The oncogenic ΔNp73 isoform contributes to aggressiveness in acute promyelocytic leukemia

The road to developing effective therapies against acute promyelocytic leukemia (APL) took a significant leap forward with the emergence of *all-trans*-retinoic acid (ATRA) and arsenic trioxide (ATO).[1] However, in low- and middle-income countries, the access to ATO as front-line therapy is limited,[2] thus maintaining the ATRA-chemotherapy regimen as the first option, with consequent prognostic implications for APL patients. In this scenario, a deeper understanding of the molecular mechanisms of ATRA sensitivity and the identification of biomarkers markers continues to be relevant.

Our group has previously described the association between higher *ΔNp73/TAp73* transcript ratios and the unfavorable treatment outcome in Brazilian patients treated with ATRA + chemotherapy.[3] Both transcripts are encoded by the *TP73* gene, which shares significant sequence homology with *TP53*.[4] Functionally, TAp73 and p53 have similar effects because of their transactivation domains (TAD), that have higher homology. In contrast, ΔNp73 lacks the TAD, and binds to DNA at the same regions of TAp73 and p53 but does not induce gene transcription of their targets. While the role of both isoforms in tumor aggressiveness is well known in solid tumors,[5,6] the functional consequences of ΔNp73 and TAp73 overexpression in leukemias are poorly understood. Here, we confirmed the prognostic role of p73 isoforms in APL patients treated with ATRA + chemotherapy and evaluated the effect of ΔNp73 and TAp73 expression on ATRA and ATO response using *in vitro* and *in vivo* models.

First, we analyzed diagnostic bone marrow (BM) samples of patients (N=98) enrolled in the International Consortium of Acute Promyelocytic Leukemia (IC-APL) from October 2006 through September 2011.[7] For comparison, BM samples from healthy individuals (N=14) without hematologic abnormalities were included. Written informed consent was obtained from all patients according to the Declaration of Helsinki (CAAE: 819878.5.1001.5440). When comparing patient BM samples with healthy BM, we found a reduced expression of *TAp73* and a high expression of *ΔNp73* in APL primary blasts (Figure 1A). Subsequently, we evaluated the prognostic significance of both isoforms in APL patients treated with ATRA + chemotherapy. We found that higher transcript levels of *ΔNp73* were associated with lower OS rates (Figure 1B). The OS rate at five years of patients with higher *ΔNp73* expression was 77.8% (95%CI: 68.5-88.4%) *versus* 96.6% (95%CI: 90-100%) in those with expression values below the cutoff. The frequency of early deaths was higher in the group of patients with higher *ΔNp73* expression values (*Online Supplementary Table S1*). On the other hand, *TAp73* expression was not associated with prognosis (Figure 1B) or with any clinical-epidemiological factors (such as sex and age) or genetic characteristic (*data not shown*).

To explore the difference of *TP73* isoforms in APL cells, we induced stable overexpression (OE) of the ΔNp73α or TAp73α isoforms in NB4 (ATRA-sensitive) and NB4-R2 cells (ATRA-resistant) by using a lentivirus system. A tagged GFP was included to express together the p73 isoforms in our strategy. Similar levels of gene expression of two isoforms were observed (*Online Supplementary Figure S1A*) in the modified cells. However, at the protein level, the abundance of ΔNp73α was superior to TAp73α in both cell lines (*Online Supplementary Figure S1B*), suggesting regulation at protein level. The cells harboring ΔNp73α presented higher proliferation rates compared to their controls, whereas no differences were observed for TAp73α over-expressing cells (*Online Supplementary Figure S1C*). We then evaluated whether these isoforms could regulate the apoptosis response, after treatment with ATO (1 μM) or ATO+ATRA (1 μM each). Overall, across the different time-points evaluated, we detected minor or non-significative differences in apoptosis induction on NB4 and NB4-R2 cells over-expressing ΔNp73α or TAp73α when compared to the empty vector control (*Online Supplementary Figure S1D, E*). These results contrast with our recent report analyzing primary APL blasts in which ΔNp73 OE was induced. Compared to controls, ΔNp73α OE blasts showed significantly lower rates of apoptosis induced by the *ex vivo* treatment with ATRA, ATO and ATRA+ATO.[8] These results may reflect differences between NB4 cell lines and primary cells, suggesting that the effect of ΔNp73α is more relevant in the latter.

