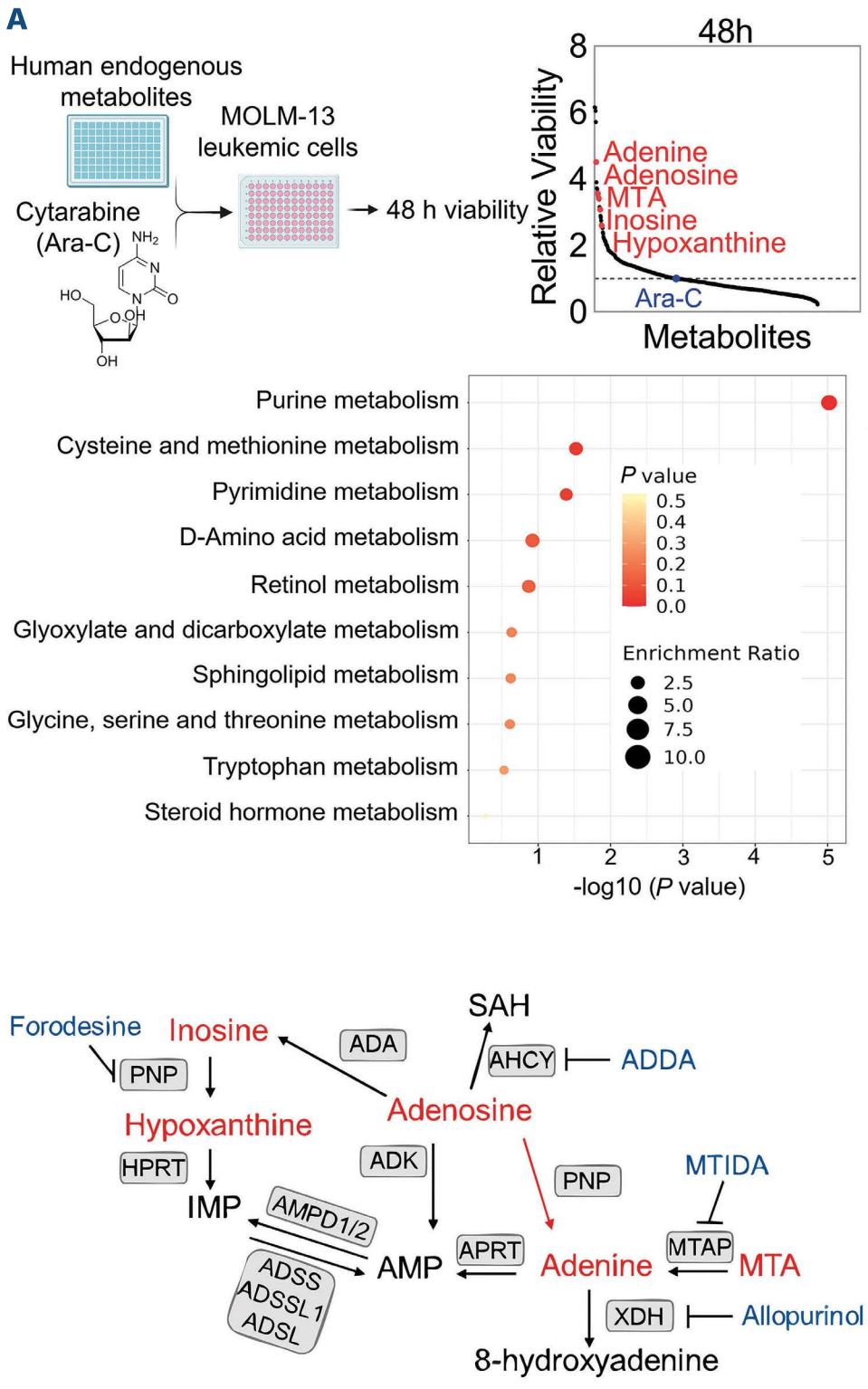
Purine metabolites regulate leukemic cell sensitivity toward cytarabine

