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Synergistic effects of PRIMA-1^{Met} (APR-246) and 5-azacitidine in *TP53*-mutated myelodysplastic syndromes and acute myeloid leukemia

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ABSTRACT

Myelodysplastic syndromes and acute myeloid leukemia with *TP53* mutations are characterized by frequent relapses, poor or short responses, and poor survival with the currently available therapies including chemotherapy and 5-azacitidine (AZA). PRIMA-1^{Met} (APR-246, APR) is a methylated derivative of PRIMA-1, which induces apoptosis in human tumor cells through restoration of the transcriptional transactivation function of mutant p53. Here we show that low doses of APR on its own or in combination with AZA reactivate the p53 pathway and induce an apoptosis program. Functionally, we demonstrate that APR exerts these activities on its own and that it synergizes with AZA in *TP53*-mutated myelodysplastic syndromes (MDS) / acute myeloid leukemia (AML) cell lines and in *TP53*-mutated primary cells from MDS / AML patients. Low doses of APR on its own or in combination with AZA also show significant efficacy *in vivo*. Lastly, using transcriptomic analysis, we found that the APR + AZA synergy was mediated by downregulation of the FLT3 pathway in drug-treated cells. Activation of the FLT3 pathway by FLT3 ligand reversed the inhibition of cell proliferation by APR + AZA. These data suggest that *TP53*-mutated MDS / AML may be better targeted by the addition of APR-246 to conventional treatments.

Introduction

Myelodysplastic syndromes (MDS) are malignant bone marrow disorders characterized by ineffective hematopoiesis leading to refractory cytopenias, and by an increased risk of progression to acute myeloid leukemia (AML).[1] They are prognostically stratified on the basis of the percentage of blasts in the bone marrow, the karyotype, and the number of cytopenias present according to an International Prognostic Scoring System (IPSS),[2] which was recently revised (revised IPSS, IPSS-R).[2] Current studies are integrating data on somatic gene mutations into prognostic indices to further refine risk stratification.[3-6] At the genetic level, it is now widely recognized that most of the clinical and pathological features of MDS and AML are the direct result of recurrent acquired somatic genetic lesions. Among these, *TP53* gene mutations have been shown to occur in 5-10% of all MDS and AML cases,[3-5,7-9] including 20-25% of the low-risk MDS with isolated del 5q,[7] and 40-50% of the MDS and AML with complex karyotypes.[8-10] Among MDS with complex karyotypes, the presence of *TP53* mutations has been correlated with a lower number of other mutations and a poorer outcome.[11] Furthermore, the allelic burden of the *TP53* mutations has been

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shown to be a strong predictor of poor overall survival.[12] Mutations of p53 proteins generally result in a loss of their normal functions (including cell cycle and apoptosis control), but can also result in a deleterious "gain of function". In addition, at least in case of complex karyotypes, *TP53*-mutated MDS/AML generally acquire del17p, i.e. loss of the remaining *TP53* allele. *TP53* mutations correlate with poor overall outcomes in MDS and AML, independently[4,5,13] or not independently[14] of a complex karyotype. *TP53*-mutated MDS/AML are associated with resistance to treatment, including anthracycline + aracytine combinations, low-dose cytarabine,[14] and allogeneic bone marrow transplantation (BMT),[15] while the hypomethylating agents (HMA) 5-azacitidine (AZA)[7,15] and decitabine[16] yield somewhat better, albeit transient, results. For this reason, HMA are generally considered to be the first-line treatment of MDS/AML with a *TP53* mutation.

As with many other types of cancer, mutated p53 protein, therefore, appears to be one of the most important therapeutic targets in MDS and AML, and restoration of its biological function could prove to be highly beneficial. PRIMA-1^{Met} (APR-246, APR) is a methylated derivative of PRIMA-1, a compound that induces apoptosis in human tumor cells through restoration of the transcriptional transactivation function to mutant p53.[17] Mechanistically, APR is a prodrug that forms an active moiety that covalently binds to thiol groups of the core domain of mutated p53 protein,[18] thereby resulting in a structural change that restores its active conformation. APR induces dose-dependent apoptosis in various tumor models, mostly in combination with other drugs.[19-28] A phase I/II clinical trial combining AZA and APR in *TP53*-mutated MDS/AML is ongoing at US centers and at our center.[29-31]

In this study, we show that APR is efficient on its own, while it also synergizes with AZA in *TP53*-mutated MDS/AML cell lines and in *TP53*-mutated primary cells from MDS/AML patients. We also identified a functional pathway involved in the synergy between these two drugs.

Methods

Reagents and drugs

APR was provided by Aprea Therapeutics AB, Stockholm, Sweden, and kept frozen (-20°C) as a stock solution. AZA was provided by Celgene Corporation (Summit, NJ, USA) and was kept frozen at -80°C as a stock solution. As previously described,[32] AZA was added once at day 0 of cell culture and APR was used in the same manner. FLT3 ligand (FLT3-L) was from PeproTech (Neuilly-sur-Seine, France).

Cell lines and primary cells

The MDS cell line SKM1 (kindly provided by Thomas Cluzeau, University Hospital of Nice, France) is an MDS-derived cell line that has undergone detailed characterization in terms of its phenotype and genotype.[33] We confirmed the presence of a homozygous mutation of *TP53* (p.R248Q). SKM1 cells were grown in RPMI 1640 medium (GIBCO; Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% FBS (GIBCO), 1% Penicillin/Streptomycin (P/S), and 1% GlutaMAX. All of the other leukemic cell lines (K562, KG1a, THP-1, and HL60) were cultured under the same conditions. Cells in the logarithmic phase of growth that had been seeded at a density of $2x10^5$/mL were used for all of the experiments.

Primary cells from MDS and AML patients with complex karyotypes, del5q, or known or suspected to have mutated *TP53* were provided by the Service d'Hématologie of the Hôpital Saint-Louis, Paris, France, after informed consent had been received. Finally, primary cells were isolated from healthy blood donors as controls. This study was approved by the Groupe Francophone des Myélodysplasies (GFM) review board.

Cytotoxicity assay

The following concentrations were used for each drug: APR from 10^{-8} M to 10^{-5} M and AZA from 10^{-8} M to 10^{-5} M. Proliferating cells were plated, incubated for three days, and then counted. Experiments were performed at least in triplicate. FLT3-ligand was added at final concentrations varying from 0.1 to 100 ng/mL for specific experiments.

Clonogenic assays

Due to partial blastic infiltration in the majority of samples, as generally reported in MDS/AML with a complex karyotype and *TP53* mutation,[9] liquid cultures were not consistently successful. We used semi-solid cultures to test for the impact of drugs on progenitor clonogenicity. Briefly, $3x10^5$ peripheral blood mononuclear cells (PBMC) or $1x10^5$ bone marrow-derived mast cells (BMMC) were cultured in cytokine-containing methylcellulose (MethoCult™, 84434, STEMCELL Technologies) in the presence of the IC_{50} of each drug (0.5 μM APR or 3 μM AZA) on their own or in combination. After 14 days at 37°C, the erythroid and myeloid colonies were counted. For patients with *TP53* mutations, isolated colonies were picked, and the DNA was extracted and Sanger sequenced to determine the *TP53* genotype at the single progenitor level.

Statistical analysis

All of the results are expressed as means±standard deviation (SD). All of the single-parameter measurement comparisons were determined using the Mann-Whitney test (PRISM 18964 software, GraphPad, La Jolla, CA, USA). All tests were two-sided; $P<0.05$ was considered statistically significant. Combination indices were calculated by CompuSyn software according to the Chou-Talalay method for drug synergy studies.

