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The long non-coding RNA CDK6-AS1 overexpression impacts on acute myeloid leukemia differentiation and mitochondrial dynamics

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Highlights
CDK6-AS1 acts in concert with CDK6
High CDK6-AS1 levels trigger RUNX1 early differentiation arrest in myeloid cells
CDK6-AS1 controls mitochondrial mass of AML blasts
CDK6-AS1 levels impact on mitochondrial-targeted agents sensitivity

CDK6-AS1 confers therapeutic sensitivity to mitochondrial drugs in pediatric AML
The long non-coding RNA CDK6-AS1 overexpression impacts on acute myeloid leukemia differentiation and mitochondrial dynamics

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SUMMARY
Patients with acute myeloid leukemia (AML) carrying high-risk genetic lesions or high residual disease levels after therapy are particularly exposed to the risk of relapse. Here, we identified the long non-coding RNA CDK6-AS1 able to cluster an AML subgroup with peculiar gene signatures linked to hematopoietic cell differentiation and mitochondrial dynamics. CDK6-AS1 silencing triggered hematopoietic commitment in healthy CD34+ cells, whereas in AML cells the pathological undifferentiated state was rescued. This latter phenomenon derived from RUNX1 transcriptional control, responsible for the stenness of hematopoietic precursors and for the block of differentiation in AML. By CDK6-AS1 silencing in vitro, AML mitochondrial mass decreased with augmented pharmacological sensitivity to mitochondria-targeting drugs. In vivo, the combination of tigecycline and cytarabine reduced leukemia progression in the AML-PDX model with high CDK6-AS1 levels, supporting the concept of a mitochondrial vulnerability. Together, these findings uncover CDK6-AS1 as crucial in myeloid differentiation and mitochondrial mass regulation.

INTRODUCTION
Pediatric acute myeloid leukemia (AML) is a molecularly heterogeneous disorder characterized by a large number of chromosomal translocations, resulting in the production of chimeric proteins, which confer a block of differentiation and an abnormal proliferation potential to hematopoietic stem cells. In the last decade, pediatric trials have incorporated numerous markers useful for stratification of patients in different risk groups (Bacher et al., 2014; Baldini et al., 2017; Inaba et al., 2012; Jongen-Lavrencic et al., 2018; Pession et al., 2013; Pigazzi et al., 2015; Rubnitz et al., 2010; Zwaan et al., 2015). Nevertheless, current diagnostic protocols partially fail to detect poor-risk patients (McNeer et al., 2019), suggesting the need to implement functional markers and treatments to more efficiently eradicate leukemia stem cells, whose survival and escape from the lytic action of cytotoxic agents are thought to be a major cause of treatment failure (Ng et al., 2016). Genome-scale studies have identified potentially targetable mutations in adult de novo AML (Papaemmanuil et al., 2016), whereas no remarkable driver mutations have been found in pediatric AML (Bolouri et al., 2018). Indeed, much AML research has focused on somatic mutations in protein coding genes, with few efforts in understanding the role played by long non-coding RNAs (lncRNAs). Recently, lncRNAs have been linked to several pathways in physiological cell biology and cancer, with recent findings demonstrating their aberrant hyper-activation (Quinn et al., 2016) leading to resistance mechanisms in different neoplasia, including acute leukemia (Bester et al., 2018; Salmena et al., 2011; Schmitt and Chang, 2016). LncRNAs are transcripts of more than 200 nucleotides, which can be localized into the nucleus or cytoplasm, playing diverse roles, acting as intergenic transcripts, enhancers, sense/antisense transcripts, regulators of nuclear domains (Deveson et al., 2017; Dykes and Emanuelli, 2017), and transcriptional regulators both in cis or trans (Quinn et al., 2016). In this study, we focused on a lncRNA, known as CDK6-AS1 (LOC101927497), to define its contribution to AML pathogenesis, since it was previously prioritized from differentially expressed lncRNAs and mRNAs in acute lymphoblastic leukemia (Fernando et al., 2015), as well as in human gastric epithelial tumorigenesis (Luo et al., 2018).

We dissected the role of CDK6-AS1 in AML samples and in healthy CD34+ hematopoietic cells discovering its transcriptional activation and regulatory action over the chromosomally adjacent gene CDK6. This...
network is able to impair the hematopoietic differentiation process through RUNX1 signaling and maintain an aberrant mitochondrial status. These latter findings were recognized in patients with AML with the highest CDK6-AS1 expression, who might be included in a subgroup of cases with mitochondrial vulnerabilities to be exploited. Hence, CDK6-AS1 levels arise as unique molecular determinant, which characterize those cases that, both in vitro and in vivo experiments, proved to benefit from a mitochondrial-oriented therapeutic strategy.

**RESULTS**

**CDK6-AS1 expression distinguishes a subgroup of patients with AML**

We retrospectively analyzed the expression of the IncRNA CDK6-AS1 in leukemia blasts of 132 pediatric patients with de novo AML enrolled in the AIEOP AML 2002/01 trial (Pession et al., 2013) harboring different genetic abnormalities including CBF rearrangements, i.e., t(8;21)RUNX1-RUNX1T1 and inv(16)CBFB-MYH11 (n = 20), MLL-translocations (n = 37), NUP98-translocations (n = 15), FLT3-ITD (n = 15), rare translocations (n = 9), and 36 patients without any recurrent molecular lesion (see Table S1A). Overall, CDK6-AS1 expression was heterogeneous but significantly higher (*p value<0.05) in all AML samples when compared with healthy mononuclear cells from cord blood (used as calibrator, RQ = 1, see Figure S1A) and with healthy bone marrow (HBM, see Figure S1B). Thus, we subdivided patients according to the increasing expression levels from quartile 1 to 4 of CDK6-AS1 (Q1, Q2, Q3 (Q123-AML) and Q4, see Figure S1C). CDK6-AS1 quartile distribution was maintained also subdividing patients according to the genetic abnormalities identified at diagnosis (*p value<0.05, **p value<0.01, see Figure S1D). This latter observation prompted us to investigate the biological role of CDK6-AS1. We examined the gene expression profiles (GEPs) of 58 of the 132 patients with AML, of whom 18 had the highest CDK6-AS1 expression (Q4-AML), whereas 40 belonged to Q123-AML quartiles (GEO: GSE75461). Of note, the unsupervised analysis showed a statistical significant clustering of Q4-AML as compared with the Q123-AML cases, independently of their genetic lesions for 57 coding and 12 ncRNAs with a Fold Change |2| (p value<0.01, coding genes in Figure 1A, for ncRNAs see also Figure S2A) or 247 coding genes and 83 ncRNAs with a Fold Change |1.5| (p value<0.01; see Figure S2B, see Tables S2A and S2B). t-Distributed stochastic neighbor embedding (t-SNE) visualization using the 57 most variably expressed transcripts revealed a clear classification of AML consistent with CDK6-AS1 expression quartiles (Figure 1B, upper panel). Applying the same method for the different genetic lesions as classifier, we noted that different high-risk genetic lesions, including FLT3-ITD, MLL-r, NUP98-r, and no markers, were present in Q4-AML (Figure 1B, lower panel). We observed that patients with Q4-AML had a greater risk of not achieving complete remission when compared with patients with Q123-AML (Table S1A, p value = 0.03), suggesting a potential role of CDK6-AS1 in determining patients’ response to therapy. The same result was confirmed in another independent cohort of pediatric AML cases (TARGET, n = 110), where high-CDK6-AS1 patients (Q4) had significantly higher minimal residual disease (MRD) levels after induction therapy with respect to low-CDK6-AS1 cases (Q123, p value = 0.034; see Table S1B).