Based on previously published data showing that TAp73 isoform is implicated in cell cycle arrest and apoptosis induced by cellular stress in cell lines of lung carcinoma,[9] we questioned whether increased TAp73α OE would restore the ATO sensitivity in ATO-resistant (ATOr) NB4 cell lines (carrying a PML S214L mutation). These cells were generated by long-term exposure to increasing concentrations of ATO, until cell death was inferior to 10%. TAp73α-OE in ATOr cells resulted in increased ATO-induced apoptosis, both in monotherapy and in combination with ATRA (*Online Supplementary Figure S1F*).

Next, we aimed to determine if p73 isoforms could affect the ATRA-induced myeloid differentiation. First, we quantified the expression of CD11b, CD14 and CD15 markers by flow cytometry in ATRA-treated NB4 cells. We found that ΔNp73α prevented the ATRA-induced up-

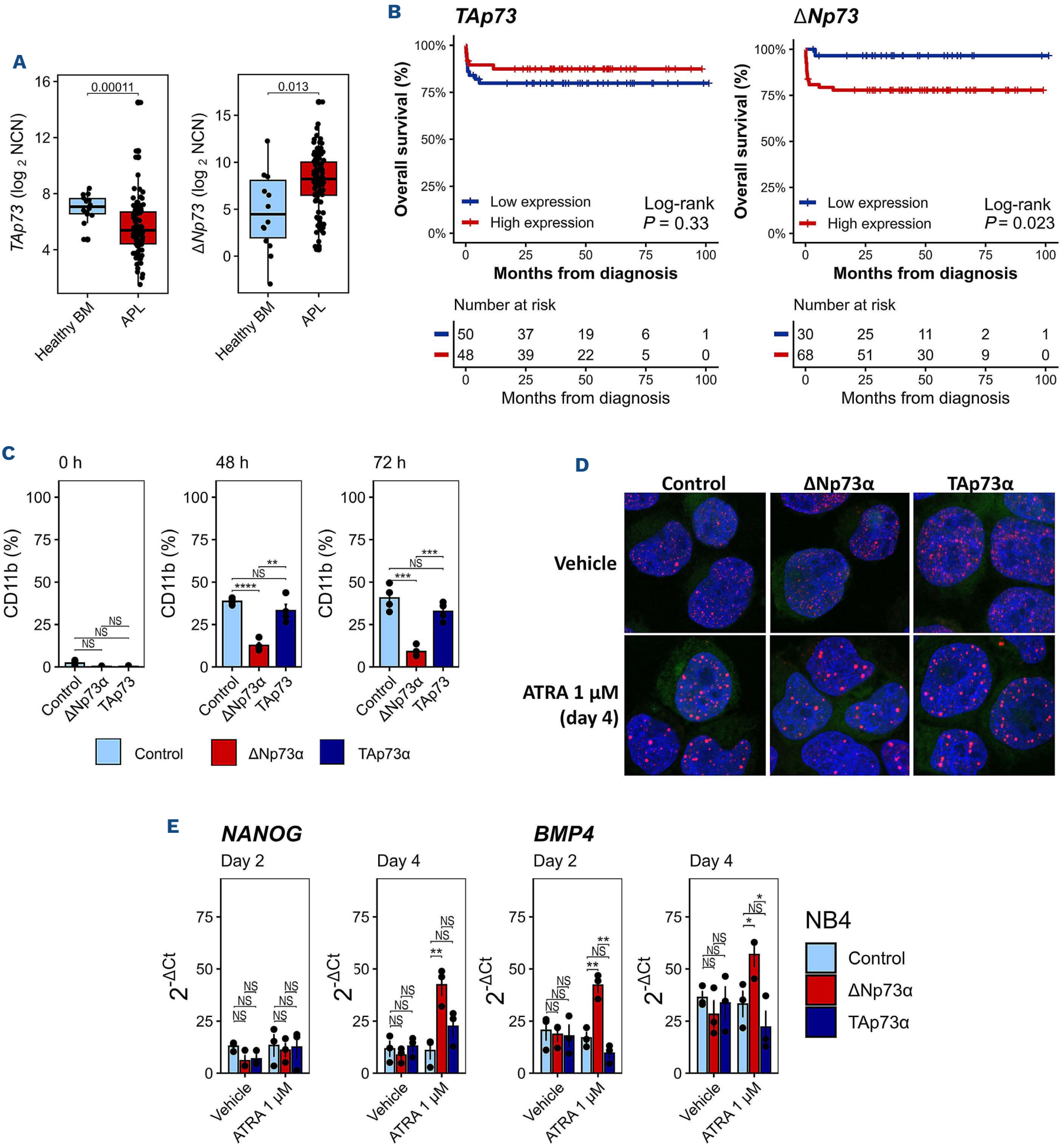

Figure 1. Expression levels, survival association and effect on ATRA-induced differentiation of *TAp73α* and *ΔNp73α* in acute promyelocytic leukemia. (A) Normalized copy numbers (NCN, numbers of copies of target gene/copies of *ACTB*) of *TAp73* (left) and *ΔNp73* (right) in acute promyelocytic leukemia (APL) samples and healthy bone marrow. (B) Overall survival (OS) (Kaplan-Meier) according to expression levels of *Tap73* (left) and *ΔNp73α* (right). The cutoffs used to establish patients with high and low expression were calculated from OS data by using the receiver operating characteristic (ROC) curve; these were 43.4 and 105.6 for *TAp73* and *ΔNp73*, respectively. (C) Percentage levels of the CD11b marker in ATRA-treated NB4 cells over-expressing p73 isoforms. (D) PML-nuclear body restoration evaluated by immunofluorescence microscopy after incubation with the PG-M3 monoclonal antibody in NB4 cells treated with ATRA for 96 hours (h). (E) *BMP4* and *NANOG* expressions were evaluated in NB4 cells. It was found that ΔNp73 modulates positively the BMP4/NANOG axis during ATRA treatment. NS: not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

regulation of CD11b, CD14, and CD15, suggesting possible disruption of ATRA-induced differentiation (Figure 1C, *Online Supplementary Figure S1G*). However, when we evaluated the restoration of PML nuclear bodies and cell morphology after ATRA exposure, no differences were observed in comparison with control cells, indicating that canonical ATRA effect was not affected (Figure 1D, *Online Supplementary Figure S1H*). In this line, p73 isoforms did not impact ATRA-induced expression of *CEBPA*, *CEBPE*, and *ELANE*, well-known ATRA targets involved in myeloid differentiation (*Online Supplementary Figure S2A*). Taken together, these results suggest that p73 isoforms do not affect ATRA-induced differentiation. Next, because it was recently described that the BMP4-ΔNp73-NANOG signaling cascade could increase the stem cell-like features of AML cells,[10] we measured the expression of *BMP4* and *NANOG*. We found that these genes were up-regulated in ΔNp73α-OE cells, but only during ATRA treatment, with no differences observed in untreated cells (Figure 1E), suggesting a possible role of this signaling axis in the ATRA response. In addition, ATRA treatment led to an increase in ΔNp73α protein levels (*Online Supplementary Figure S2B*), indicating that ΔNp73α gains protein stability in the context of ATRA treatment.