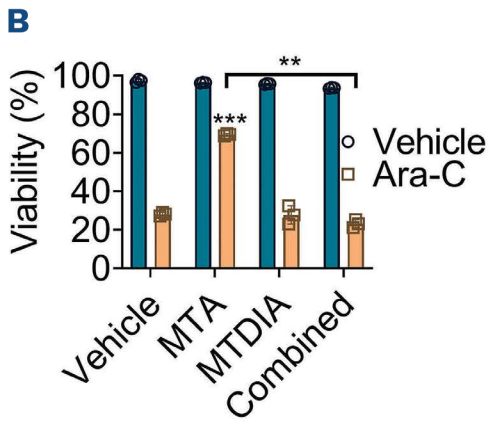
While cytarabine (Ara-C) remains a first-line chemotherapy drug for treating acute myeloid leukemia (AML), resistance to Ara-C and poor prognosis continue to be an unresolved clinical problem. Metabolites are involved in regulating leukemic cell chemosensitivity.[1-4] However, due to the complexity and diversity of metabolites, few chemosensitivity-modulating metabolites have been identified. To this end, we employed a library composed of 686 human endogenous metabolites and performed a high-throughput screen (HTS) to examine the viability of leukemic cells upon Ara-C treatment in the presence or absence of these metabolites (Figure 1A). We first focused on the metabolites that promote the survival of MOLM-13 leukemic cells. We identified 25 metabolites increasing the cell viability by at least 2.5-fold upon Ara-C treatment (*Online Supplementary Table S1*). Of these metabolites, 5'-methylthioadenosine (MTA), adenine, adenosine, inosine, and hypoxanthine are all involved in purine metabolism. Enrichment analysis also demonstrated that purine metabolism represents the most significantly enriched group (Figure 1A). Protective effects of these five metabolites are confirmed in leukemic cell lines and in leukemia stem cells (LSC) enriched $CD34^+$ AML patient cells (*Online Supplementary Figure S1A*) (specimen acquisition was approved by Shenzhen People's Hospital Review Board, approval date August 9, 2024; #LL-KY-2023089-01). Further, these metabolites are found to be enriched in the bone marrow (BM) serum of MOLM-13 leukemic mice following Ara-C treatment (*Online Supplementary Figure S1B*) (animal experiments in this study were approved by Institutional Animal Care and Research Advisory Committee at Fudan University, approval date January 7, 2024; #IDM2024017). This enrichment may result from metabolites released during chemotherapy-induced cell death, indicating that Ara-C chemotherapy might create a protective metabolic niche. Additionally, in two cohorts of AML patients, higher levels of related purine metabolism enzymes are associated with poorer survival outcomes (*Online Supplementary Figure S1C, D*).

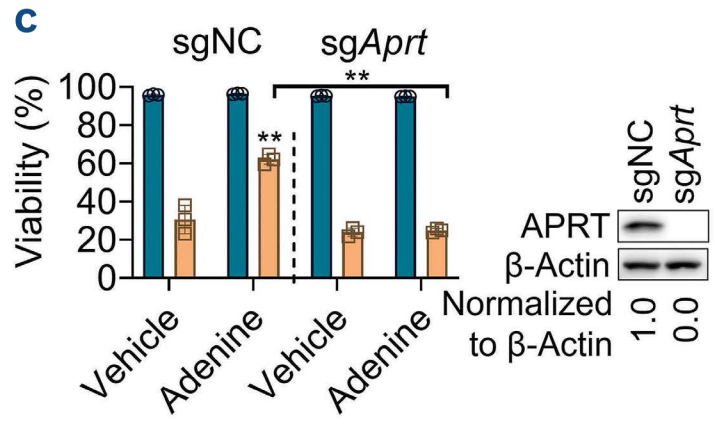
We then investigated the underlying mechanisms for purine metabolite-induced chemoresistance. We first focused on MTA, which can be converted to adenine by methylthioadenosine phosphorylase (MTAP). Since adenine protects leukemic cells from Ara-C, we tested whether the protective effect of MTA depends on converting to adenine. Treatment with MT-DADMe-ImmA (MTDIA), an MTAP inhibitor, eliminates MTA's protection against Ara-C (Figure 1B). Similar results were observed in MTAP-knockout (KO) and MTAP-knockdown (KD) cells (*Online Supplementary Figure S1E, F*). Adenine can be further converted to AMP by adenine phosphoribosyltransferase (APRT) or to 8-hydroxyadnine by xanthine dehydrogenase (XDH). Allopurinol, an XDH inhibitor, does not impact adenine-induced chemoresistance (*Online Supplementary Figure S1G*). In contrast, adenine no longer protects leukemic cells against Ara-C in APRT-KO and APRT-KD cells (Figure 1C; *Online Supplementary Figure S1H*).