Gene Set Enrichment Analysis (GSEA) highlighted that Q4-AML were negatively enriched in genes regulated by the Runt domain of the Runt-related transcription factor 1 (RUNX1), essential for the early hematopoietic differentiation process (Fujimoto et al., 2007). In parallel, a concomitant hyper-expression of genes related to mitochondrial translation processes was found to be upregulated in Q4-AML (Figure 1C).

Among the most differentially expressed genes in Q4-AML, we identified CDK6-AS1 adjacent gene CDK6, located at 7q21.2. Given that IncRNAs are known to transcriptionally regulate other genes in cis, we interrogated neighbor genes within a window of 10 Mb, finding a strong expression correlation exclusively between CDK6 and CDK6-AS1, whereas other pairwise correlations were not significant (see Figure S3A). An overall significant positive correlation between CDK6-AS1 and CDK6 expression was confirmed in our AML cohort (Figure 1D, Pearson correlation r = 0.7, p value<0.001, see also Figure S3B, *p value<0.05, ***p value<0.0001) not influenced by the presence of recurrent molecular markers (see Figure S3C, Pearson correlation respectively r = 0.79, 0.84, 0.96 and 0.75; p value<0.05). With the goal of further investigating this correlation, we stably knocked down CDK6-AS1 (CDK6-AS1 KD) in SHI-1 AML cell line (documented to have high CDK6-AS1 expression at the same extent of patients with Q4-AML), finding that CDK6 mRNA transcript and protein levels significantly decreased (Figures 1E and 1F), whereas other neighbor genes (SAMD9, GATAD1, PEX1, FAM133B) were not altered (see Figure S3D). These findings indicated that CDK6-AS1 might coordinate CDK6 gene expression. Indeed, bioinformatic analysis revealed that the two genes share an overlapping untranslated first exon transcribed in opposite directions, with a
Figure 1. CDK6-AS1 expression and correlation with CDK6 in bone marrow samples of pediatric patients with AML

(A) Hierarchical clustering analyses of patients with AML. High-CDK6-AS1 expression (Q4) patients clustered independently (black square; Pearson correlation distance, t test, p value<0.01, Fold Change FC>|2|) by 57 coding genes (see Table S2A). Genetic AML subtypes and quartile distribution are indicated in the legend with color codes.

(B) t-SNE visualization of patients with AML utilizing the 57 top differentially expressed genes.

(C) Gene Set Enrichment Analysis (GSEA) performed on the 337 gene signature revealed genes of the pathway involved in the upregulation of RUNX1 target genes in patients with Q1-AML versus patients with Q4-AML (false discovery rate [FDR] = 0.044, normalized enrichment score (NES) = 1.41) and in mitochondrial synthesis and function being upregulated in the patients with Q4-AML (FDR = 0.0, NES = 2.28 for mitochondrial translation; FDR = 8.12x10⁻⁵, NES = 2.17 for metabolic process).

(D) Correlation between CDK6-AS1 and CDK6 mRNA expressions (Pearson correlation, r = 0.7, p value<0.0001) plotted in the scatter diagram and fitted in linear regression.

(E) RQ-PCR expression of CDK6-AS1 and CDK6 measured in shCDK6-AS1 cells with respect to shRNA NEG (RQ = 1) (n = 4, **p value<0.01, ***p value<0.001). Data are represented as mean ± SEM.

(F) Western blot of CDK6 after stable CDK6-AS1 silencing with relative densitometric analysis.
promoter region identified by in silico analysis for its enrichment in transcription factor binding sites, within exon 1 and intron 1 of CDK6 (see Figures S4A–S4C). To further corroborate the hypothesis of a common bidirectional promoter for CDK6-AS1 and CDK6 controlled by CDK6-AS1, we generated a single (only at 3') and dual (both at 5' and 3') luciferase reporter vectors containing 1,400 nucleotides of the predicted promoter. The luciferase expression in transfected cell lines increased using the dual vector with respect to the single luciferase vector, confirming promoter bidirectionality. When we transfected these vectors in CDK6-AS1 KD we measured a lowered luciferase activity in both constructs, demonstrating that CDK6-AS1 reduction modulated the promoter in two directions, thus regulating the expression of both CDK6-AS1 and CDK6 (see Figure S4D, *p value<0.05). Consistently, we showed that CDK6-AS1 was present in both nuclear and cytosolic cell fractions (see Figure S4E, *p value<0.05) and that in the CDK6-AS1 stable silenced cells its expression levels were decreased both in the nuclear and cytosolic compartments (see Figure S4F, *p value<0.05, **p value<0.01).

**CDK6-AS1 correlates with differentiation of hematopoietic progenitors**

In order to confirm the contribution of CDK6-AS1/CDK6 axis in hematopoietic cell differentiation process as suggested by GSEA, we ranked all the transcripts that correlated with CDK6-AS1 expression in AML cases according to the absolute Spearman’s rank correlation coefficient. We used the top 1% probes and identified a 337-tran- script signature used to interrogate 33 healthy donor-derived human blood cell samples (GEO: GSE98633) (Schwarzer et al., 2017). By unsupervised clustering we observed that these genes were able to subdivide the healthy immature CD34+ from the myeloid granulocyte-monocyte progenitors (CMP/GMP), and from the more mature hematopoietic pools (granulocytes, erythroblasts, monocytes, and megakaryocytes); this clustering correlates with CDK6-AS1 expression levels (expressed in quartiles, Figure 2A). t-SNE analysis confirmed that the more immature healthy samples had the highest levels (Q4) of CDK6-AS1, whereas more committed populations showed lower CDK6-AS1 levels (Q123) (Figure 2B, see also Figure S5A, *p value<0.05). These findings highlight that CDK6-AS1 expression reflects the hematopoietic differentiation stage, in both primary AML samples and healthy hematopoietic cells. At support we investigated CDK6-AS1 levels in CD34+/CD38- cells from primary AML samples confirming an increased RNA expression in sorted CD34+/CD38- blasts (RQ = CDK6-AS1 = 1.3, CDK6 = 5.2) with respect to the rest of blasts (RQ = 1), as well as an increased fluorescence detected by flow cytometry with respect to the non-CD34+/CD38- (see Figure S5B). In line with this observation, we documented an increased percentage of CD34+ cells in samples of patients with AML collected at diagnosis showing higher CDK6-AS1 expression when compared with AML samples of Q1 for CDK6-AS1 expression (Figure 2C). To support this observation, we found a higher CDK6-AS1 and CDK6 expression (RQ CDK6-AS1 = 11.9 and RQ CDK6 = 9.5) in isolated primary normal CD34+ cells when compared with the more mature CD34-counterpart (RQ = 1, Figure 3A, left, *p value<0.05). Finally, we transduced CD34+ cells with shCDK6-AS1, documenting no effects on cell viability (see Figure S5C) but a 20% downregulation of CD3 expression 10 days after silencing (Figure 3A, right, *p value<0.01). On the contrary, we promoted CD34+ cell differentiation by 10-day treatment with ATRA 5 μM, documenting that both CDK6-AS1 and CDK6 expression decreased (Figure 3B, *p value<0.05, see also Figure S5D). In addition, CDK6 silencing enhanced CD34+ cell differentiation upon ATRA exposure. By contrast, CDK6 overexpression delayed CD34+ differentiation (Figure 3C, *p value<0.05, **p value<0.01).

