. (**b**) Left panel: basal activity of the P2p73 promoter and its deletion mutants. H1299 cells were transiently co-transfected, using the calcium phosphate method, with the indicated luciferase reporter plasmids and with the Renilla-encoding pRL-null plasmid. At 24 h after transfection, cells were lysed and luciferase activity quantified using the Dual Luciferase Reporter kit (Promega Inc.). Renilla activity was used to normalize the transfection efficiency. Results are expressed as fold activation relative to the basal activity of P2p73-RARE-luc–5800. Error bars represent two standard deviations calculated on two independent experiments. Right panel: dose-dependent repression on the full-length P2p73 promoter by PML/RARα and its effects on the different promoter deletion mutants. H1299 cells were co-transfected with 0.5–1 μg of the pSG5-PML/RARα expression vector together with the indicated P2p73 promoter constructs. Results are expressed, after normalization to the renilla luciferase activity, as fold activation relative to the basal activity of each P2p73 deletion mutant. (**c**) Chromatin immunoprecipitation (ChIP) analysis of the P2p73 promoter in PR9 ZnSO4 (Zn^+) induced cells and NB4 promyelocytic cells before and after RA treatment. Quantitative real-time PCR analysis (qRT-PCR) of anti-PML immunoprecipitated chromatin in PR9 cells (upper panel) and NB4 cells (lower panel). Results are expressed as relative enrichment as compared to the input. Negative control (no antibody) values were subtracted from the corresponding samples. Standard curves and absolute quantification were obtained by serial dilutions of the input DNA samples. The oligonucleotide sequences for both conventional PCR and qRT-PCR are available upon request. (**d**) H1299 cells were transiently co-transfected with the P2-p73-RARE-luc (–5800) reporter construct with or without the PML/RARα expression vector. At 18 h after transfection, cells were either left untreated or treated with RA (2 μM) for 24 h. Cells were lysed and luciferase activity quantified as described in (**b**). Results are expressed as fold activation relative to the basal activity of P2-p73-RARE-luc–5800 promoter. (**e**) PML/RARα (left panel) and ΔN-p73 (right panel) mRNA levels in non-induced and Zn^+-induced (100 μM) PR9 cells either untreated or treated with RA (2 μM). cDNAs were amplified by qRT-PCR using primer pairs and TaqMan probe sets specific for PML/RARα or ΔN-p73. ABL mRNA transcript was used as control to correct for RNA quality differences. The sequence of primer pairs and probe sets are available upon request. Upper boxes in (**e**): immunoblotting analysis of PML/RARα induction by Zn^+ in PR9 cells. **(f)** Effect of ΔN-p73 expression on APL cell line NB4 differentiation. NB4 cells were electroporated either with an empty vector (pDsRed1, Clontech) or with the corresponding ΔN-p73 expression vector (pDsRed1-ΔN-p73) and analyzed for cell morphology (left panel) and membrane phenotype (right panel). Using a single-pulse protocol (voltage 260 V and capacitance 1050 F) and the Gene Pulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA), we consistently reached a transfection efficiency of 80% or more without significant reduction of cell viability (data not shown). At 24 h after transfection, cells were treated with RA for 1 or 2 days. In left panel, the morphological analysis of MGG-stained cytospin preparations of NB4-pDsRed1 and NB4-pDsRed1-ΔN-p73 cells either untreated (NT) or exposed for 24 hours to 2 μM RA (+RA, 24 h). Original magnification $\times$400. Table (right panel) of a representative flow cytometry analysis of untreated and RA (2 μM)-treated NB4-pDsRed1 and NB4-pDsRed1-ΔN-p73 cells (+RA, 24 h) stained either with irrelevant mouse Igs or with fluorochrome-labeled anti-CD11b, CD11c, CD13, CD15, CD54 and G-CSFR antibodies. Percentages indicate positive staining

very low in pre-treatment samples and it is strongly increased in 18 out of 22 patients (81.8%) after therapy (Supplementary Figure 3).

Altogether, our findings show that ΔN-p73 is a transcriptional target of the PML/RARα oncogene. This results in the transcriptional repression of ΔN-p73 providing one potential molecular basis underlying the lack of ΔN-p73 expression in a large subset of APL leukemias. The role of PML/RARα in ΔN-p73 repression is confirmed by the ability of RA to restore its expression both *in vitro* and *in vivo*. The observation that ΔN-p73 expression induces a number of differentiation markers in APL cells and cooperates with RA-induced differentiation *in vitro* suggests that ΔN-p73 might be necessary for proper myeloid differentiation. Indeed, ΔN-p73 expression is modulated during muscle and kidney differentiation.[8,9] Although ΔN-p73 has been mainly involved in the inhibition of p53-, TAp63- and TAp73-dependent transcription of target gene promoters containing p53REs, a series of recent evidences indicates that ΔN-p73 may directly and indirectly activate transcription from a number of target genes.[10] Thus, the ability of RA to remove the differentiation block of APL leukemias and to restore ΔN-p73 expression might result in the activation of a specific subset of yet unidentified genes involved in myeloid differentiation.

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Role of EndoG in development and cell injury

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Dear Editor,

Recent studies have presented contradictory evidence about the role of endonuclease G (EndoG) in early development and cell injury. While multiple data suggest that this enzyme is important in embryogenesis[1] and injury-induced cell death (see Supplementary Table 1), two recent reports claim that EndoG is dispensable in both processes.[2,3]

EndoG is currently identified as one of the most active cell death endonucleases. It is a nuclear DNA-coded mitochondrial endonuclease that has a unique site selectivity, initially attacking poly(dG).poly(dC) sequences in double-stranded DNA.[4] EndoG's expression varies among the tissues.[5] The enzyme was first localized in the intermembrane space of mitochondria, while later found to be tightly attached to the inner membrane of mitochondria.[6] Mammalian EndoG is synthesized as an inactive 32 kDa propeptide. The mitochondrial signal peptide is cleaved off by an unknown proteinase upon entering the mitochondria and the mature active 27 kDa EndoG can be released from mitochondria during apoptosis. Even though the protein does not have a known nuclear localization signal, it moves to the nucleus, where it cleaves DNA apparently without sequence specificity. Unlike other cell death endonucleases, EndoG can be often seen in the nucleus at the moment of DNA fragmentation as measured by the TUNEL assay.[7] EndoG is highly cytotoxic. Overexpression of extramitochondrially active EndoG in HeLa and CV1 cells induced cell death by acting alone, while the expression