To deepen our understanding of how p73-dependent pathways could affect APL cells, we analyzed the total proteomic profile of ΔNp73α- or TAp73-NB4 cells, with or without ATRA exposure. Briefly, 100 μg of protein of each sample was subjected to reduction, alkylation, and overnight tryptic digestion. Then, peptides were purified, dried, and labeled with TMT reagents according to the manufacturer's protocol (Thermo Fisher Scientific). Labeled peptides were separated by nanoflow liquid chromatography and analyzed using an Orbitrap Tribrid Eclipse mass spectrometer (Thermo Fisher Scientific).[11] Protein identification and quantification were performed with MaxQuant, referencing the human proteome (Swiss-Prot, 20,366 entries, 2020).[12] Next, to evaluate the cellular pathways, enrichment scores were calculated based on Kolmogorov-Smirnov statistics after applying Spearman co-efficient as metric for ranking genes and tested for significance (false discovery rate <0.05) using 1,000 permutations. As a result, the top upregulation pathways in ΔNp73α-OE NB4 cells were related to the RNA splicing process, whereas these pathways were down-regulated when TAp73α was over-expressed (*Online Supplementary Figure S2C*). In contrast, this pathway was down-regulated during the ATRA treatment independent of the presence of the p73 isoforms (*Online Supplementary Figure S2D*). To look for protein interactions and individual proteins altered under different conditions, the proteins differentially abundant under different conditions were evaluated by STRING (https://string-db.org/).[13] Seven clusters of proteins were identified after comparing cells treated with ATRA with controls (Figure 2). This analysis confirms that p73 isoforms regulate splicing-related proteins (clusters 2 and 3), but also shows that some of the proteins are differently modulated by p73 isoforms even during ATRA treatment (cluster 2 and 3). Additionally, motor proteins (like DYNC1H1), clathrin-binding elements (CLTCL1, CLTC and COPG1), nucleotides and fatty acid metabolism enzymes (FASN and CAD), and proteins related to ion transporter (ATP1A1, ATP2A2, VDAC3 and SLC25A3) were down-regulated in ΔNp73α-OE cells while they were up-regulated in TAp73-OE cells. Finally, we found that NAP1L1 and ANP32E (cluster 5), proteins involved in chromatin accessibility during embryonic stem cell differentiation[14] by interaction with sox motifs,[14,15] are up-regulated in ΔNp73α-OE cells only during the ATRA treatment. This last finding is in line with the upregulation of *NANOG* observed in ATRA-treated ΔNp73α-OE cells. This suggests that, although ΔNp73α does not block the core molecular differentiation process induced by ATRA, it represses surface markers related to mature leukocytes and up-regulates stemness-associated factors upon ATRA exposure. This suggests that ΔNp73α could maintain a degree of stemness even under differentiation pressure.