**CDK6-AS1/CDK6 axis controls RUNX1 transcriptional program in AML**

In line with these results, we explored CDK6-AS1 role in AML differentiation in AML cell lines. CDK6-AS1 KD cells acquired CD11b expression, a marker of myeloid maturation with respect to negative control (shNEG) cells (79.5% versus 55.2%, respectively, Figure 4A, *p value<0.05; same results were found in transient transfection for HL-60 and U-937 cell lines, see also Figures S6A–S6C, *p value<0.05, **p value<0.01). Functionally, CDK6-AS1 KD cells showed a significantly increased phagocytic capacity (Figure 4A, right and lower panels, **p value<0.01, see also Figures S6D and S6E, *p value<0.05) that was further augmented when cells were stimulated with GM-CSF (see Figure S6F, *p value<0.05). Moreover, CDK6-AS1 KD cells showed a reduction of more than 30% in their colony-forming capacity (see Figure S6G, *p value<0.05) and an increase in PU1 protein, a transcription factor implicated in early myeloid differentiation (see Figure S6H), supporting the involvement of CDK6-AS1 in the block of myeloid differentiation without effects on both cell viability and cell cycle (see Figures S6I and S6J).

Since CDK6-AS1 depletion in AML cells reduced both CDK6 RNA and protein expression (Figures 1E and 1F), we observed a deregulation in the complex formed by CDK6 with RUNX1, allowing RUNX1 to work as transcription factor, an essential step of the early hematopoietic differentiation process (red spots in Figure 4B, see also Figure S6K) (Fujimoto et al., 2007). We looked at RUNX1 transcriptional program monitoring the 81 RUNX1 target
genes (deposited in ChEA database [Lachmann et al., 2010] and annotated to myeloid cell differentiation by GO term 0030099) expression in Q1 versus Q4-AML cases. Most of the RUNX1 target genes including PU.1, FOS, MEIS1, and PRDM16 were significantly de-regulated in Q4-AML as shown by PCA, clustering, and GSEA analyses (Figures 1C and 4C, see also Figure S6L, see also Table S3). Vice versa, a pro-differentiating effect was obtained by silencing CDK6 as confirmed by the increased RUNX1 target genes expression, as well as by CD11b/CD16 markers positivity and cell morphology (see Figures S7A–S7D, *p value<0.05, **p value<0.01, ***p value<0.001). Furthermore, the immature phenotype was rescued when we exogenously expressed CDK6 in CDK6-AS1 KD cells, as shown by a reduced CD11b expression and phagocytic ability (see Figures S7E–S7G, **p value<0.01). These findings clearly support the hypothesis that aberrant CDK6-AS1 expression, by controlling CDK6 levels, triggers the block of early hematopoietic differentiation process, an essential step of the hematopoietic cell transformation.

CDK6-AS1 controls mitochondrial dynamics in AML and healthy CD34+ cells

Since gene expression suggested that differentiation and mitochondrial signatures were related to the CDK6-AS1 Q4-AML (Figure 1C, right, see also Table S4A) and to healthy hematopoietic cells (see Table S4B), we further investigated this association between hematopoietic differentiation and mitochondrial...
We examined mitochondrial membrane potential (ΔΨ) by tetramethylrhodamine, ethyl ester (TMRE) staining in the healthy CD34+ cell population, which was characterized to include several steps of hematopoietic hierarchy (Pellin et al., 2019) (including hematopoietic stem cells CD34+/38-/90+ and other precursor cells, see Figure S8). The ΔΨ regulates ATP synthesis, ROS production, calcium homeostasis, import-export of mitochondrial proteins, mitochondrial membrane dynamics for biogenesis, mitochondrial proton conductance, and respiratory chain capacity. We revealed a mitochondrial substructure in CD34+ cells by using ΔΨ values: we identified and sorted two different subpopulations, TMRE low and TMRE high.

Figure 3. CDK6-AS1/CDK6 is strongly associated to differentiation in healthy CD34+ cells
(A) CDK6-AS1 expression in healthy CD34+ and CD34- fractioned population (left, n = 3, *p value<0.05). Decrease in the percentage of CD34+ positive cells upon CDK6-AS1 depletion (shCDK6-AS1 that are GFP+) evaluated by flow cytometry (right, n = 3, **p value<0.01).
(B) Expression level of CDK6-AS1 and CDK6 in normal CD34+ cells at day 0 and after cell differentiation induced by ATRA 5 μM, at day 10 (left, n = 3, *p value<0.05). Expression of several surface markers during ATRA-induced cell differentiation (right).
(C) Percentage of positive cells for several differentiation markers in siRNA CDK6 and pCDK6 transfected healthy CD34+ cells, respect to negative controls siRNA NEG and p∅, respectively, after 7 days with ATRA (5 μM) treatment (n = 2, *p value<0.05, **p value<0.01). Data are represented as mean ± SEM.

We examined mitochondrial membrane potential (ΔΨ) by tetramethylrhodamine, ethyl ester (TMRE) staining in the healthy CD34+ cell population, which was characterized to include several steps of hematopoietic hierarchy (Pellin et al., 2019) (including hematopoietic stem cells CD34+/38-/90+ and other precursor cells, see Figure S8). The ΔΨ regulates ATP synthesis, ROS production, calcium homeostasis, import-export of mitochondrial proteins, mitochondrial membrane dynamics for biogenesis, mitochondrial proton conductance, and respiratory chain capacity. We revealed a mitochondrial substructure in CD34+ cells by using ΔΨ values: we identified and sorted two different subpopulations, TMRE low and TMRE high.
high, finding that the CD34+/TMRE low were more quiescent, as shown by Pyronin Y/Hoechst staining, and mainly CD38- with respect to the CD34+/TMRE high counterpart being more proliferating and mostly CD38+ cells (Figure 5A, *p value < 0.05, **p value < 0.01). Concomitantly, CDK6-AS1/CDK6 expression is reduced in CD34+/TMRE high after exiting from quiescence to differentiate (RQ = 0.5 for CDK6-AS1 and RQ = 2.29 for CDK6, versus CD34+/TMRE low RQ = 1.32 for CDK6-AS1 and RQ = 14.71 for CDK6; Figure 5A, lower panel). These data support the observation that an association between CDK6-AS1 levels, hematopoietic differentiation, and mitochondrial dynamics exists.

To decipher this latter finding in AML cases, we performed a single-sample GSEA revealing that specific mitochondrial biogenesis signatures were enriched according to CDK6-AS1 expression (see Figure 5A, *p value < 0.05, **p value < 0.01, see also Table S4B). Consistently, mitochondrial mass in CD6K-AS1 KD cells decreased in terms of mitochondrial area (by TOMM20, Figure 5B, left, ****p value < 0.0001, see also Figure S9C), mitochondrial DNA content (Lazarou et al., 2015) (see Figure S9C, ****p value < 0.0001), and ΔΨ (Figure 5B, lower right, *p value < 0.05), which was not observed upon CDK6 silencing (see Figures S9D–S9F). Furthermore, we analyzed the more immature CD34+ cells isolated from patients with bone marrow-derived primary AML at diagnosis and counted a significantly higher number of mitochondria and a 2.5-fold increased ΔΨ with respect to the more differentiated CD34- blasts (Figure 5C, *p value < 0.05, ****p value < 0.0001). This latter finding reflects the hypothesis that AML-CD34+ had higher CD6K-AS1 levels and mitochondria content than the more differentiated CD34- AML blasts (fold change 2.03, black line inside the histogram in Figure 5C). Altogether, these data confirm that CDK6-AS1 overexpression...
positively correlates with mitochondrial biogenesis signatures and mass in leukemia cells, and particularly in the CD34+ cell compartment.