Results

APR inhibits the proliferation of myeloid cell lines

To evaluate the efficacy of APR-mediated growth inhibition of MDS/AML cells, we tested increasing doses of the drug on a series of MDS/AML cell lines with mutant forms of the *TP53* gene (i.e. SKM1, K562, KG1a, THP-1, and HL60 cells). We first verified that all of these cell lines harbor *TP53* mutations. The SKM1 cell line derived from an MDS patient carries the recurrent homozygous *TP53* mutation p.R248Q (c.743G>A). The four other cell lines carry a frameshift or a splicing mutation (K562: c.406dupC, HL60: c.1_1182del1182, KG1a: c.672+1G>A, or THP-1: c.520_545del26). The p53 protein was detectable in the SKM1 cell line only (*Online Supplementary Figure S1*). Treatment with APR led to a pronounced and dose-dependent reduction in cell proliferation of all of the cell lines tested (Figure 1A), with 50% inhibitory concentrations (IC_{50}) that were in the micromolar range (Figure 1B). Of note, the IC_{50} was at least 2-fold lower for the SKM1 cell line compared to the other AML lines (Figure 1B and *Online Supplementary Table S1*).

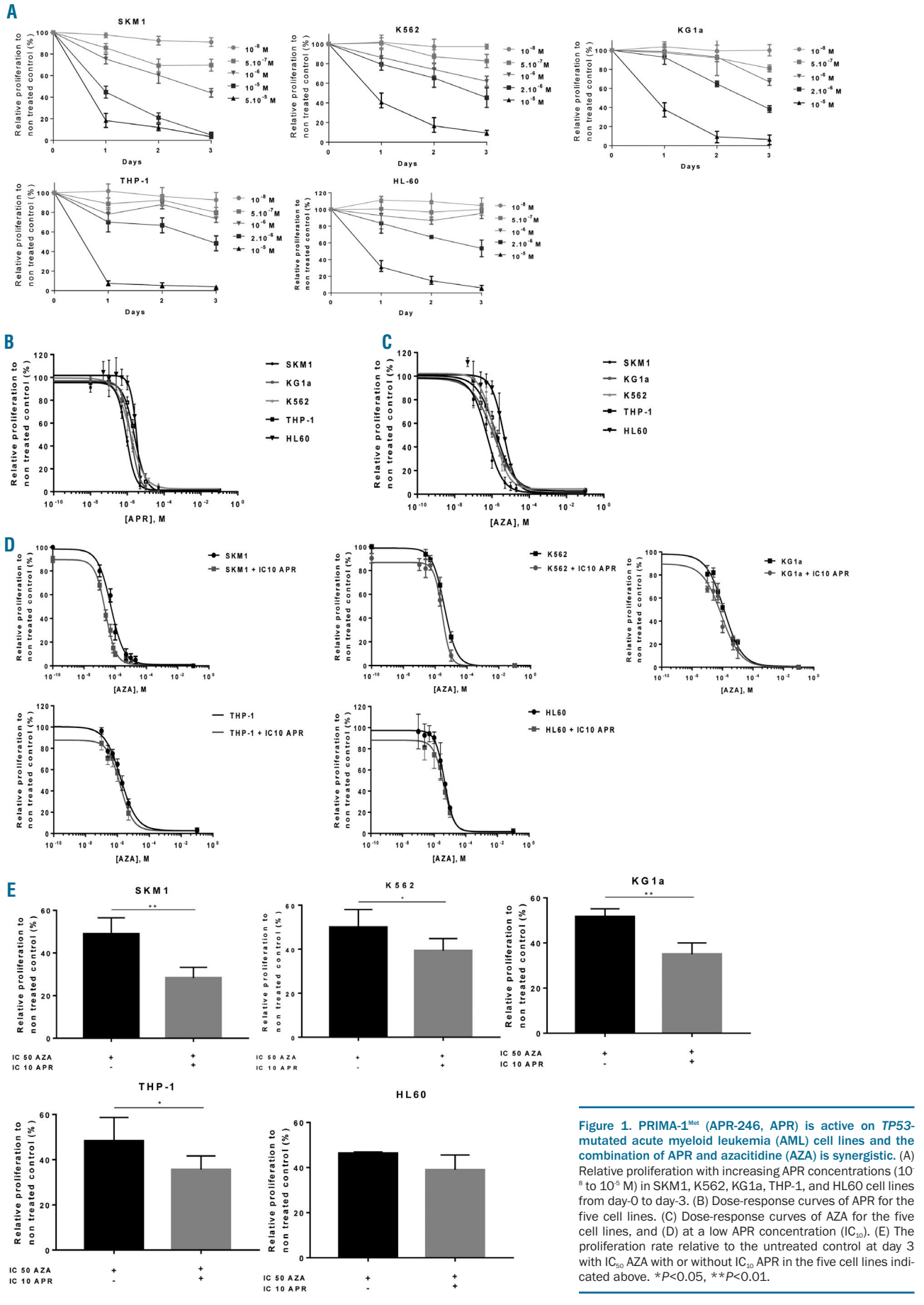

Figure 1. PRIMA-1^{Met} (APR-246, APR) is active on *TP53*-mutated acute myeloid leukemia (AML) cell lines and the combination of APR and azacitidine (AZA) is synergistic. (A) Relative proliferation with increasing APR concentrations (10^{-8} to 10^{-5} M) in SKM1, K562, KG1a, THP-1, and HL60 cell lines from day-0 to day-3. (B) Dose-response curves of APR for the five cell lines. (C) Dose-response curves of AZA for the five cell lines, and (D) at a low APR concentration (IC_{10}). (E) The proliferation rate relative to the untreated control at day 3 with IC_{50} AZA with or without IC_{10} APR in the five cell lines indicated above. **P*<0.05, ***P*<0.01.

The combination of APR and AZA synergistically reduces the proliferation of myeloid cell lines

AZA is considered to be a first-line treatment for most patients with high-risk MDS, but its effect is relatively limited in MDS patients who have *TP53* mutations. After we evaluated the activity of AZA on *TP53*-mutated AML lines (IC_{50} ranging from $0.54x10^{-6}$ M for SKM1 to $4.5x10^{-6}$ M for HL60 cells) (Figure 1C), we investigated whether its efficacy could be enhanced by combination with APR. The addition of low doses of APR (IC10) (*Online Supplementary Table S1*) to increasing doses of AZA reduced the proliferation of the five cell lines compared to AZA on its own, with the exception of HL60 cells (Figure 1D and E). SKM1 cells were the most sensitive to the combination, as the IC_{50} of AZA in these cells was significantly reduced in the presence of low doses of APR. This synergistic effect was confirmed at different concentrations of each drug by calculating the combination index (CI < 1) for all of the tested cell lines, but not at low concentrations of both drugs (i.e. both drugs at their IC_{10}) (*Online Supplementary Table S1*).

The combination of APR and AZA promotes G0/G1 cell cycle arrest and apoptosis

To elucidate the mechanisms underlying the antiproliferative effect of the combination compared with each drug on their own, we performed apoptosis and cell cycle studies using low dose APR (i.e. at the IC_{10}) with a standard dose of AZA (i.e. at the IC_{50}). Other associations are shown in the *Online Supplementary Figures S2-S4*. Under these conditions, APR alone did not induce apoptosis, while AZA on its own induced low levels of apoptosis in some of the cell lines. The combination of APR with AZA significantly increased apoptosis compared to the individual drugs in every cell line that was tested. This effect was more pronounced in SKM1 cells (39% apoptotic cells with APR + AZA *vs*. 19% with AZA alone, $P<0.01$) (Figure 2A-E).