To validate our data, we evaluated p73 isoforms in an APL transgenic murine model. A total of $1x10^4$ ΔNp73α or TAp73α OE GFP^+ murine APL blasts ($CD117^+Gr1^{dim}$) (Figure 3A) obtained from leukemic hCG-PML::RARA mice were transplanted intravenously to 8-week old NOD/SCID gamma (NSG) mice. Fourteen days post transplant, we divided the animals into 3 treatment groups: ATRA, ATRA+ATO, and control (Figure 3B). Males and females were used in similar proportions in all groups. Then, after 21 days of treatment, spleen and bone marrow tissues were analyzed by flow cytometry to assess leukemic burden by measuring GFP^+ cell content (Figure 3B). All procedures were performed under pathogen-free conditions and approved by the Animal Ethics Committee of the University of São Paulo (protocol no. 097/2018). As a result, we found a persistence of leukemic cells in BM and spleen after the ATRA treatment in animals transplanted with ΔNp73α over-expressing blasts (Figure 3C, D). In contrast, ATRA drastically reduced the leukemic burden in blasts over-expressing TAp73α or control cells. Of note, we observed a similar response to the combination of ATO plus ATRA regardless of which isoform was expressed.

Overall, our results validate the clinical relevance of TP73 isoforms in APL, confirming that high expression of ΔNp73α is associated with lower OS and a higher rate of early death. Our *in vivo* model corroborates that ΔNp73α favors disease persistence following ATRA treatment, conferring a resistance phenotype not observed with the TAp73 isoform. Mechanistically, this persistence is not driven by blocking canonical granulocytic differentiation. Unlike other molecular alterations such as FLT3-ITD,[16] ΔNp73α prevents neither the restoration of PML nuclear bodies nor morphological differentiation, indicating a mechanism