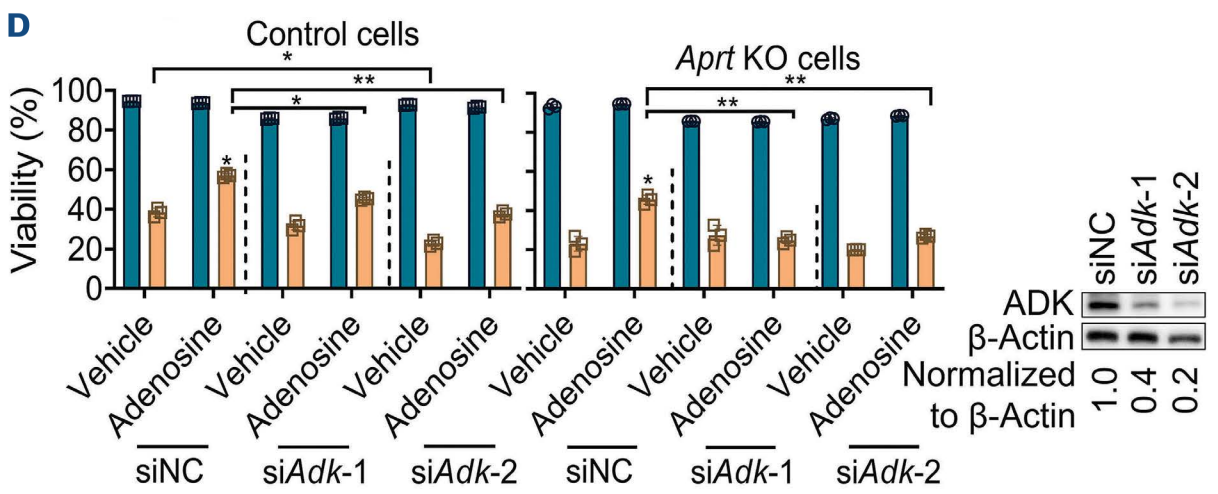
Adenosine can enter multiple metabolic pathways. Given that inosine is identified as a protective metabolite, we postulated that adenosine promotes survival through the adenosine-to-inosine metabolic pathway. Surprising, pentostatin, an adenosine deaminase inhibitor (ADAi), does not diminish the protective effect of adenosine; rather, it enhances adenosine-induced chemoresistance (*Online Supplementary Figure S1I*). We then explored alternative metabolic pathways. Treatment with adenosine dialdehyde (ADDA), an adenosyl homocysteinase inhibitor (AHCYi), does not affect the protective effect of adenosine (*Online Supplementary Figure S1J*). In contrast, knocking down adenosine kinase (ADK) reduces the protective affect of adenosine (Figure 1D). Intriguingly, when the purine nucleoside phosphorylase inhibitor (PNPi), forodesine, is employed, the protective effect of adenosine is also significantly reduced (Figure 1E). PNP is responsible for converting inosine to hypoxanthine. When PNP is inhibited, inosine-induced protection disappears (*Online Supplementary Figure S1K*), indicating that conversion to hypoxanthine is necessary for inosine-induced chemoresistance. When ADK is knocked down in APRT-KO cells, a condition in which both ADK- and APRT-mediated AMP production is blocked, adenosine no longer promotes survival (Figure 1D). These results indicated that in leukemic cells, PNP also converts adenosine to adenine, which APRT then converts to AMP to induce Ara-C resistance. More importantly, PNPi can be utilized to chemo-sensitize leukemic cells *in vivo* (Figure 1F). Therefore, we identified two adenosine-mediated chemoprotective pathways, one involving PNP and the other ADK. Our results suggest that MTA, adenine and adenosine must be converted to AMP to protect leukemic cells from Ara-C. Similarly, inosine and hypoxanthine require conversion to AMP to induce Ara-C resistance, as KD of hypoxanthine guanine phosphoribosyltransferase (HPRT) or adenylosuccinate lyase (ADSL) eliminates their protective effects (*Online Supplementary Figure S1L, M*).