We then analyzed the cell cycle distribution after treatment with IC_{10} APR, IC_{50} AZA, or the combination of these two drugs at these concentrations. At this low concentration, APR on its own did not affect the cell cycle distribution of the various cell lines tested, while AZA tended to increase the proportion of cells in the G0/G1 phase. However, SKM1 cells treated with the combination of AZA and APR underwent cell cycle arrest in G0/G1 as early as 24 hours after exposure (83% of the cells were in G0/G1 phase following APR + AZA treatment *vs*. 62% for APR-treated cells, $P<0.01$). In parallel, the proportion of cells in the S and G2/M phases was significantly reduced (Figure 2A). Similar results, although less pronounced, were observed in the other cell lines (Figure 2B-E), while various combinations of these drugs confirmed the observed synergism between the two drugs in terms of cell cycle arrest and the induction of apoptosis (*Online Supplementary Figures S2-S4*). Altogether, although the effect is more pronounced on apoptosis than on cell cycle, these results suggest that the addition of APR to AZA in various *TP53*-mutated cell lines potentiates the AZA antiproliferative effect by increasing G0/G1 arrest and a pro-apoptotic effect.

APR is active on primary cells of *TP53*-mutated MDS/AML samples

We then investigated the effect of these drugs on primary cells from MDS/AML patients. Of the 34 patients included in our study, 15 patients had mutations in the *TP53* gene (10 had a complex karyotype, 2 had an isolated 5q deletion, and 14 had deletion of the other *TP53* allele). All mutations were located in the DNA-binding domain hotspots (DBD) (Table 1).

We performed clonogenic assays in semi-solid medium using the previously determined IC_{50} for SKM1 cells. AZA on its own at 3 μM induced only a modest reduction (30%) of myeloid and erythroid colony growth, while APR at 0.5 μM significantly inhibited colony growth by approximately 60% (Figure 3A and B).

We then sought to determine whether there was a targeted effect on cells from patients with mutated *TP53*. When compared to AZA on its own, the drug combination always had a greater inhibitory effect on the growth of myeloid or erythroid progenitor colonies, irrespective of the *TP53* genotype of the patients (*Online Supplementary Figure S5*). However, when compared to APR on its own, the APR + AZA combination significantly reduced the number of myeloid and erythroid colonies formed by progenitors derived from patients with mutated *TP53*, while this was not observed with wild-type *TP53* patients (Figure 3C and D). This indicates that the drug combination may be more beneficial to patients with *TP53* mutations. However, the molecular analysis of the residual colonies from four patients with mutated *TP53*, irrespective of the treatment conditions, only revealed mutant *TP53* colonies (Table 2 and *data not shown*), thus making it impossible to conclude that cells with mutated p53 are targeted specifically. To further study the specificity of the combination, we performed liquid cultures of $CD34^+$ cells isolated from *TP53*-mutated or wild-type MDS/AML patients and studied proliferation and apoptosis when treated by APR and/or AZA. A significant efficacy of the combination compared to drugs given alone was observed in *TP53*-mutated patients only (Figure 4A-D). Accordingly, $CD34^+$ cells isolated from healthy blood donors did not show increased sensitivity to the combination compared to isolated drugs (Figure 4E and F).

In vivo efficacy of APR and AZA in a xenotransplantation model

In order to evaluate the *in vivo* antiproliferative effect of APR and AZA, we developed a xenograft model of SKM1-Luciferase cells in NSG mice that allowed us to use bioluminescence to measure tumor volumes before and after APR and AZA treatment. Intravenous injections of 10^7 cells yielded highly reproducible tumor engraftment and growth over time (100% of the mice had engraftment) (Figure 5A). The mice were treated with the drugs as soon as the tumor bioluminescence signal reached 10^6 p/sec/cm^2/sr.

Treatment with low doses of APR in combination with AZA resulted in pronounced inhibition of disease progression as early as four days after starting the drug treatment compared to APR or AZA treatment on their own (median tumor volume: $91.1x10^6$ p/sec/cm^2/sr in untreated mice, $64.4x10^6$ p/sec/cm^2/sr with APR on its own, $103.2x10^6$ p/sec/cm^2/sr with AZA on its own *vs*. $18.9x10^6$ p/sec/cm^2/sr mm^3 in the APR + AZA group; $P<0.05$) (Figure 5A). There was still a beneficial impact of the drug combination after eight days of treatment (Figure 5B). These results confirmed *in vivo* the efficacy of the combination of a low dose of APR with standard AZA doses that we had previously shown *in vitro*.