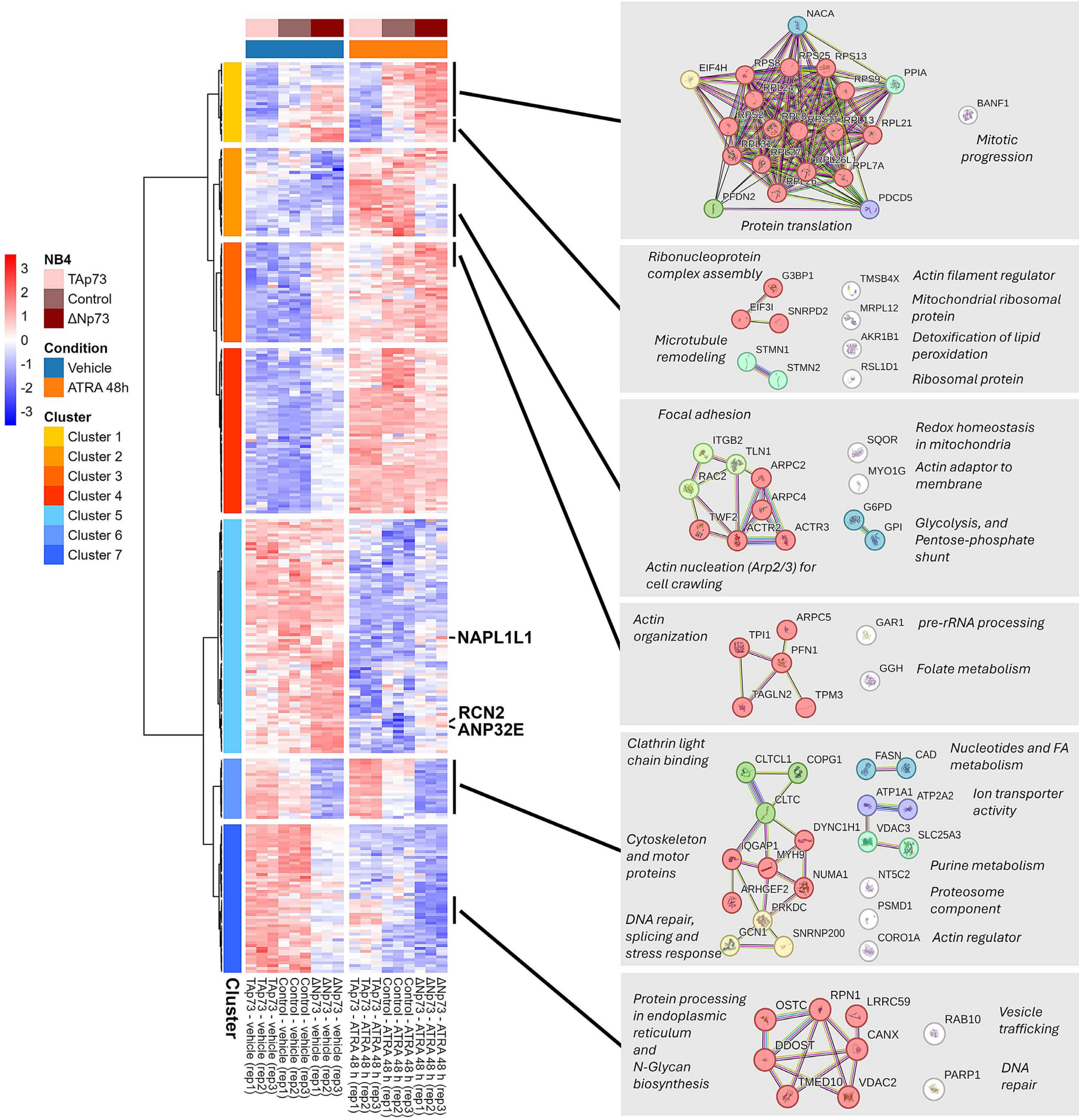

Figure 2. Proteomic profiling and protein-protein interaction networks regulated by p73 isoforms in ATRA-treated NB4 cells. Hierarchical clustering heatmap showing the total proteomic profile of NB4 cells with stable overexpression of TAp73α, ΔNp73α, or empty vector control, treated with either vehicle or ATRA (1 μM) for 48 hours (h). The color scale represents row-scaled protein abundance (Z-score), with red indicating high expression and blue indicating low expression. Seven distinct protein clusters based on expression patterns are identified by the color bars on the left. Specific proteins involved in chromatin accessibility and stress response, such as NAP1L1, RCN2, and ANP32E, are highlighted with arrows. The diagrams on the right show STRING protein-protein interaction networks derived from selected clusters, illustrating enriched biological processes and functional pathways differentially modulated by p73 isoforms and ATRA.

