Targeting of AML1-ETO in t(8;21) leukemia by oridonin generates a tumor suppressor-like protein

Running title: Oridonin targets AML-ETO and inhibits LIC activity

Tao Zhen[1*], Chuan-Feng Wu[1, 2*], Ping Liu[1*], Hai-Yan Wu[2*], Guang-Biao Zhou[3*], Ying Lu[1], Jian-Xiang Liu[1], Yang Liang[1], Keqin Kathy Li[1], Yue-Ying Wang[1], Yin-Yin Xie[1], Miao-Miao He[1], Huang-Ming Cao[1], Wei-Na Zhang[1], Li-min Chen[1, 4], Kevin Petrie[5], Sai-Juan Chen[1, 2, 5#], Zhu Chen[1, 2, 5#]

[1]State Key Laboratory of Medical Genomics, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (CAS) & Shanghai Jiao Tong University School of Medicine (SJTUSM) and Shanghai Institute of Hematology, Rui-Jin Hospital, 200025, Shanghai, China; [2]Shanghai Center for Systems Biomedicine, SJTU, 800 Dong Chuan Road, 200240, Shanghai, China; [3]Laboratory of Molecular Carcinogenesis and Targeted Therapy for Cancer, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, CAS, 100101, Beijing, China; [4]Drug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, CAS, 201203, Shanghai, China; [5]Samuel Waxman Cancer Research Foundation Joint Translational Research Center, Shanghai Institute of Hematology, Rui-Jin Hospital, SJTUSM, 200025, Shanghai, China.

*These authors contributed equally to this work.

[#]To whom correspondence should be addressed. E-mail: zchen@stn.sh.cn or sjchen@stn.sh.cn.

ABSTRACT

Nearly 60% of acute myeloid leukemia (AML) patients with the t(8;21)(q22;q22) translocation fail to achieve long-term disease-free survival. Our previous studies demonstrated that oridonin selectively induces apoptosis of t(8;21) leukemia cells and causes cleavage of AML-ETO oncoprotein resulting from t(8;21), but the underlying mechanisms remain unclear. Here, we show that oridonin interacts with glutathione (GSH) and thioredoxin/thioredoxin reductase (Trx/TrxR) to increase intracellular reactive oxygen species (ROS), which in turn activates caspase-3 in t(8;21) cells. Moreover, oridonin binds AML1-ETO, directing the enzymatic cleavage at D188 via caspase-3 to generate a truncated AML1-ETO (ΔAML1-ETO), and preventing the protein from further proteolysis. ΔAML1-ETO interacts with AML1-ETO and interferes with the trans-regulatory functions of remaining AML1-ETO oncoprotein, thus acting as a tumor suppressor that mediates the anti-leukemia effect of oridonin. Furthermore, oridonin inhibits the activity of c-Kit+ leukemia-initiating cells (LICs). Therefore, oridonin is a potential lead compound for molecular target-based therapy of leukemia.

INTRODUCTION

Acute myeloid leukemia (AML) is the most common malignant myeloid disorder in adults and a heterogeneous clonal disorder of hematopoietic progenitor cells through the acquisition of chromosomal rearrangements and multiple gene mutations. The t(8;21)(q22;q22) translocation is the most frequent chromosomal rearrangement in AML (12–20%), especially in French-American-British (FAB) subtype M2 (AML-M2, 40–50%) (1). The resultant AML1-ETO chimeric protein comprises the N-terminal 177 amino acids (a.a.) of AML1 (known as RUNX1, CBFα2 or PEBP2$\alpha\beta$) and almost the entire protein sequence of ETO (also known as MTG8/RUNX1T1). AML1-ETO retains the DNA-binding specificity of AML1 and the capacity to recruit ETO-associated regulators, which promotes the self-renewal of hematopoietic stem/progenitor cells (1). These combined functions immortalize hematopoietic progenitors (2, 3) and induce a myeloproliferative disorder in mice (4). Additional mutations cooperate with this critical leukemogenic role of AML1-ETO, resulting in full-blown disease (2, 5, 6). An isoform of AML1-ETO with an alternatively spliced exon (exon 9a) of ETO (AML1-ETO9a) can induce leukemia in mice (7). Therefore agents that target AML1-ETO could be useful in treating t(8;21) AML (8, 9).