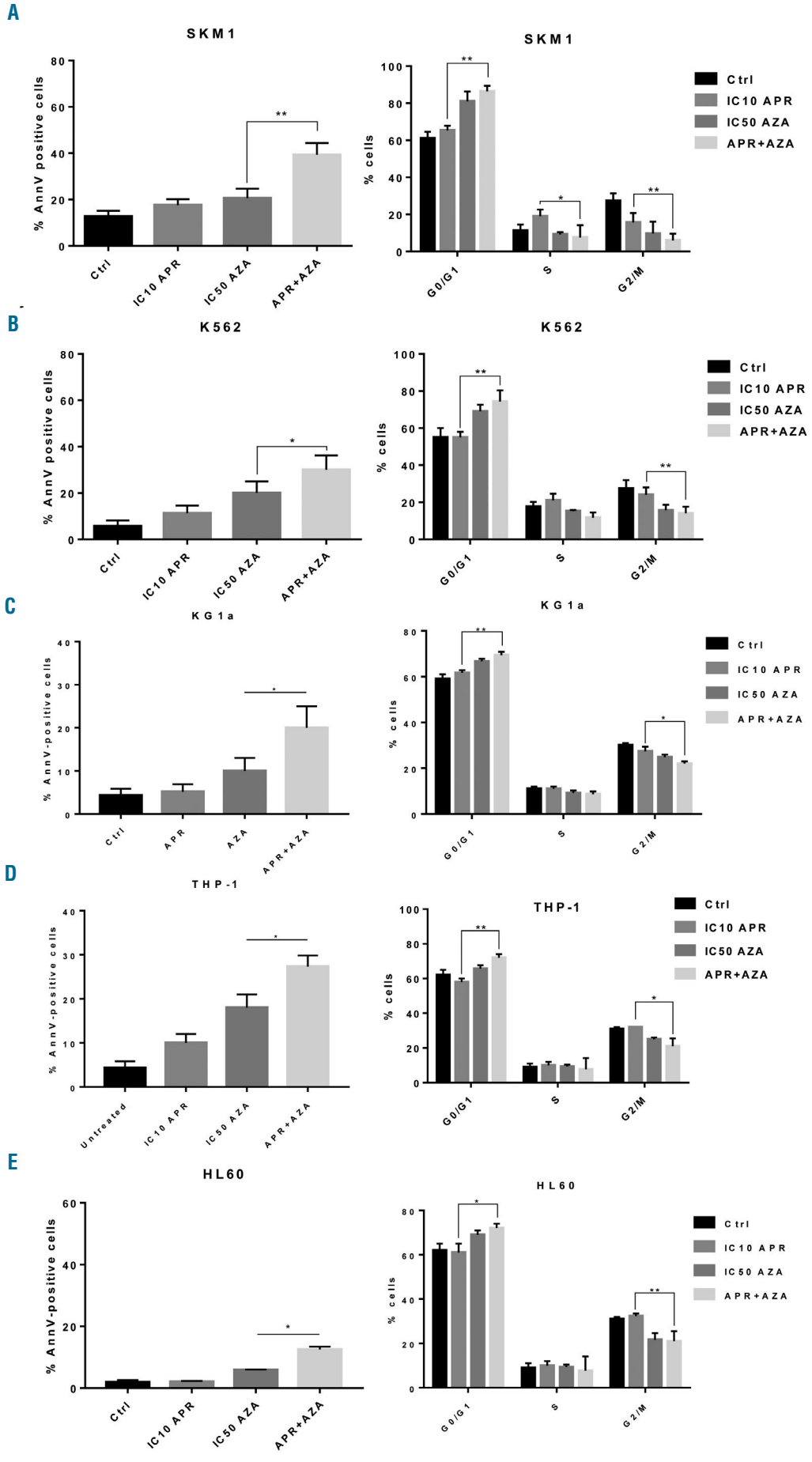

Figure 2. The combination of PRIMA-1^{Met} (APR-246, APR) and azacitidine (AZA) promotes G0/G1 arrest and apoptosis in various *TP53*-mutated acute myeloid leukemia (AML) cell lines. (Left) Percentage of Annexin V-positive cells at day 3 post treatment with IC_{10} APR, IC_{50} AZA, or the combination of these two drugs at these same concentrations. (Right) Proportion of cells in G0/G1, S, or G2/M phase 24 hours after treatment with IC_{10} APR, IC_{50} AZA or the combination of these two drugs at these same concentrations. (A) SKM1, (B) K562, (C) KG1a, (D) THP-1, and (E) HL60 cell lines. **P*<0.05, ***P*<0.01.

Treatment with the APR + AZA combination is characterized by a specific gene expression profile

We conducted a transcriptomic analysis of the SKM1 cell line following treatment with APR, AZA, or a combination of these two drugs. A large number of genes were differentially expressed ($P<0.05$) under treatment by AZA on its own or by APR + AZA (n=4,620 and n=4,672, respectively), while the number of genes differentially expressed under APR alone was relatively small (n= 691) (Figure 6A). Accordingly, the number of genes deregulated in common for APR and APR + AZA treatments was small (n=201) while this was very high for the AZA and the APR + AZA treatments (n= 3,461). As expected, the Gene Set Enrichment Analysis (GSEA) (Figure 6B) and DAVID analyses of the genes differentially expressed by APR identified the p53 pathway as one of the main deregulated pathways [Normalized Enrichment Score (NES) = 1.2; false discovery rate (FDR) = 0.15], with increased expression of p53-target genes such as *CDKN1A*, *CASP1*, *BAX*, and *FAS*. These differential expression patterns were confirmed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis (Figure 6D). In accordance with the involvement of some of these genes in apoptosis, the GSEA analysis also revealed activation of an early apoptotic program (NES= 1.1; FDR= 0.2) (Figure 6B). Importantly, similar results on p53 and pro-apoptotic pathways were found to occur when AZA was added to APR (Figure 6C and E), thus suggesting that AZA did not suppress the transcriptional modifications associated with APR treatment. Of note, enrichment of ROS-induced genes was found to occur with APR on its own (FDR= 0.06; NES= 1.2) (*Online Supplementary Figure S6A*) and APR + AZA (FDR= 0.02; NES= 1.52) (*Online Supplementary Figure S6B*), possibly confirming a p53-independent mechanism of action of APR on oxidative stress, as has already been described in other tumor models.[27,34,35] Using Interpretative Phenomenological Analysis (IPA) analysis and a selection of genes with a significant level of differential expression ($P<0.05$) (*Online Supplementary Table S2*) on the basis of the difference in fold change (FC), we found 5,428 transcriptionally-regulated genes with APR + AZA treatment compared to untreated cells. Eight hundred and seventy-five genes appeared to be up- or down-regulated with the combined treatment only ("synergistic only" genes) and not by either drug on their own. GSEA analysis of the "synergistic only" genes revealed activation of the p53 pathway, induction of an apoptotic program, and downregulation of the MYC pathway (*Online Supplementary Table S3*). Surprisingly, in this group of "synergistic only" genes, we identified decreased expression of several FLT3-pathway genes, including FLT3 and FLT3-L (*Online Supplementary Table S2*). This finding was confirmed using the comparative GSEA analysis, which similarly revealed downregulation of the FLT3 pathway (Valk, FLT3-ITD representative of activation of the FLT3 pathway) (Figure 7A) with the combination treatment compared to either of the drugs on their own. Using RT-qPCR, we were able to confirm the significant downregulation of both the *FLT3* and the *FLT3*-L gene with the combined treatment (Figure 7B).

Downregulation of the FLT3 pathway contributes to the APR + AZA-induced anti-proliferative effect

To analyze the relevance of downregulation of the FLT3 signaling pathway to the synergistic effect of the APR + AZA combination, SKM1 cells were cultured with these drugs in the presence or absence of FLT3-L at different concentrations. The addition of FLT3-L on its own did not affect SKM1 cell proliferation, but, when increasing amounts of FLT3-L were added to the APR + AZA combi-

Table 1. World Health Organization classification of cytogenetic and molecular characteristics of TP53-mutated myelodysplastic syndromes / acute myeloid leukemia patients.

Patient	*TP53* mutation	Karyotype	Colonies sequenced	Variant allele frequency (VAF, %)
1	p.V157F	45,XX,add(1)(q4?3),del(5)(q1?3q3?3),der(6)t(6;14)(p2?2;q1?2)x2,-11,-14,+?15,der(16)t(?;16)(?;p1?1)[11q13?],-17,+r(11)[24]	71	90%
2	p.P190L	45,XX,-7,add(3)(p2?6),del(4)(q2?4),del(5)(q1?q3q3?3),del(12)(p13)[10]/47,XX,del(4)(q2?4),del(5)(q1?q3q3?3),del(12)(p13),+21[6]/47,XX,del(4)(q2?4),del(5)(q1?q3q3?3),del(12)(p13),+21,+r(?)[4]	72	92%
3	p.C238F	45,XX,-7[4]/46,XX[6]	71	22%
4	p.R248Q	44,XY,del(5)(q1?3q3?3),-7,add(17)(p1?1),-19[11]/44,idem,del(11)(p1?4p1?5)/46,XY[3]	50	30%
5	p.V216M + p.R248Q	46,XX,del(5)(q1?3q3?3)[11]/46,XX[13]	ND	NA
6	p.S241C	45,XX,t(4;13)(p1?3;q1?2),del(5)(q15q34),del(7)(q21),dic(12;16)(p11;?p1?2),-18,der(?21)(?),+r(?)[cp21]	ND	NA
7	p.R248Q	46,XX,ins(3;12)(p2?1;q13q2?4),del(12)(q1?3),-17,+mar1[cp10]/46,idem,-7,+mar2,+min[cp7]/46,XX[6]	ND	NA
8	p.M246R	47,XY,+8[21]/46,XY[1]	ND	NA
9	p.R248Q	43,XX,del(5)(q1?4q3?3),del(7)(q?21),der(12)t(12;13)(p1?1;q1?3),der(13)t(7;13)(q21;q13),del(18(q21)[21]	ND	77%
10	p.A276T	46,XX,del(5)(q1?3q3?3)[15]/46,XX[7]	ND	NA
11	p.R248T + p.L330R	46,XY,i(17)(q10)[25]	ND	NA
12	p.C275Y	44-47,XY,del(5)(q13q33),+8,+9,del(9)(q2?2),t(12;15)(p13;q1?2),-13,-17,+r(?)[3], +1-4mar[cp19]/46,XY[3]	ND	9%
13	p.R273C	46,XY,del(2)(q1?1),add(10)(p1?1),de1(11)(q2?1),del(16)(p1?1)[19]/46,idem,add(17)(q2?5)[3]/46,XY,-12,-17,+2mar[3]	ND	NA
14	p.C238Y	44,XX,-5,del(7)(q2?1),add(8)(q2?4),-18,?der(21)t(8;21)(q?13;q2?1)[21]	ND	NA
15	c.675-2A>G	45,XY,der(1;5)(q10;p10),del(4)(q2?4q?21),add(17)(p1?1),-21,+r(?1)[21]	ND	NA