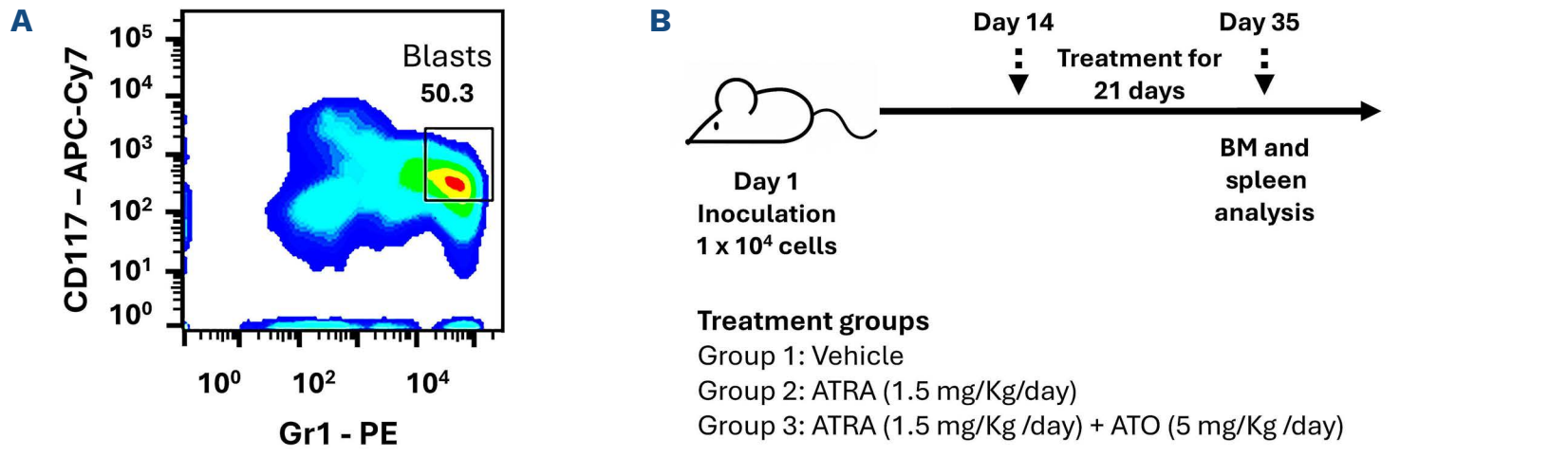

Figure 3. ΔNp73α reduces blast clearance induced by ATRA in an acute promyelocytic leukemia mice model. (A) To evaluate the effect of p73 isoforms *in vivo*, over-expressing ΔNp73α or TAp73α, or control acute promyelocytic leukemia (APL) blasts (from leukemic hCG-PML-RARA) were transplanted to NSG mice. After lentiviral infection, the modified cells were enriched by sorting the GFP⁺ cells, achieving more than 98% of purity. CD117⁺ cells were the main cell population in the GFP⁺ fraction. (B) Treatment scheme and groups of animals evaluated in the experiment. (C and D) Leukemic burden, evaluated by GFP⁺ cells, in bone marrow and spleen after 21 days of treatment, showing that ΔNp73α reduces the blast-clearance induced by ATRA in comparison with the control group. NS: not significant; *P<0.05.

independent of the PML::RARA fusion. However, we observed that ATRA paradoxically stabilizes ΔNp73α, which agrees with the promotion of expression of proteins related to stem-like phenotype only under the pressure of ATRA treatment. This suggests that while ATRA triggers differentiation signaling, in APL cases with higher expression of ΔNp73α, it can activate stemness and alternative splicing programs, allowing cells to survive despite differentiation pressure. These findings highlight the importance of ΔNp73α not only as a prognostic biomarker, but also as a key regulator of cellular plasticity.

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https://doi.org/10.3324/haematol.2025.288595

Received: September 8, 2025.
Accepted: May 19, 2026.
Early view: May 28, 2026.

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Disclosures

No conflicts of interest to disclose.

Contributions

EMR conceived and designed the study, performed experiments, analyzed and interpreted data, performed the statistical analyses, drafted the article, and gave final approval of the submitted version. CAOR, DAP-M, EA, AB and FG conceived and designed the study, performed experiments, analyzed and interpreted data, performed the statistical analyses, and drafted the article. CHT, GAF, TB, IW, and CLAS conceived and designed the study, and reviewed the paper. GH, SP, VMF, JJS, FT and LLFP reviewed the paper. FT, LLFP, ASG, PSS, ABG, KP and ECN provided patient samples and clinical data, and reviewed the paper.

Funding

This investigation was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant #2013/08135-2). CAOR received a fellowship from FAPESP (Grant #2023/02952-0). DAP-M received a fellowship from FAPESP (Grant #2017/23117-1). IW received a fellowship from FAPESP (Grant #2015/09228-0). IW and DAP-M were sponsored by the Abel Tasman Talent Program (ATTP) of the Graduate School of Medical Sciences of the University of Groningen/University Medical Center Groningen (UG/UMCG), The Netherlands.

Data-sharing statement

The data generated from this work can be shared on request to the authors.

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