These findings supported the idea that CDK6-AS1 aberrant hyper-expression maintains blasts, including the more immature CD34+ subpopulation, to meet the challenges of high energy demand and promotes drug resistance to evade apoptosis (Lapalombella, 2017; Yoshida, 2015), suggesting that approaches targeting mitochondrial dependencies (Roth et al., 2019) should impact particularly on the CDK6-AS1 Q4-AML cases. We confirmed that three of the tested compounds targeting mitochondria processes, Tigecycline (TIGE, mitochondrial translation inhibitor), Oligomycin (OLIG, mitochondrial complex V inhibitor), and Rotenone (ROT, mitochondrial complex I inhibitor), were more effective in reducing viability in CDK6-AS1 KD cells. On the other hand, Venetoclax (VEN, anti-apoptotic mitochondrial proteins),
Figure 6. TIGE sensitization to chemotherapy in high-CDK6-AS1 AML

(A) Detection of hCD45+ AML cells in bone marrow (BM) of NSG mice inoculated with shCDK6-AS1 SHI-1 cell line (low-CDK6-AS1 AML mice, left) or shNEG SHI-1 cell line (high-CDK6-AS1 AML mice, right), after 2-week treatment with TIGE 75 mg/kg or ARA-C 25 mg/kg and combination therapy (COMBO). Synergistic effect in shNEG (Combination Index CI = 0.9; mice n = 3/5 per group, *p value<0.05, **p value<0.01, ***p value<0.001, ****p value<0.0001). Data are represented as mean ± SEM.

(B) Percentage of hCD45+ AML cells detected in peripheral blood (PB) of low- (left) and high- (right) CDK6-AS1 PDXs with TIGE, ARA-C, or COMBO (after 3-week treatment *p value<0.05, ***p value<0.001 of treated mice with respect to DMSO). Monitoring of hCD45 during 3 weeks after treatment discontinuation (mice n = 4/5 per group; *p value<0.05, **p value<0.01). Synergistic effect of COMBO in high-CDK6-AS1 PDX (CI = 0.19).
Enasidenib (ENA, anti-mitochondrial IDH-2 drug), and Ivosidenib (IVO, anti-cytoplasmic IDH-1 drug) did not show differential efficacy in CDK6-AS1 KD with respect to control cells (see Figure S10, **p value<0.01, ***p value<0.001, ****p value<0.0001) probably because they are indirectly targeting mitochondria.

Mitochondrial targeting sensitizes high CDK6-AS1 AML to chemotherapy

Since TIGE was previously reported to produce preclinical results against AML (Jin et al., 2019; Skrtić et al., 2011), we focused on this compound and tested its potency as an anti-mitochondrial agent. AML cells with low (shCDK6-AS1) and high (shNEG) CDK6-AS1 expression were inoculated in NSG mice and treated with low-dose Cytarabine (25 mg/kg ARA-C) (Wunderlich et al., 2013), TIGE (75 mg/kg), and a combination strategy (ARA-C + TIGE, COMBO). We found that all treatments mediate the same leukemia reduction in shCDK6-AS1 mice (having reduced mitochondria, Figure 6A, left, **p value<0.01, ***p value<0.001), whereas the COMBO treatment decreased the percentage of CD45+ AML cells with high CD6-AS1 levels (shNEG) when compared with ARA-C and TIGE treatment alone (Figure 6A, right, *p value<0.05, **p value<0.01, ****p value<0.0001; Synergistic Combination Index<1, CI = 0.9; confirmed by spleen length, see Figure S11A, **p value<0.01, ****p value<0.0001). Then, we moved to primary AML samples previously studied for CDK6-AS1 expression levels and mitochondrial mass (see Figures S11B and S11C, ****p value<0.0001). We found that primary AML samples with low CDK6-AS1 were sensitive to all treatments, and COMBO treatment was the most effective treatment in reducing high CDK6-AS1 blasts viability (see Figure S11D, *p value<0.05, **p value<0.01, ****p value<0.0001, CI = 0.85 for high-CDK6-AS1 patient). In vivo, after engraftment, low- and high-CDK6-AS1 patient-derived xenografts (PDXs) were treated for 3 weeks showing that ARA-C and COMBO strategies were effective in reaching AML remission (Figure 6B, gray area, *p value<0.05, ****p value<0.0001). After treatment discontinuation both models relapsed, but faster in the high-CDK6-AS1 PDX animals treated with ARA-C alone with respect to the COMBO that delayed leukemia progression thanks to mito-mass targeting (Figure 4B, right, *p value<0.05, **p value<0.01, CI = 0.19). Moreover, in CD34+ cells sorted from primary AML samples mitochondrial targeting was effective and synergistically active in vitro when combined with ARA-C (Figure 4C, *p value<0.05, **p value<0.01, CI = 0.86). Finally, we observed that mice with high-CDK6-AS1 AML treated with COMBO benefited from a prolonged survival (Figure 6D). These results provide the rationale for further considering the CDK6-AS1 levels as a marker in defining those AML cases that could benefit from combined mitochondrial-targeting therapies to prevent leukemia progression.

DISCUSSION

Front-line therapy in most cancer is still mainly based on the use of chemotherapy, but, in many patients, drug resistance and persistent disease after treatment discontinuation result in tumor recurrence and poor outcome (Pession et al., 2013; Ramos et al., 2015). Our study was designed with the goal of discovering markers able to improve stratification of pediatric patients with AML, with great interest toward the non-coding transcriptome, shown to cluster pediatric AML with the same accuracy as protein coding genes and found to have the potential to dissect unexplored AML networks (Deveson et al., 2017; Schwarzer et al., 2017; Zampini et al., 2017). In this study, we identified that the lncRNA CDK6-AS1, together with its neighbor gene CDK6 (Lai et al., 2013), is a robust marker of a specific AML subgroup with features similar to those found in healthy CD34+ cells, including an early myeloid differentiation arrest and enhanced mitochondria biogenesis. Functionally, CDK6-AS1 depletion increased differentiation of CD34+ cells, this finding being consistent with the idea that CDK6-AS1 is important in regulating the balance between differentiation potential and self-renewal capacity in both normal and leukemic cells. Indeed, we produced data showing that, at the first stages of CD34+ differentiation, the more quiescent CD38- cells expressed higher CDK6-AS1/CDK6 levels, which decrease when differentiation occurs in CD38+ cells. This observation highlights that CDK6-AS1 is important in the characterization of early versus late progenitors, including CD34+ versus CD34- hematopoietic cells of both healthy and AML subjects. This finding supports the concept that aberrant CDK6-AS1/CDK6 expression during hematopoietic commitment could provide a key transforming event inducing the block of myeloid differentiation (Schmoeller et al., 2020). In the AML context, we found a clue as to how this might occur: CDK6-AS1, being hyper-expressed, modulates...
CDK6 levels and CDK6-RUNX1 interaction, inhibiting several RUNX1 promoters (Fujimoto et al., 2007) with decreased expression of differentiation factors, including PU.1, MEIS1, and RUNX1 itself. In line with these findings, reduced CDK6-AS1/CDK6 expression in AML led to the emergence of more differentiated cells with reduced in vitro clonogenic potential. This confirms CDK6-AS1/CDK6 axis playing a role in the hematopoietic differentiation program, suggesting that CDK6-AS1 aberrant hyper-expression might concur during the leukemogenesis process (Corrà et al., 2018; Laurenti et al., 2015; Schwarzer et al., 2017).