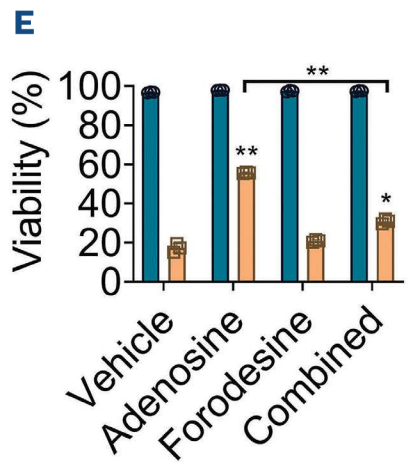
To uncover why converting to AMP is critical for purine metabolites-induced Ara-C resistance, we labeled leukemic cells with either $^{13}C_1$-adenine or $^{13}C_{10}$, $^{15}N_5$-adenosine and analyzed the final metabolites produced, both with and without Ara-C treatment. dATP is the most increased











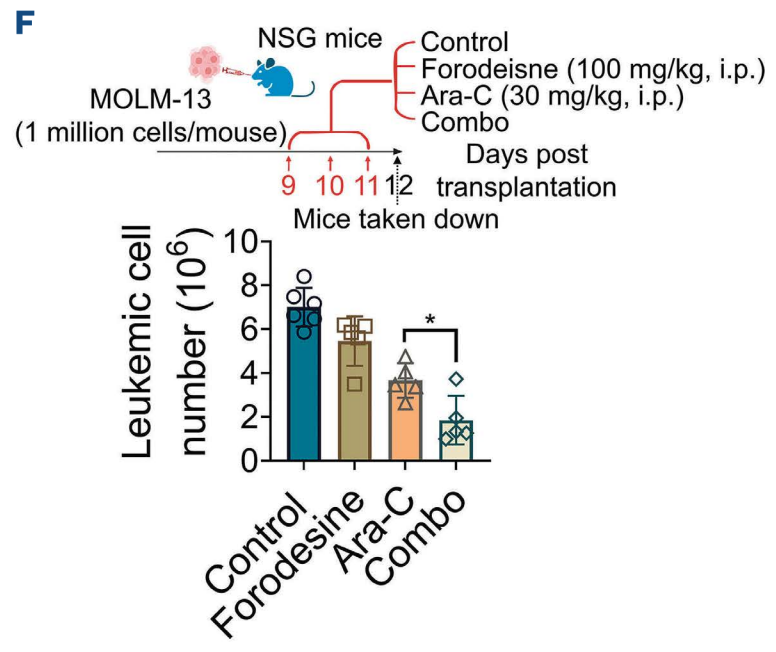


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Figure 1. Purine metabolites induce cytarabine resistance. (A) A high-throughput screen (HTS) was conducted to identify metabolites with chemosensitivity regulatory functions. The relative viability of leukemic cells post cytarabine (Ara-C) (0.2 μM) treatment was examined. Enrichment analysis was performed on the 25 identified metabolites promoting survival of leukemic cells. The metabolic pathways of the 5 selected purine metabolites are shown, where red indicates the selected metabolites, blue denotes enzyme inhibitors, and red arrow represents pathways identified in this study. (B) Viability of MOLM-13 leukemic cells treated with Ara-C combined with 5'-methylthioadenosine (MTA) and the methylthioadenosine phosphorylase (MTAP) inhibitor MTDIA (10 nM) (N=3). (C) Viability of control and adenine phosphoribosyltransferase (APRT) knockout (KO) cells treated with Ara-C combined with 10 μM adenine (N=3). (D) ADK was knocked down by small interfering RNA (siRNA) in control and APRT-KO leukemic cells. Cells were then treated with Ara-C combined with 10 μM adenosine. Viability was accessed 48 hours (h) post-treatment (N=3). (E) The 48-h viability of leukemic cells treated with Ara-C combined with purine metabolites and the purine nucleoside phosphorylase (PNP) inhibitor forodesine (100 nM) (N=3). (F) MOLM-13 leukemic mice were treated with Ara-C or forodesine or Ara-C combined with forodesine (combo) for 3 consecutive days. Leukemic cell number in 1 hindleg (tibia + femur) was accessed (N=5-6). All data are represented as mean ± standard deviation. Quantification is provided for each gel image. $^{*}P\leq0.05$; $^{**}P\leq0.01$; $^{***}P\leq0.001$; $^{****}P\leq0.0001$.

labeled metabolite upon Ara-C treatment (Figure 2A). Interestingly, although GMP production from adenine is elevated, inhibiting inosine-5'-monophosphate dehydrogenase (IMPDH) does not impact the protective effect of adenine and supplementing with guanosine or guanine does not affect Ara-C toxicity toward leukemic cells (*Online Supplementary Figure S2A, B*).