ND: not determined; NA: not available.

nation, we observed a dose-dependent reversal of the inhibition of cell proliferation (Figure 7C). The relative proliferation of APR + AZA treated cells at day 3 was significantly higher when FLT3-L was added (P<0.01) (Figure 7D). This reversal of the inhibitory effect on proliferation was associated with a decrease in apoptosis, since 40% of the cells stained positive for Annexin V with the drug combination treatment without FLT3-L compared with

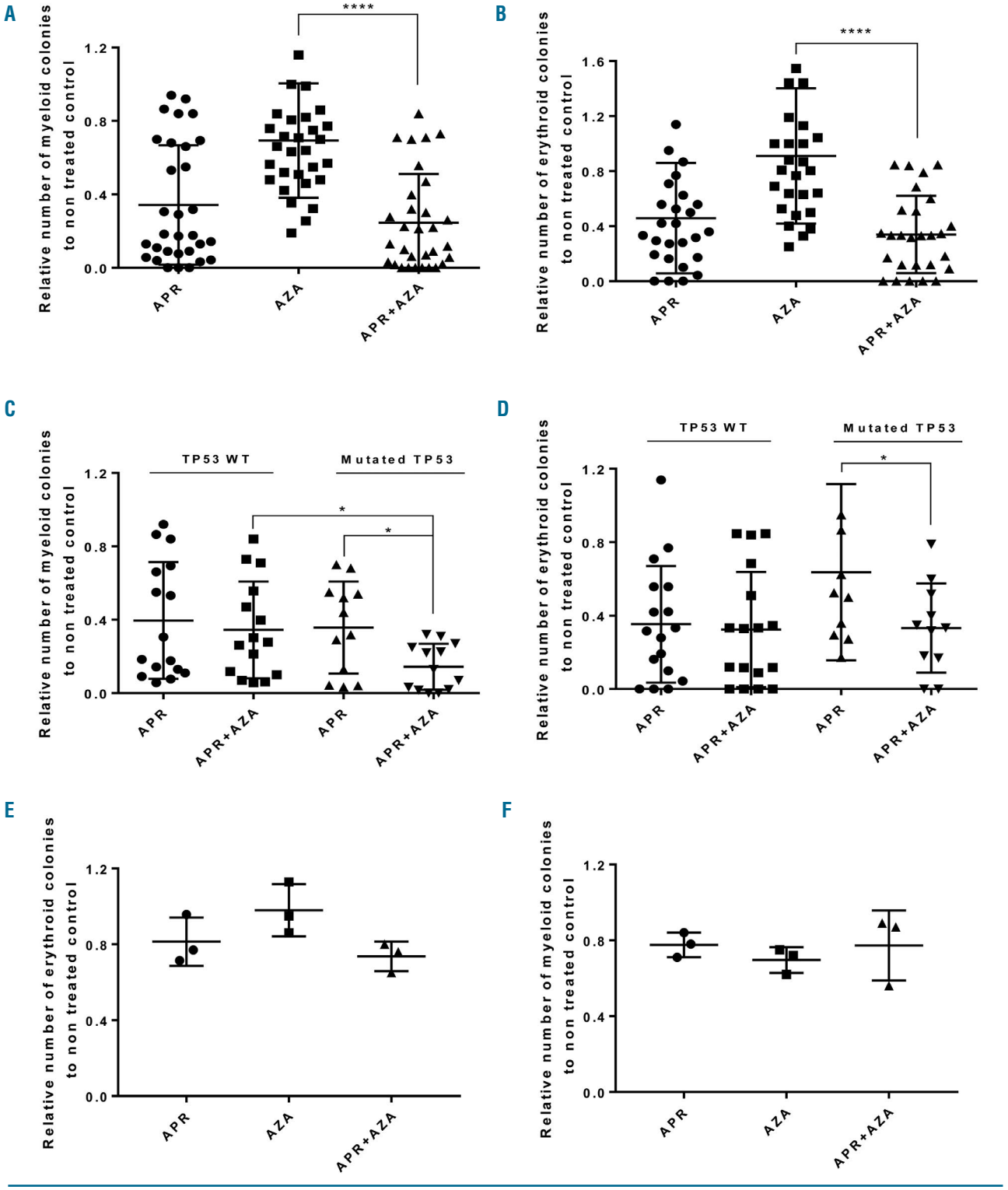

Figure 3. Effects of PRIMA-1^{Met} (APR-246, APR) on primary cells from *TP53*-mutated myelodysplastic syndromes (MDS) / acute myeloid leukemia (AML) patients and healthy donors. The median numbers of (A) myeloid and (B) erythroid colonies relative to the untreated control for 34 bone marrow samples from MDS/AML patients treated with APR, azacitidine (AZA), or the combination APR + AZA in semi-solid medium (methylcellulose). Relative numbers of (C) myeloid and (D) erythroid colonies according to the *TP53* status (WT: wild-type) treated with APR or the combination of APR + AZA. Median numbers of (E) myeloid and (F) erythroid colonies relative to untreated control for 3 CD34$^+$ cells from healthy donors treated with APR, AZA or the combination APR + AZA. *P<0.05, ****P<0.0001.

only 26% when FLT3-L was added (Figure 7E). These results suggest that the synergistic effect observed with the APR + AZA combination on proliferation and apoptosis correlates with downregulation of the FLT3 pathway.

Discussion

APR has been shown to have efficacy on its own as well as when combined with other drugs in solid tumors[19,20,23-25] and lymphoid tumors.[21,27,28,36] In this study, we observed that APR inhibited the proliferation of several TP53-mutated myeloid cell lines, as previously suggested by other studies on myeloid[26,34] and lymphoid cell lines.[27,28] Of note, the additive/synergistic impact of the combination was more robust with regards to apoptosis compared to cell cycle arrest, suggesting that most of the anti-proliferative effect may be mediated by hypomethylating agent (AZA) while the pro-apoptotic effects of the combination was clearly due to APR.