In addition, we found an association between high CDK6-AS1 levels and mitochondrial biogenesis pathway activation. Sorting healthy CD34+ cells according to their mitochondrial membrane potential, we confirmed that CDK6-AS1 levels decreased along the hematopoietic differentiation process together with the concomitant CDK6-AS1/C20 axis playing a role in the hematopoietic differentiation program, suggesting that CDK6-AS1 aberrant hyper-expression might concur during the leukemogenesis process (Corrà et al., 2018; Laurenti et al., 2015; Schwarzer et al., 2017).

To the best of our knowledge, this is the first work that demonstrates the potency of a lncRNA in controlling an axis that regulates mitochondrial homeostasis and, in concert with its neighbor gene CDK6, the myeloid differentiation process. Our findings indicate that the measurement of a single lncRNA at diagnosis might orient clinicians to specific therapeutic strategies. Further studies aimed at investigating the efficacy of mitochondrial drugs, either alone or in combination with conventional chemotherapy, will be instrumental to further corroborate our data and to pursue novel strategies able to reduce relapse occurrence in children with high risk of relapse in AML.

Limitations of the study

Since this study is based on retrospective analyses, the potential bias from different cohorts of patients is inevitable. The investigation of the CDK6-AS1 expression profile and the validation of its role in resistance to therapy in a larger population is recommended. Furthermore, additional efforts should be performed to deeply analyze the CDK6-AS1 mechanism in leukemia. Moreover, our findings encourage the use of other anti-mitochondrial agents to significantly improve AML sensitivity to standard chemotherapy.

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- Mitochondrial membrane potential
- PLA analysis
- Immunoblotting
- In vivo experiments

QUANTIFICATION AND STATISTICAL ANALYSIS
- Statistical analyses

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103350.

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AUTHOR CONTRIBUTIONS
Conceptualization, M.P., E.P., M. Benetton, V.B.; methodology, A.D.R., E.P., V.B., M. Benetton; formal analysis, C.Z., E.P., M. Benetton, V.B.; investigation, E.P., M. Benetton, V.B., G.B., A.D.R., C.T., G.G., M. Bordi, S.C.; resources, M.P., F.L., D.S.R., S.M.; writing – original draft, M.P., E.P., M. Benetton, V.B., D.S.R., F.L.; writing – review & editing, M.P., E.P., M. Benetton; supervision, M.P.; funding acquisition, M.P., F.L.

DECLARATION OF INTERESTS
D.S.R. is an inventor on a patent, assigned to UCLA, relating to “Methods and compositions involving lincRNA and leukemia; all other co-authors declare no competing financial and non-financial interests.
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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CDK6+RUNX1 Protein Interaction Ab Pair (for PLA) | Abcam | Cat# DI0411 |
| anti-CDK6           | Santa Cruz Biotecnology | Cat# sc-7961 |
| TOMM20             | Abcam | Cat# ab56783 |
| PU1                | Santa Cruz Biotecnology | Cat# sc-352 |
| β- ACTIN           | Sigma-Aldrich S.r.l. | Cat# A5316 |
| α-TUBULIN          | Sigma-Aldrich S.r.l. | Cat# T9026 |
| GAPDH              | GeneTex | Cat# GTX627408 |
| anti-rabbit horseradish peroxidase–conjugated secondary antibody | Santa Cruz Biotecnology | Cat# sc-2357 |
| anti-mouse horseradish peroxidase–conjugated secondary antibody | PerkinElmer | Cat# NEF822001EA |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 | ThermoFisher Scientific | Cat# A-11032 |
| CD34-PC7           | Becton Dickinson | Cat# 348811 |
| CD38-APC-A750      | Becton Dickinson | Cat# B49200 |
| CD33-APC           | Beckman Coulter | Cat# IM2467 |
| CD11b-PE           | Beckman Coulter | Cat# IM2581U |
| CD15-FITC          | Beckman Coulter | Cat# 332778 |
| CD14-PC7           | Beckman Coulter | Cat# A22331 |
| CD66b-PE           | Becton Dickinson | Cat# 561650 |
| CD45-EC            | Beckman Coulter | Cat# A07784 |
| CD16-PC5           | Beckman Coulter | Cat# A07767 |
| CD164-PerCP Cy5.5  | Becton Dickinson | Cat# 561196 |
| Lin1-FITC anti-human Lineage cocktail 1 | Becton Dickinson | Cat# 340546 |
| CD90-PC7           | Becton Dickinson | Cat# 561558 |
| CD45-RA-PE         | Becton Dickinson | Cat# MHCD458A04 |
| CD10-PC7           | Becton Dickinson | Cat# A46527 |
| CD7-FITC           | Becton Dickinson | Cat# 332773 |
| CD135-PE           | Becton Dickinson | Cat# IM2234U |
| CD38-eFluor450 for PrimeFlow RNA Assay | Invitrogen | Cat# 48-0389-42 |
| CD34-APC-eFluor780 for PrimeFlow RNA Assay | Invitrogen | Cat# 47-0349-42 |
| CDK6-AS1 RNA probe for PrimeFlow RNA Assay | Invitrogen | Cat# VA1-3024816-PF |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Trizol             | Invitrogen | Cat# 15596026 |
| human GM-CSF       | Milteny Biotec | Cat# 130-093-864 |
| interleukin3 (rhIL-3) | Milteny Biotec | Cat# 130-093-909 |
| interleukin6 (rhIL-6) | Milteny Biotec | Cat# 130-093-931 |
| stem cell factor (rhSCF) | Milteny Biotec | Cat# 130-096-694 |
| thrombopoietin (rhTPO) | Milteny Biotec | Cat# 130-095-747 |

(Continued on next page)
### REAGENT or RESOURCE  | SOURCE  | IDENTIFIER  
--- | --- | ---  
Fms-like tyrosine kinase-3 ligand (rhFLT-3 ligand)  | Milteny Biotec  | Cat# 130-096-479  
Fibronectin  | Corning  |  
Methylcellulose semisolid (Methocult)  | StemCell Technologies  | Cat# H4230  
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyldtetrazolium bromide (MTT)  | Sigma-Aldrich S.r.l.  | Cat# M5655  
Nocodazole  | Sigma-Aldrich S.r.l.  | Cat# M1404  
RNase A  | Qiagen  | Cat# 1045705  
ATRA (Retinoid Acid)  | Sigma-Aldrich S.r.l.  | Cat# R2625  
Tigecycline (TIGE)  | Selleckchem  | Cat# S1403  
Oligomycin (OLIG)  | Sigma-Aldrich S.r.l.  | Cat# O4876  
Rotenone (ROT)  | Sigma-Aldrich S.r.l.  | Cat# R8875  
Venetoclax (VEN) ABT-199  | Sigma-Aldrich S.r.l.  | Cat# ADV465750285  
Enasidenib (ENA)  | Selleckchem  | Cat# S8205  
Ivosidenib (IVO)  | Selleckchem  | Cat# S8206  
Cytarabine (ARA-C)  | Sigma-Aldrich S.r.l.  | Cat# PHR1787  
DAPI  | Sigma-Aldrich S.r.l.  | Cat# 10236276001  
Hoechst33342  | Invitrogen  | Cat# H3570  
Pyronin Y  | Sigma-Aldrich S.r.l.  | Cat# 83200  
Latex Beads, fluorescent red  | Sigma-Aldrich S.r.l.  | Cat# L3030  
Propidium Iodide (PI) Staining Solution  | Becton Dickinson  | Cat# 556463  