With dual labeling by ^{13}C and ^{15}N, we traced adenosine metabolic pathways in leukemic cells. Consistent with Figure 1 results, adenosine can be converted to AMP by multiple pathways (Figure 2B). Interestingly, we found that ADK-generated AMP is converted to IMP, which can then be recycled back to AMP (Figure 2B). Further, in the presence of Ara-C, AMP production via the ADA-mediated pathway decreases, while AMP production through alternative pathways increases (Figure 2B). These data align with our findings that inhibiting ADK or PNP impairs adenosine-induced Ara-C resistance, while ADA inhibition does not affect this resistance. More importantly, dATP produced from all these pathways, including the ADA-mediated one, is increased in the presence of Ara-C (Figure 2B), suggesting that the machinery for dATP production from AMP is activated.

Next, we investigated whether dATP synthesis is essential for purine metabolite-mediated Ara-C resistance. Ribonucleotide reductase regulatory subunit M2 (RRM2) is a key protein for dATP synthesis.[5] Treatment with hydroxyurea (HU), an RRM2 inhibitor (RRM2i), sensitizes leukemic cells to Ara-C and weakens purine metabolites-induced Ara-C resistance (Figure 2C). Knocking down RRM2 produces similar effects, while RRM2 overexpression confers resistance to Ara-C (*Online Supplementary Figure S2C, D*). Notably, the chemo-sensitizing effect of RRM2i is consistent with previous findings.[6] These results suggest that purine metabolites-induced chemoresistance is RRM2 dependent. We then investigated the mechanisms underlying RRM2-mediated Ara-C resistance. RRM2 activation produces dATP from purines. An unbalanced increase in a single dNTP, such as dATP, acts as a cellular stress signal[7] that may trigger dNTPase activity in leukemic cells. SAMHD1 is a dNTPase that degrades dNTP into nucleosides and functions as a detoxifier of Ara-CTP by converting it back to Ara-C.[8] Therefore, we reasoned that SAMHD1 may contribute to RRM2-mediated Ara-C resistance. We treated leukemic cells with ^{2}H labeled Ara-C and found that $^{2}H_2$-Ara-CTP levels are greatly reduced in the presence of purines metabolites (Figure 2D). Further, when SAMHD1 is KD or KO, leukemic cells are more sensitive to Ara-C and purine metabolites lose their protective effects (Figure 2E; *Online Supplementary Figure S2E*). SAMHD1 demonstrates the maximal dNTPase activity in its homotetramer form.[9] We found that purine metabolites promote the formation of SAMHD1 tetramer, while the overall SAMHD1 protein levels remain unchanged by purine metabolites (Figure 2F; *Online Supplementary Figure SF, H*). Leukemic cells that survived Ara-C treatment also display an increased level of SAMHD1 tetramer and RRM2i significantly reduces SAMHD1 tetramerization induced by purine metabolites and Ara-C (Figure 2F; *Online Supplementary Figure S2F, G*). To directly demonstrate that elevated cellular dATP induces SAMHD1 tetramerization, we electroporated dATP into leukemic cells and observed that dATP indeed promotes SAMHD1 tetramer formation (*Online Supplementary Figure S2I, J*). Additionally, we found that higher expression levels of *Rrm2* and *Samhd1* are associated with poorer survival in AML patients (*Online Supplementary Figure S2K*). These results suggest that purine metabolites induce Ara-C resistance via promoting SAMHD1 tetramerization.