These inhibitory effects were more pronounced in the *TP53*-mutated MDS-derived SKM1 cell line, and may be related to the structure of the mutant protein in the different cell lines. Indeed, APR binding to the cysteine residues of the DBD has been associated with the reformation of an active structure by p.R175H and p.R273H mutant p53

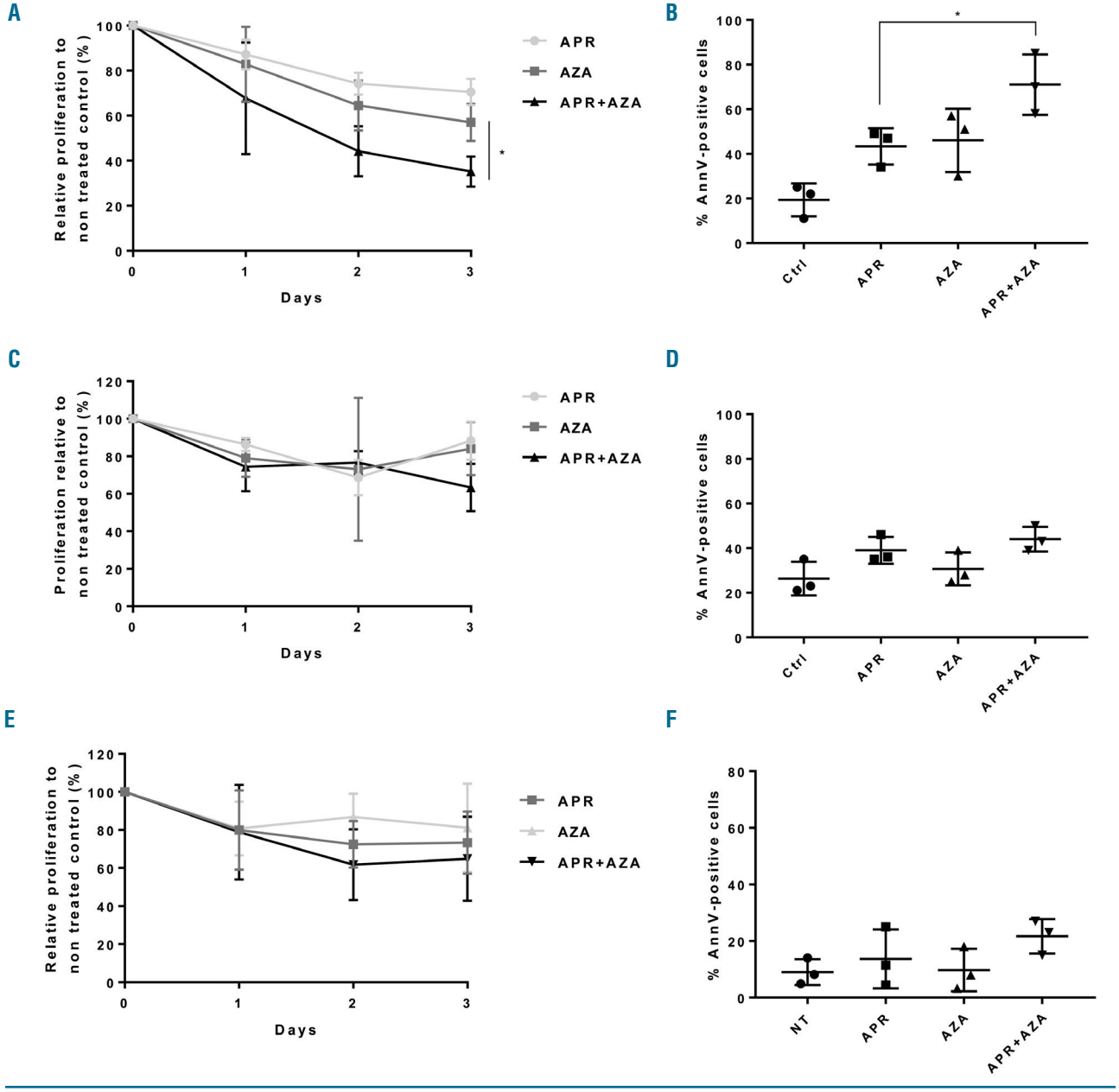

Figure 4. Effects of PRIMA-1^{Met} (APR-246, APR) on primary cells from *TP53* wild-type or mutated myelodysplastic syndromes (MDS) / acute myeloid leukemia (AML) patients and healthy donors. (Left) Relative proliferation of CD34$^+$ cells treated with 1 μM APR, 1 μM AZA or the combination APR + AZA at these concentrations. (Right) Percentages of Annexin V-positive cells at day 3 post treatment with 1 μM APR, 1 μM of AZA or the combination APR + AZA at these concentrations. (A) *TP53*-mutated MDS/AML samples (n=3), (B) Wild-type *TP53* MDS/AML samples (n=3), and (C) healthy donors (n=3). *P<0.05.

proteins.[37] The SKM1 cell line has a p.R248Q mutation located in the DBD, while the other myeloid cell lines tested in this study have various *TP53* truncation mutations that result in a lack of detectable p53 protein in all four cell lines.[38,39] APR may not be able to restore an active conformation to the truncated p53 protein in these cell lines, thereby explaining the lower efficacy of APR compared with SKM1 cells. This also suggests that the effects of APR in these cell lines may be due in part to p53-independent processes.[27] We then demonstrated that the inhibitory effect of the APR + AZA combination was synergistic in the five *TP53*-mutated cell lines that were tested, although, again, this effect was more pronounced in the SKM1 cells for presumably the same reason. To confirm this efficacy, we showed that APR + AZA combination significantly antagonized development of the disease in an *in vivo* model.

APR exhibited a pronounced anti-clonogenic effect on hematopoietic progenitor cells from MDS/AML patients irrespective of the *TP53* mutational status. This suggests that APR on its own targets not only *TP53*-mutated cells (at least at the doses that we used in our experiments), and that its inhibitory effects are partly p53-independent, as had already been shown previously.[27,34,35,40] On the other