#### Critical commercial assays  
SuperScript II Reverse Transcriptase  | Invitrogen  | Cat# 18064014  
SYBR Green PCR master mix  | Applied Biosystems  | Cat# 4309155  
Cytoplasmic & Nuclear RNA Purification Kit  | Norgen biotek corp.  | Cat# 21000  
anti-human CD34 microbeads  | Miltenyi Biotec  | Cat# 130-046-702  
Dual-Luciferase Reporter Assay  | Promega Corporation  | Cat# E1910  
Duolink® In Situ Red Starter Kit Mouse/Rabbit (for PLA)  | Sigma-Aldrich S.r.l.  | Cat# DUO92101-1KT  
CellTiter-Glo® assay  | Promega Corporation  | Cat# G7572  
PrimeFlow RNA Assay  | Invitrogen  | Cat# 88-18005-204  
TMRE-Mitochondrial Membrane Potential Assay Kit  | Abcam  | Cat# ab113852  

#### Deposited data  
HTA expression data  | Tregnago et al. (2016)  | GSE75461  
Microarray-based profiling of ncRNAs in normal hematopoiesis  | Schwarzer et al. (2017)  | GSE98633  
ChiP-seq data  | McKeown et al. (2017)  | PRJNA381888  

#### Experimental models: Cell lines  
SHI-1  | DSMZ  | Cat# ACC 645  
HL-60  | DSMZ  | Cat# ACC 3  
U-937  | DSMZ  | Cat# ACC 5  

#### Experimental models: Organisms/strains  
NSG (NOD/scid IL-2Rgnull)  | Charles River laboratories  | 005557  

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martina Pigazzi (martina.pigazzi@unipd.it).

Materials availability
All developed expression plasmids produced in this study can be made available upon request to the lead contact.

Data and code availability
This paper analyses existing, publicly available data. These accession numbers for the datasets are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients’ samples
Patients with de novo AML (N=132) other than acute promyelocytic leukemia, aged 0–18 years, diagnosed between 2002 and 2014 were included in this study (see Table S1A). These children were treated according to the same protocol, namely the AIEOP AML 2002/01. FAB morphological classification, immunophenotypic analysis and molecular characterization were centrally reviewed. Bone marrow (BM) samples were processed for RNA isolation and PCR analysis. PCR for recurrent rearrangements, such as those involving CBF, MLL, FLT3, and other rare mutations, were performed (Masetti et al., 2013; Pigazzi et al., 2011, 2012). In accordance with the Declaration of Helsinki, informed consent to provide samples for biological studies was obtained from patient’s parents or legal guardians.

Cell lines
SHI-1 leukemia cell line was bought at DSMZ-Collection of Microorganisms and Cell Cultures, Germany and cultured in DMEM 10% FBS (Invitrogen, Carlsbad, CA) for around 20-30 passages (maximum) from thawing. Analogously HL-60 and U-937 were cultured in RPMI 10% FBS (Invitrogen, Carlsbad, CA). Mycoplasma absence was tested using a commercial kit (Minerva Biolabs, Berlin, DE).

Primary cell cultures
Human CB was collected after obtaining a written informed consent for research use from the delivering women. Mononuclear cells were isolated from CB samples by density gradient centrifugation at 400 g for 20 min, using Ficoll solutions. In order to isolate CD34+ and CD34− cells, samples were incubated with anti-human CD34 microbeads. Primary healthy CD34+ cells were cultured in RPMI1640 10% FBS supplemented with recombinant human (rh) cytokines: interleukin6 (rhIL-6; 20 ng/mL), stem cell factor (rhSCF;
100 ng/mL), thrombopoietin (rhTPO; 20 ng/mL) and fms-like tyrosine kinase-3 ligand (rhFLT-3 ligand; 100 ng/mL).

Primary AML cells were cultured in RPMI1640 10% FBS with the addition of recombinant human (rh) cytokines: interleukin3 (rhIL-3; 20 ng/mL), rhIL-6; 20 ng/mL, rhSCF; 50 ng/mL, rhTPO; 50 ng/mL and rhFLT-3 ligand (50 ng/mL) (Tregnago et al., 2020). All cell cultures were maintained at 37°C, 5% CO2.

Animal models
Procedures involving animals and their care were in accordance with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December 1987) and with “ARRIVE” guidelines (Animals in Research Reporting In Vivo Experiments). Ministry authorization approved: 622/17-PR, 512/19-PR. NSG mice (of 4-8 weeks old, randomized for sex, 20-25 g/mouse, maximum 5 animals/cage) were injected intravenously (tail vein) with 1x10^6 human CD45 (hCD45) cells derived from PDX-P3 (biobank).

METHOD DETAILS
Quantitative real-time PCR
Total RNA was isolated using Trizol. One microgram RNA was reverse-transcribed into cDNA using SuperScript II. Expression of mRNA were measured by Real Time PCR (RQ-PCR) on an ABI 7900HD platform (Applied Biosystems, Foster City, CA) using the SYBR Green PCR master mix and normalized on GUSB using the 2^-ΔΔCt method. Cytoplasmic & Nuclear RNA Purification Kit was used for cytoplasmic or nuclear RNA fractionation. Ratio were calculated as 2^-ΔCt (Schwarzer et al., 2017).

GEP and correlation analyses
RNA from bone marrow (BM) cells collected at diagnosis from 58 patients was hybridized on the GeneChip® Human Transcriptome Array 2.0 (HTA, Affymetrix, Santa Clara, CA, USA). Microarray expression data are deposited in GEO database under accession number GSE75461 (Tregnago et al., 2016).

Briefly, 100 ng of total RNA were labeled and hybridized to Affymetrix GeneChip for 16 h at 45°C using a rotational oven and washed according to Affymetrix standard protocols using a GC450 Fluidics Station. RNA quality was assessed on an Agilent2100 Bioanalyzer (Agilent Technologies).

The GeneChips were scanned with an Affymetrix 7G scanner and the CEL files generated were analyzed through Affymetrix Expression Console Software (version 1.3) which normalizes array signals using a robust mult array averaging (RMA) algorithm. Probes were re-annotated using GENECODE v.19 gene annotation database (www.genecodegenes.org) in order to identify both coding and non-coding RNAs covered by probes. Transcripts were included if at least the 95% of nucleotides overlapped with probes. In miRNAs analysis, for every probe which recognizes pre-miRNA, we used both associated mature miRNA, where available. Normalized data were analyzed using GENE-E analysis platform (Broad Institute of Harvard and MIT, Boston, MA). Arraystar Human LncRNA microarray V2.0 probes (GPL23434 platform) were mapped to RefSeq genes. Microarray expression data are deposited in GEO database under accession number GSE75461 (Tregnago et al., 2016).