Our study demonstrates that both RRM2 and SAMHD1 are promising targets to chemo-sensitize leukemic cells and are critical for purine metabolite-induced chemoresistance. However, currently reported SAMHD1 inhibitors lack cellular activities or exhibit extremely high half maximal inhibitory concentration (IC_{50}) values.[6] Therefore, agents inhibiting SAMHD1 tetramerization could provide an alternative strategy to counteract SAMHD1-induced Ara-C resistance. Our HTS experiment also identifies metabolites sensitizing leukemic cells to Ara-C. We then focused on metabolites enhancing Ara-C toxicity 24 hours post-treatment (Figure 3A). Among these, two adenosine derivatives, 1-methyladenosine (1-MA) and N6-methyladenosine (N6-MA), display potent chemo-sensitizing effects (Figure 3A). We validated that both derivatives promote Ara-C killing effect in leukemic cell lines and $CD34^{+}$ AML patient cells (Figure 3B; *Online Supplementary Figure S2L*). Notably, 1-MA is more

potent than N6-MA and chemo-sensitizes an Ara-C-resistant MOLM-13 line (*Online Supplementary Figure S2M*). Further, 1-MA decreases SAMHD1 tetramerization upon Ara-C treatment (Figure 3C; *Online Supplementary Figure*

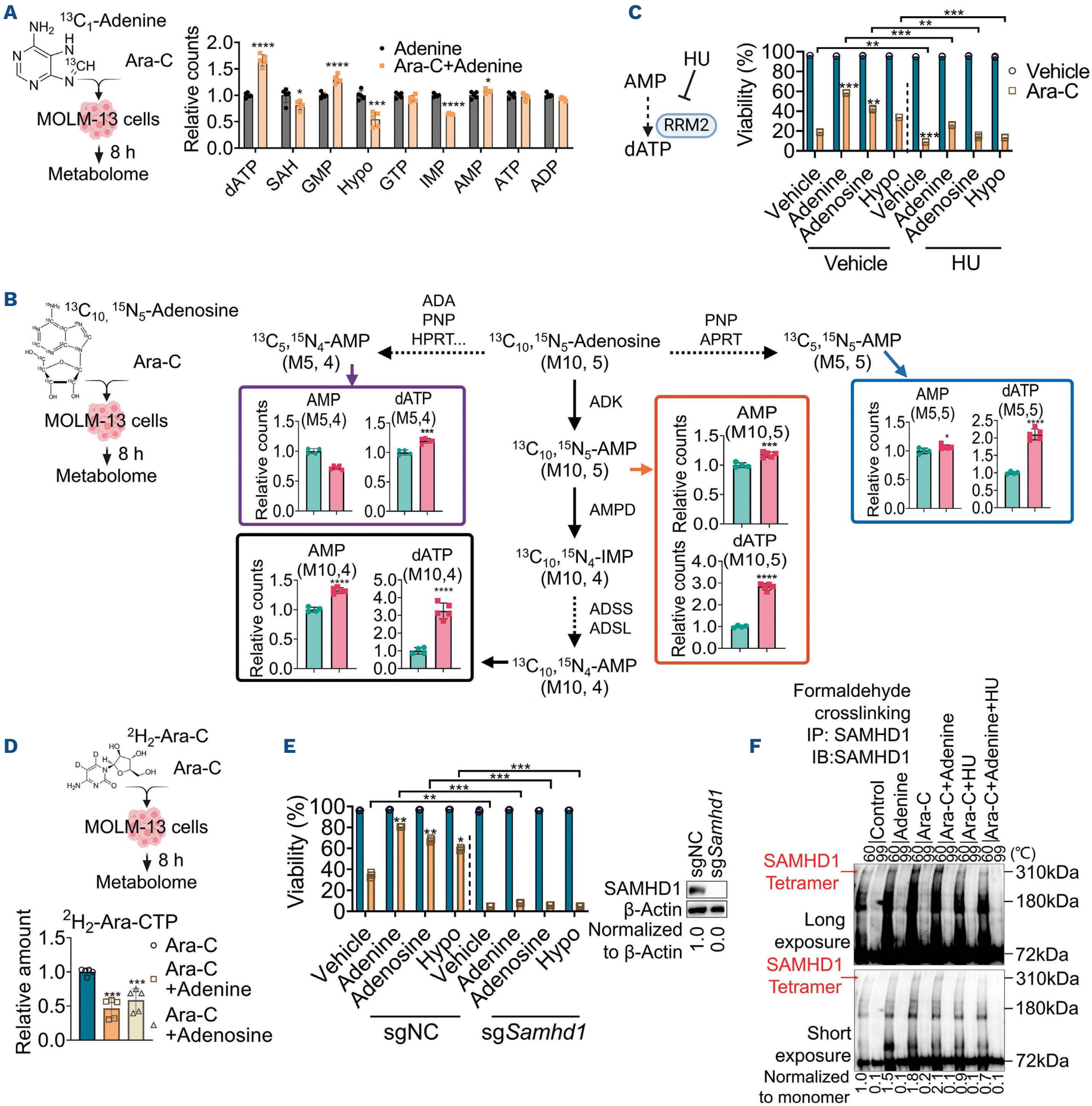

Figure 2. Purine metabolites are converted to dATP to induce SAMHD1 tetramerization in leukemic cells treated with cytarabine. (A, B) MOLM-13 cells were labeled with $^{13}C_1$-adenine or $^{13}C_{10}$, $^{15}N_5$-adenosine with or without the presence of cytarabine (Ara-C). Eight hours post-labeling, leukemic cells were harvested for metabolic analyses (N=4-5). Hypo: hypoxanthine. (C) Viability of MOLM-13 cells treated with Ara-C combined with purine metabolites and the RRM2 inhibitor hydroxyurea (HU) (20 μM) (N=3). (D) MOLM-13 cells were treated with $^2H_2$-Ara-C with or without the presence of purine metabolites. $^2H_2$-Ara-CTP levels were assessed 8 hours post-labeling (N=5). (E) SAMHD1 was knocked out by single guide RNA (sgRNA) in leukemic cells. Protection of purine metabolites was assessed (N=3). (F) SAMHD1 tetramerization was examined in leukemic cells under indicated conditions by protein crosslinking. All data are represented as mean ± standard deviation. Quantification is provided for each gel image. $^*P$≤0.05; $^{**}P$≤0.01; $^{***}P$≤0.001; $^{****}P$≤0.0001.