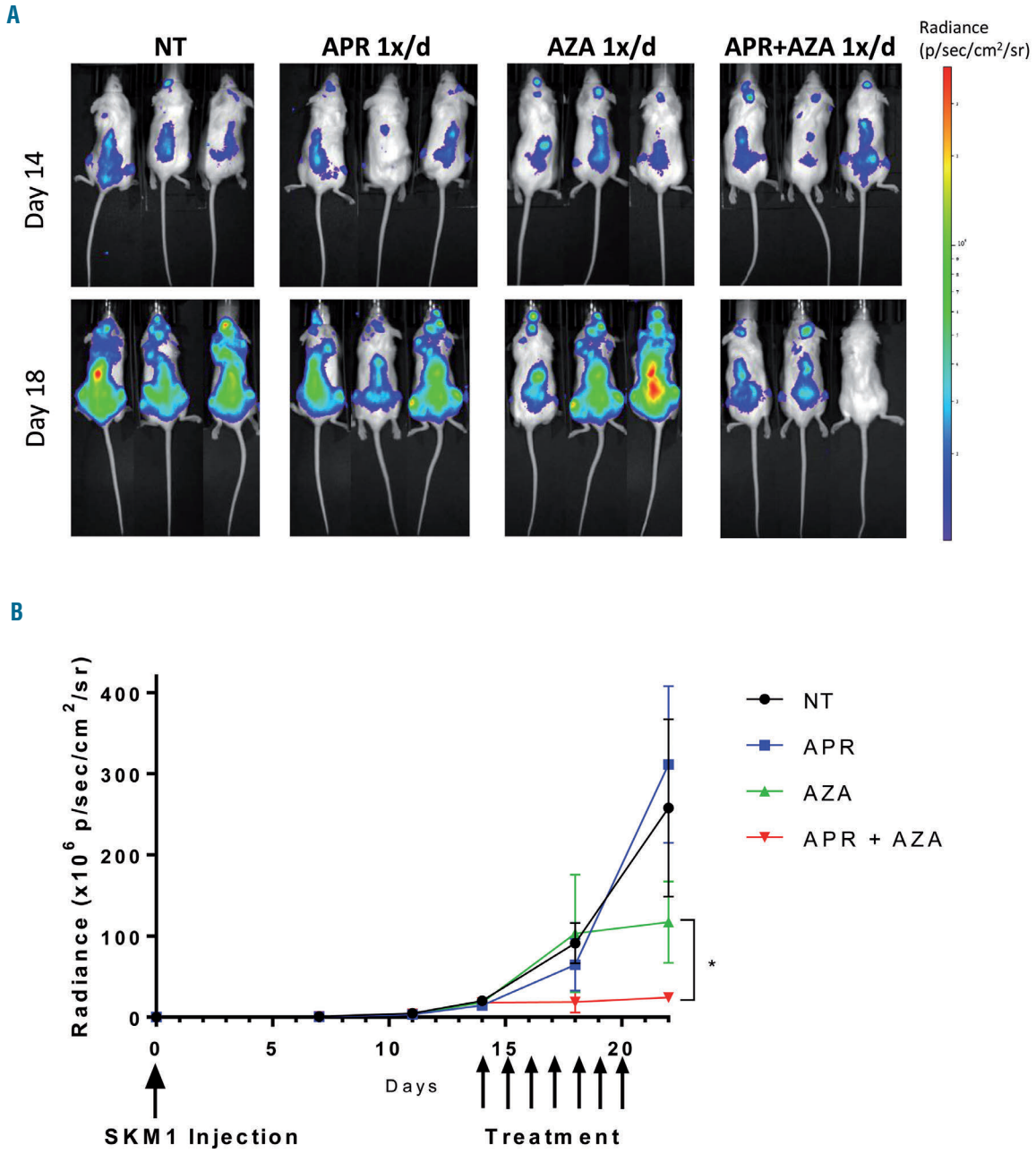

Figure 5. ***In vivo*** **efficacy of PRIMA-1^{Met} (APR-246, APR) and azacitidine (AZA) in a xenotransplantation model.** (A) Disease development monitored by luciferase activity and bioluminescence imaging. Images of untreated mice at day 14 (day 1 of treatment) and treated mice at day 18 (day 5 of treatment) following injection of SKM1-Luc cells. (B) The tumor volume (p/sec/cm^2/sr) during and after treatment with PBS, APR, AZA, or the APR + AZA combination (treatment days are indicated by the solid arrows). The drug treatments were started when the tumor volume had reached 10^6 p/sec/cm^2/sr. *P<0.05.

hand, the combination of the two drugs (APR + AZA) had a similar effect as APR on its own in progenitor cells from patients with WT *TP53*, whereas the combination had a significantly more pronounced effect than APR on its own on progenitor cells from patients with mutant *TP53*. This strongly suggests a selective effect on mutant p53 cells, although we were unable to demonstrate the formation of wild-type *TP53* colonies under treatment, which

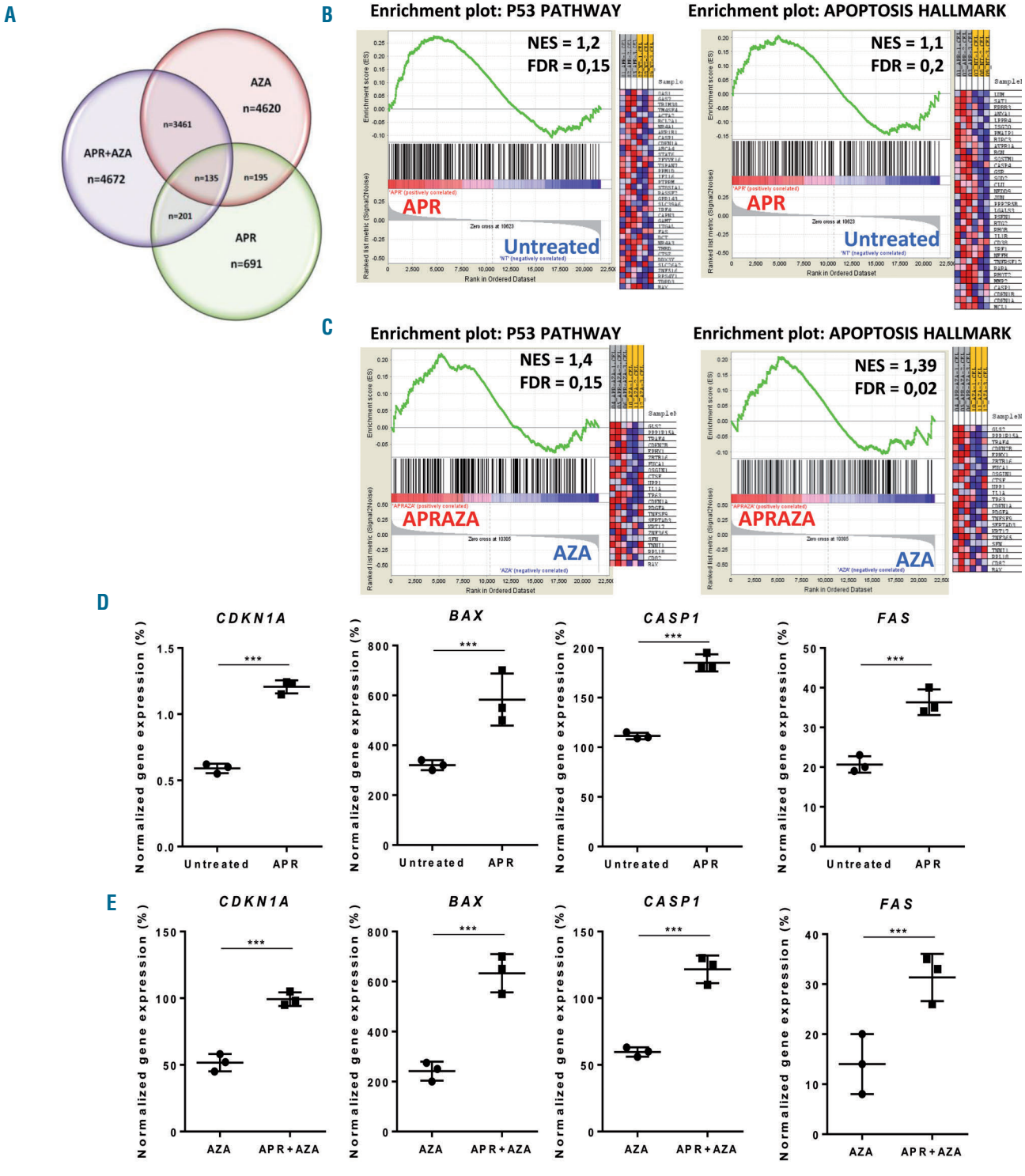

Figure 6. Changes in gene expression induced by treatment with PRIMA-1^{Met} (APR-246, APR) + azacitidine (AZA). (A) A Venn diagram representing the number of deregulated genes with APR on its own, AZA on its own, or the combination of APR + AZA. (B) Gene enrichment plots and associated heatmaps showing reactivation of the p53 pathway and induction of an apoptosis program in SKM1 cells treated with IC_{10} APR compared to untreated cells. (C) Gene enrichment plots and associated heatmaps showing reactivation of the p53 pathway and induction of an apoptosis program in SKM1 cells treated with the combination of APR IC_{10} + AZA IC_{50} (APR + AZA) compared to AZA on its own (AZA). (D) RT-qPCR expression of the main p53 targets (*CDKN1A* and *BAX*) and pro-apoptotic factors (*CASP1* and *FAS*) in SKM1 cells treated with APR compared to untreated cells. (E) RT-qPCR expression of the main p53 targets (*CDKN1A* and *BAX*) and pro-apoptotic factors (*CASP1* and *FAS*) in SKM1 cells treated with the combination of APR + AZA *versus* AZA on its own. ***P<0.001. NES: Normalized Enrichment Score; FDR: False Discovery Rate.