Coding and noncoding gene expression data of human blood cells from healthy donors were downloaded as Series Matrix File of the GSE98633 dataset (Schwarzer et al., 2017) from the GEO database. Thirty-three samples from the dataset were selected and distinguished into “mature Granulocytes purified from peripheral blood”, “CD14+ Monocytes purified from peripheral blood”, “purified erythroblasts in vitro derived”, “purified megakaryocytic progenitors in vitro derived”, “CMP purified from cord blood”, “GMP purified from cord blood”, “CD34+ CD38- HSPC purified from cord blood”, “CD14+ CD38- HSPC purified from fetal liver” and “G-CSF-mobilized CD34+ CD38-HSPC purified from peripheral blood”.

For CDK6-AS1 interaction network from gene expression data, we ranked the genes according to their absolute Spearman’s rank correlation coefficient across all the selected samples with the expression profile of the probe detecting the first exon of both CDK6 and CDK6-AS1 genes, using the ‘cor’ function within the ‘stats’ package of the R software. The top 1% correlated values associated to 251 probes, and mapping to
337 RefSeq transcripts, were then used to perform an unsupervised clustering by using GSE98633 dataset (Schwarzer et al., 2017).

Unsupervised clusterings were created using the ‘heatmap.plus’ function from the heatmap.plus’ R package with the ‘canberra’ as distance method and ‘ward.D2’ as the clustering algorithm and visualized using the ‘heatmap3’ function from the ‘hetamap3’ package.

Boxplots were produced using the ‘boxplot’ function of the ‘graphics’ R package with the Wilcoxon test p-values calculated by means of the ‘wilcox.test’ function of the ‘stats’ package.

For each sample from PRJNA381888 (McKeown et al., 2017) and GSE75461 (Tregnago et al., 2016) datasets, expression and ChIPseq signals within 5 Mb from the CDK6/CDK6-AS1 genes were firstly mapped within overlapping 100 kb intervals and the resulting binned values were subsequently correlated to the value of the bin harboring the CDK6/CDK6-AS1 shared exon1. Smoothed averages were calculated using the ‘smooth.spline’ function form the ‘stats’ R package.

**t-SNE**

Dimensionality reduction clustering by means of t-distributed stochastic neighbor embedding (t-SNE) was performed using the ‘Rtsne’ function of the ‘Rtsne’ R package after calculating inter-samples distances with the ‘dist’ function of the ‘stats’ R package by means of the ‘maximum’ distance method and plotted using the ‘plot’ function of the ‘graphics’ R package.

**Gene Set Enrichment Analysis (GSEA)**

GSEA was performed using the ‘Signal2Noise’ metric for ranking genes and tested against the c5.all.v6.2.symbols.gmt Gene Ontology sets database with 5000 permutations and a ‘weighted_p2’ enrichment statistic. A leading edge analysis was then performed on the resulting gene sets hits showing both nominal p-values lower than 0.05.

**Cell treatments**

Cells were treated with Tigecycline (TIGE), Oligomycin (OLIG) Rotenone (ROT), Venetoclax (VEN), Enasidenib (ENA), Ivosidenib (IVO), Cytarabine (ARA-C) for dose-response evaluation. After GI50 calculation, cells were treated with 0.6 \( \mu \text{M} \) of ARA-C, 60 \( \mu \text{M} \) of TIGE or COMBO (ARA-C+TIGE).

**Cell transfection and lentiviral transduction**

Three specific p2CZL short hairpin RNAs (shRNAs1, 2 and 3) were kindly provided by Dr. Rao (Fernando et al., 2015) and used to silence CDK6-AS1 both transiently (pool of three) and stably.

Transient cell transfection was performed using the Cell Line Nucleofector® Kit V.

Two specific siRNAs, one mapping at 3’UTR and one in the coding region, were adopted to silence CDK6. We used the combination of the two oligos for a better silencing effect. Transfected cells were screened by RQ-PCR for CDK6 and CDK6-AS1 expression, CDK6 protein level was checked by western blot, and cells were used for in vitro experiments.

Third-generation lentiviral vectors were produced to generate CDK6-AS1 stable knockdown constructs (Fernando et al., 2015). A total of 14x10⁶ SHI-1 and 1x10⁵ CD34+ cells were spin-infected at 37°C overnight in the presence of polybrene & µg/ml (Manara et al., 2014). Transduced cells were single-cell sorted for GFP expression (MoFlow XDP FACS (Beckman Coulter, Brea, CA). Cells were screened by RQ-PCR for CDK6-AS1 expression and best clones were selected for expansion and in vitro experiments: we referred to shRNA 1 colony 2 (namely “shCDK6-AS1”) for the best downregulation of both CDK6-AS1 and CDK6, but other independent clones were used and presented in Supplemental Figures.

**Plasmids construction**

For CDK6 overexpression, we generated a new construct starting from pHAGE-CDK6 plasmid (gift from Gordon Mills & Kenneth Scott (Ng et al., 2018)) and pEGFP-N1 (Pigazzi et al., 2008). In detail, we cut pHAGE-CDK6 with Ncol and pEGFP-N1 with EcoRI, used DNA Polymerase I Large (Klenow) Fragment
(Promega) to generate blunt ends, then cut both plasmids using NotI. After this second digestion, we subcloned the CDK6 fragment into pEGFP-N1 backbone, obtaining pCDK6 plasmid. Both pCDK6 and pEGFP-N1, used as control vector and indicated as pØ, were transiently transfected in SHI-1 cell line for in vitro validations.

**Plasmids construction and promoter activity assay**

We generated reporter vectors to verify CDK6/CDK6-AS1 bidirectional promoter activity. By in silico prediction, we selected a 1.17 kb genomic fragment of chromosome 7 (hg19: 5'-92464746-92465919-3'), amplified it using a pair of primers including the restriction sites for XhoI and HindIII (Forward primer-XhoI CTCCAGAGAGAAGGACAGCAATG, Reverse primer-HindIII AAGCTTCCGAGAGAGTGCTGG TAAC), and cloned it into the pGL4.28[luc2CP/minP/Hygro] luciferase vector, obtaining pGL4-CDK6-AS1/CDK6-LUC plasmid. We subsequently constructed the double luciferase vector, by amplifying the luc2CP gene of the pGL4.28 (Forward primer-KpnI GGTACCCCCGACTCTAGACTGCAGTT, Reverse primer-NheI GCTAGCGGCAATCCGGTACTGTTGGT) and cloning it in 3'-5' direction upstream the promoter region, obtaining pGL4-LUC-CDK6-AS1/CDK6-LUC plasmid. Then, we knocked-out luc2CP gene from the pGL4-CDK6-AS1/CDK6-LUC plasmid, by mutagenesis of the ATG start codon (Q5/C226 site-directed mutagenesis Kit, New England BioLabs, Ipswich, MA - Forward primer AAAGATGCCAAAAACATTAGAAG, Reverse primer ATGGTGGCTTTACCAACAG), producing the pGL4-CDK6-AS1/CDK6-ΔLUC plasmid. To test luciferase activity, we transfected SHI-1 shNEG and shCDK6-AS1 stable cell lines with 0.6 mg of each of these vectors, together with 0.06 mg of Renilla, using the Nucleofector system, and measured luciferase activity 6 hours after transfection (Dual-Luciferase Reporter Assay).