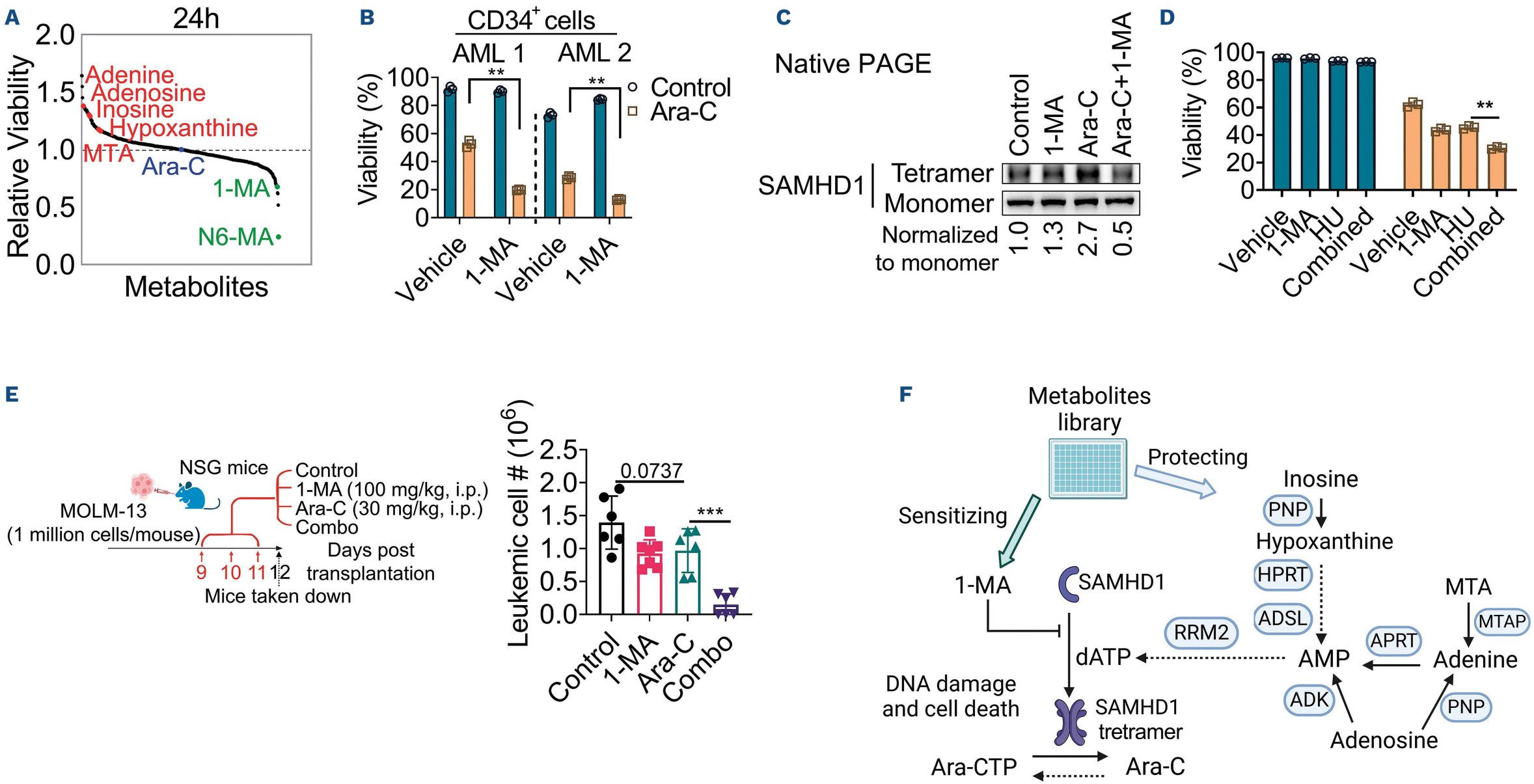

Figure 3. Purine derivatives chemo-sensitize leukemic cells by inhibiting SAMHD1 tetramerization. (A) Relative viability of leukemic cells 24 hours post cytarabine (Ara-C) treatment. (B) Viability of CD34⁺ acute myeloid leukemia (AML) patient cells treated with Ara-C combined with 1-methyladenosine (1-MA) (10 μM) (N=3). (C) SAMHD1 tetramerization was examined in cells treated with 1-MA, Ara-C, or Ara-C combined with 1-MA by native PAGE. (D) Chemo-sensitizing effect of 1-MA was assessed with or without the presence of hydroxyurea (HU) (N=3). (E) MOLM-13 leukemic mice were treated with Ara-C, 1-MA, and Ara-C combined with Ara-C (Combo). Bone marrow leukemic cell number was accessed (N=6-7). (F) Working model. Purine metabolites is converted to AMP via differential enzymes. AMP is then converted to dATP via RRM2. Increased dATP promotes SAMHD1 tetramerization and detoxifies Ara-C. Purine derivatives such as 1-MA inhibits SAMHD1 tetramerization to chemo-sensitize leukemic cells. All data are represented as mean ± standard deviation. Quantification is provided for each gel image. $^{*}P\leq0.05$; $^{**}P\leq0.01$; $^{***}P\leq0.001$; $^{****}P\leq0.0001$. AML: acute myeloid leukemia; i.p.: intraperitoneally.

S2N). Interestingly, 1-MA chemo-sensitizes cells to Ara-C even with RRM2 inhibition, suggesting RRM2 independence (Figure 3D). Importantly, 1-MA displays significantly chemo-sensitizing effects *in vivo* (Figure 3E). These data indicate that 1-MA is a chemo-sensitizer through inhibiting SAMHD1 tetramerization.

Together, our data suggest that purine metabolites can be converted to AMP via multiple metabolic pathways, which is subsequently converted to dATP via RRM2. Increased dATP levels induce SAMHD1 tetramerization, which functions as a detoxifier of Ara-CTP, thereby promoting the survival of leukemic cells. We further identified that 1-MA serves as an inhibitor of SAMHD1 tetramerization and demonstrate that 1-MA acts as a potent chemosensitizer (Figure 3F). We propose that designing future AML therapies so as to target purine metabolism may improve therapeutic outcomes.

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Disclosures

No conflicts of interest to disclose.

Contributions

HY conceived the project, designed the experiments. HY wrote the manuscript together with CTJ. XZ conducted most of the experiments and data analysis. ZZ and YW assisted with HTS experiments. JY and LC assisted with isotope labeling experiments. JZ assisted with patient sample experiments. AJ and TL assisted with gene KD and KO experiments. LH, XD, TZ and PL assisted with detection of tetramerization experiments.

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Data-sharing statement

Protocols and original data from this manuscript are available upon request by contacting the corresponding author.

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