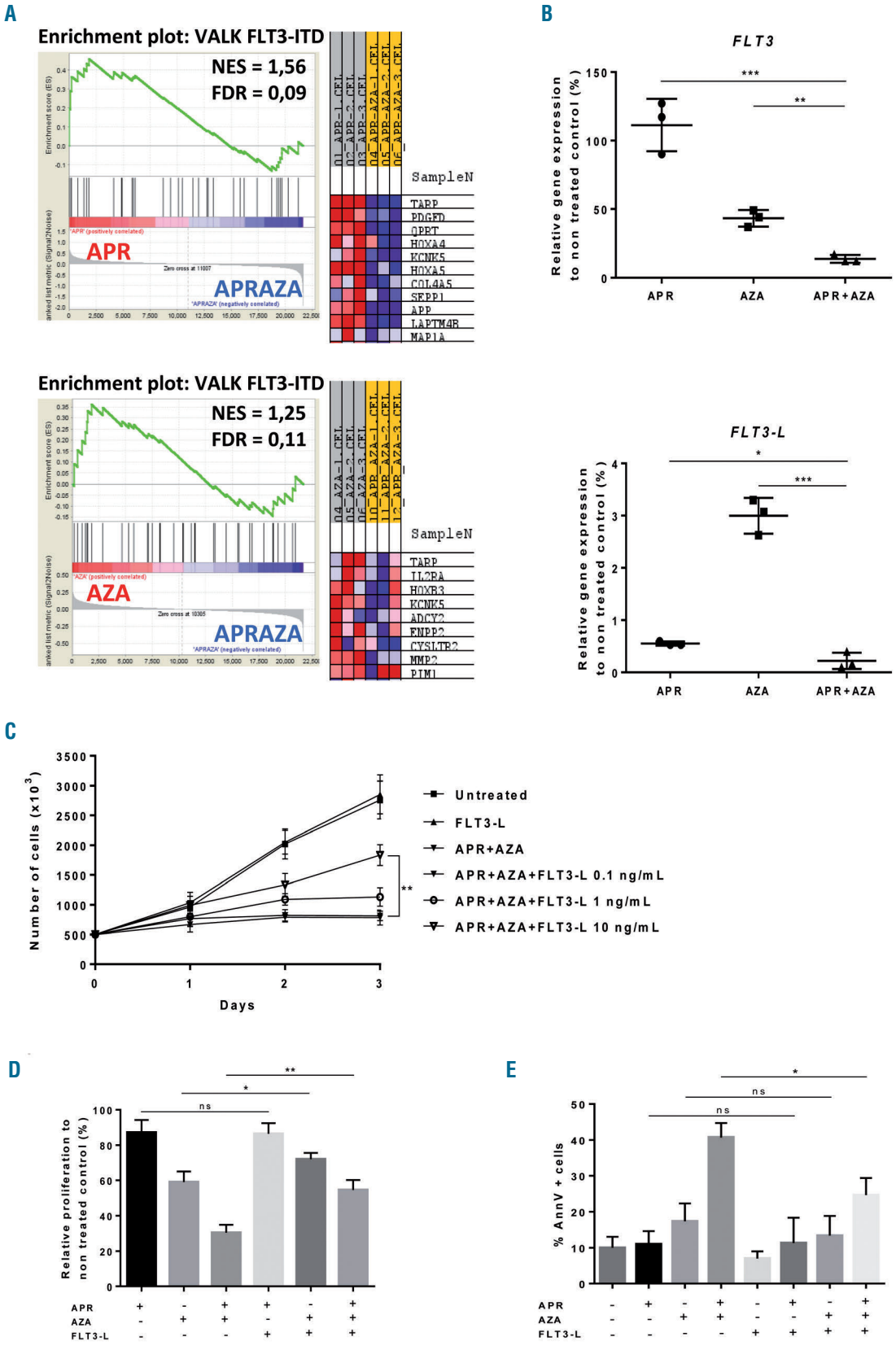

Figure 7. The antiproliferative effect of PRIMA-1^{Met} (APR-246, APR) + azacitidine (AZA) combination is related to downregulation of the FLT3 pathway. (A) Gene enrichment plots and heatmaps representing the FLT3 activated pathway (Valk, FLT3-ITD) in APR *versus* untreated SKM1 cells and APR + AZA *versus* AZA-treated cells. (B) Real-time quantitative polymerase chain reaction analysis of gene expression by *FLT3* and *FLT3*-L with APR, AZA, or APR + AZA relative to the untreated control. (C) Absolute numbers of untreated and APR + AZA-treated SKM1 cells exposed to increasing concentrations of FLT3-L. (D) Proliferation of SKM1 cells treated with APR, AZA, or the combination APR + AZA relative to controls, without or with 10 ng/mL FLT3 ligand (FLT3-L). (E) Proportion of Annexin V-positive SKM1 cells at day 3 following treatment with APR, AZA, or the combination of APR + AZA, with or without FLT3L. **P*<0.05, ***P*<0.01, ****P*<0.001.

was probably due to the very high proportion of mutant progenitor cells that grew in these conditions, as no wild-type colonies could be discerned even in the absence of treatment. An *in vivo* evaluation of the change in the *TP53* allelic burden in patients treated by the combination would be relevant to address the question of mutant *TP53* selectivity. A clinical trial (*clinicaltrials.gov identifier: 03745716*) testing the association of APR and AZA in *TP53*-mutant MDS/AML is ongoing and may help answer this question.

The molecular mechanism underlying the efficacy of the association between AZA and APR remains unknown. AZA is a DNA demethylating agent and APR is an agent that reactivates the transcription factor p53. Both drugs may act by modulating gene expression. Thus, we sought to study differences in gene expression profiles between cells treated by the single agents or with the drug combination. The results of the transcriptomic analysis provide further evidence that APR, even at low doses, reactivates the p53 pathway and that it results in activation of an apoptotic program. We also found that genes involved in FLT3 signaling were down-regulated by the combination of APR and AZA. The relevance of this observation was confirmed as the addition of FLT3 ligand reversed the inhibition of cell proliferation by the APR + AZA combination. Interestingly none of the cell lines tested had *FLT3* mutations or duplications,[41] suggesting that the drug combination effect is not dependent on the presence of such molecular alterations. However, it would be interesting to test this drug combination in cell lines with *FLT-3* mutations or duplications. In addition to providing clues for a specific mechanism of action of the drug combination through a synthetic lethality process involving the FLT3 pathway, our observations indicate that the addition of FLT3-inhibitors to the APR + AZA combination could be of relevance in *TP53*-mutated MDS/AML.[42]

In conclusion, our results suggest that APR exhibits promising synergistic effects when combined with conventional AZA therapy in the high-risk subgroup of *TP53*-mutated MDS/AML. Promising preliminary results of a phase I/II clinical trial combining AZA and APR in *TP53*-mutated MDS/AML, and activated at US centers and our center, have been reported.[29]

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