**RNA detection by flow cytometry**

To investigate the expression of CDK6-AS1 in primary AML samples by flow cytometry, we took advantage of the PrimeFlow RNA Assay, an innovative technique consisting of an in situ hybridization to detect RNA transcripts together with cell surface markers by flow cytometry. Following manufacturer's instructions, cells were stained for the surface markers using anti-CD34 and CD38 antibodies. Next, cells were fixed and permeabilized to allow the binding of the target RNA probe for CDK6-AS1 to its specific transcript inside the cell. Cells were analyzed by flow cytometry by gating on CD34+/CD38- cells to evaluate the median fluorescence intensity for CDK6-AS1.

**Cell viability and cell cycle**

Cell viability assays were performed using CellTiter-Glo® assay. Briefly, 5x10^4 cells were cultured in sterile 96-well plates and incubated for 24 to 48 hours; then, 90 μl of CellTiter-Glo® reagent was added and after 15-minute incubation at room temperature, luminescence was recorded (VICTOR, Perkin Elmer, Milan, IT) with an integration time of 1s per well. The combination index (CI = (Drug1 effect + drug2 effect) / combo effect (Fouquier and Guedj, 2015)) was calculated to evaluate the synergistic effect (CI<1). For cell-cycle analyses, 16-hour incubation with 300 nM nocodazole was used to synchronize in G2/M phase. The synchronized cells were washed with PBS and released from the mitotic block by the addition of normal serum-containing media and fixed in 70% cold-ethanol for at least 1 hour at −20 °C. Fixed cells were incubated in propidium iodide (PI) staining buffer (PBS containing RNase A (50 μg/ml), 0.1% sodium citrate, and PI (50 μg/ml) for 30 minutes at room temperature (RT). Cells were analyzed by flow cytometry and the cell distribution was determined using the FlowJo software.

**Cell differentiation**

Differentiation markers were detected by flow cytometry. Phagocytosis capability was assessed adding Latex Beads at a ratio of 1:500 with or without the addition of 30 ng/ml GM-CSF. Cells were analyzed by flow cytometry and representative images were captured after centrifugation by cytopsin or seeding cells on fibronectin (40 μg/ml)-coated chamber slides and stained by May–Grünwald Giemsa method.

At the indicated times, cells were measured by flow cytometry for the expression of CD11b and CD16 after 24-48 hours of CDK6-AS1 or CDK6 knockdown (Tregnago et al., 2016). Normal CD34+ cells were analyzed for surface markers expression by staining with CD34, CD38, CD164, Lin1 (anti-human Lineage cocktail 1, including CD3, CD14, CD16, CD19, CD20, CD56), CD90, CD45RA, CD33, CD66b, CD10, CD7, CD15, CD135.
**Colony forming assay**
After CDK6-AS1 silencing, a total of $2 \times 10^5$ SHI-1 cells were seeded onto a minimum methylcellulose semi-solid dish (Methocult) and incubated at 37°C. Fourteen days later, colonies were counted by light microscopy after incorporation of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

**Cell quiescence**
Hoechst33342 and Pyronin Y staining was used to discriminate quiescent cells, in G0 phase (Hoechst-/ Pyronin Y-), with respect to G1 and S-G2/M phases, in TMRE-sorted normal CD34+ cells. After cell sorting, cells were incubated with Hoechst33342 10 μg/ml and Pyronin Y 3 μg/ml, for 45 and 15 minutes respectively.

**Mitochondrial mass quantification**
Immunofluorescence was performed either in cytopsins of transient or stable CDK6-AS1 knockdown cells cultured in suspension or in chamber slides after 30 minutes of fibronectin (40 μg/ml) coating at 37°C. Cells were incubated overnight at 4°C with primary antibody anti-TOMM20 to visualize mitochondria and then with goat anti-mouse IgG (H+L) secondary Antibody, Alexa Fluor 594 for 1h. DAPI/Hoechst 33342 were used to label nuclei. Images were acquired using confocal microscope ZEISS LSM800. The mitochondrial area was quantified using Fiji-ImageJ.

**Mitochondrial membrane potential**
TMRE-Mitochondrial Membrane Potential Assay Kit was used for measuring the mitochondrial membrane potential. Briefly, TMRE 200 nM diluted in BSA 0.2%-PBS 1x was added at 37°C. After 20' incubation, cells were analyzed by flow cytometry using Cytomix FC500 or CytoFLEX. Normal CD34+ cells were sorted for TMRE MFI levels (MoFlow XDP FACS, Beckman Coulter, Brea, CA).

**PLA analysis**
*In situ* proximity ligation assay (PLA) experiments were performed by using DuoLink II. Cells were incubated overnight at 4°C with anti-RUNX1 and anti-CDK6 antibody in diluent buffer. Samples were then washed with PLA washing buffer and incubated with PLA probes anti-rabbit-PLUS and anti-mouse-MINUS. The subsequent washing steps, ligation, rolling-circle amplification and fluorescent labeling with DuoLink II Detection Reagent Red were performed according to the manufacturer’s instructions. Slides were mounted in vector shield Mounting Media with DAPI; Zeiss LSM 510 microscope equipped with a Zeiss image processing system was used for confocal imaging analysis processed with identical settings for all conditions.

**Immunoblotting**
Equal amounts of proteins (10 μg) were resolved by SDS PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking for at least 1 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies anti-ß-ACTIN, anti-GAPDH, anti-α-TUBULIN, anti-CDK6, TOMM20 and then incubated with horseradish peroxidase-labeled secondary antibodies, either anti-rabbit or mouse, for 60 min. All membranes were visualized using ECL Select and exposed to iBright FL1500 (ThermoFisher) (Bisio et al., 2017). Signal was quantified by Fiji-ImageJ software.

**In vivo experiments**
NSG mice (of 4-8 weeks old, randomized for sex) were injected intravenously (tail vein) with $1 \times 10^6$ human CD45 (hCD45) cells derived from PDX-P3 (biobank). After 1 month from leukemic cell injection, tumor burden was assessed weekly by flow cytometric measurement of hCD45 positive cells in peripheral blood (PB). Treatment was delayed until leukemia burden was around 5% in PB. For the *in vivo* experiment performed using shNEG and CDK6-AS1 KD cell line, NSG mice were injected intravenously with $1.5 \times 10^6$ cells and treatments started after 5 days from cells inoculation. Mice were randomized into 4 groups (n=3/5) and treated with TIGE 75 mg/kg, ARA-C 25 mg/kg (low dose), combination of TIGE 75 mg/kg + ARA-C 25 mg/kg (COMBO), and control group (DMSO). Drugs were given daily with weekend wash out (for three weeks in PDXs, whereas mice injected with SHI-1 were treated for two weeks), by intraperitoneal (i.p.) injection. Treatment efficacy was assessed by flow cytometric measurement of hCD45 positive cells; in particular for PDXs we evaluated peripheral blood (PB), whereas SHI-1-injected mice were evaluated at sacrifice for BM invasion and spleen dimension. TIGE and ARA-C powder were dissolved in DMSO to prepare stock solutions that were freshly diluted in saline solution for i.p. injection. Appropriate vehicle was used for
control group. Mice weight was monitored twice a week in order to adjust the correct dose of drugs during treatment.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analyses**

The t-test was adopted for detecting significance between differences in means; the Wilcoxon test was used as a non-parametric alternative to the Student’s t-test. Overall survival was estimated using the Kaplan–Meier method and differences between groups were compared through the log-rank Mantel-Cox test. Graphs and associated statistical analyses were generated using GraphPad Prism 8. All data are presented as mean± standard error of the mean (SEM). *p-value<0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001 were considered to be statistically significant.