The principal front-line treatment for AML-ETO positive AML consists of cytosine arabinoside (Ara-C) and anthracycline-based chemotherapy (10); however, these therapies result in a median survival of less than 2 years and a 5-year overall survival (OS) of no more than 40% (11, 12). In China, homoharringtonine has also been added as chemotherapy (13). Notably, the overall response of these therapies seems less favorable in East Asian countries with a 5-year OS of 10-40% (14-16). In our previous work, oridonin, a diterpenoid isolated from the medicinal herb Isodon rubescens, was identified to have relatively selective effect against t(8;21) leukemia cells. In t(8;21) cell lines, oridonin triggered caspase-3-dependent cleavage of AML1-ETO at D188, generating a 70 kDa fragment (ΔAML1-ETO). AML1-ETO-expressing U937 cells also generated ΔAML1-ETO upon oridonin treatment and were more sensitive to the apoptotic effect of the compound than parental cells (8).

In this study, we undertake to identify the proteins targeted and modified by oridonin and address the biological functions of ΔAML1-ETO. We also investigate the possible effects of oridonin on leukemia-initiating cells (LICs) at cellular and organism levels.

RESULTS

Oridonin induces caspase-3 activation and AML1-ETO cleavage through increasing intracellular ROS

As previously reported (8), oridonin triggered caspase-3 activation as shown by the generation of the cleaved caspase-3 and AML1-ETO cleavage with generation of C-terminal truncated protein (ΔAML1-ETO) in Kasumi-1 cells (Fig. 1, A and B), while no appearance of N-terminal band was noted using specific antibody (fig. S1A). The PML-RARα oncoprotein in an acute promyelocytic leukemia (APL) cell line and the BCR-ABL oncoprotein in a chronic myeloid leukemia cell line were resistant to oridonin over 24 hrs of treatment (fig. S1B). Expression of ΔAML1-ETO enhanced sensitivity of Kasumi-1 cells to oridonin (fig. S2), indicating the selective effect of oridonin on AML1-ETO leukemia cells may be partly mediated by the generation of □AML1-ETO.

We then explored intracellular reactive oxygen species (ROS), essential signals in initiating apoptosis and caspase-3 activation (17), under oridonin treatment. In Kasumi-1 cells, intracellular ROS was augmented as early as 0.5 hr upon effect of oridonin (Fig. 1C), whereas pre-treatment with ROS scavenger N-Acetyl Cysteine (NAC) not only decreased oridonin-induced ROS and apoptosis (fig. S3), but also inhibited caspase-3 activation and ΔAML1-ETO production (Fig. 1D). Oridonin also increased ROS and generated ΔAML1-ETO in SKNO-1 cells, another t(8;21) cell line, where these effects were also blocked by NAC (fig. S4).

We further examined the mechanisms by which ROS level was increased. Intracellular glutathione (GSH), the major molecule in maintaining cellular redox balance, was decreased after oridonin treatment in a time-dependent manner (Fig. 1E). Based on the chemical properties of oridonin, it was assumed that the compound could react with sulfhydryl (-SH) groups (fig. S5A) and *in vitro* system using dithio-bis-nitrobenzoic acid (DTNB) demonstrated an interaction between oridonin and GSH (fig. S5B). It is known that the thioredoxin system, composed of thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH, is the major disulfide reducing enzyme system in all cells responsible for maintaining the redox homeostasis (18). Since both TrxR and Trx contain cysteines and/or selenocysteines in their active sites (19), we examined whether oridonin could interact with them and affect their activities. Indeed, TrxR and Trx activities were suppressed by oridonin in Kasumi-1 cells (Fig. 1F). To examine a possible interaction between oridonin and TrxR/Trx, a biotin-labeled oridonin (biotin-oridonin) with similar effect on ROS induction and ΔAML1-ETO generation was made (fig. S6). Streptavidin agarose affinity assay showed that biotin-oridonin could bind TrxR and Trx in Kasumi-1 cells, and unlabeled oridonin competed with these interactions (Fig. 1G). The direct binding of oridonin (molecular weight (MW): 364.43) to Trx was also demonstrated by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS) (fig. S7). Thus, induction of cellular ROS by oridonin primarily results from both intracellular GSH depletion and TrxR/Trx activity inhibition.

Oridonin binds AML1-ETO to mediate a caspase-3-dependent cleavage at D188 and stabilizes ΔAML1-ETO

As reported (8), oridonin triggered caspase-3 dependent cleavage of AML1-ETO with generation of

ΔAML1-ETO, while doxorubicin, arsenic trioxide and MG132 could not do so though they were able to activate caspase-3 (fig. S8), supporting a selective effect of oridonin. Previous studies showed that caspase-3 cut AML1-ETO at D188 and D368, and D188V mutation almost abolished the generation of ΔAML1-ETO under oridonin (Fig. 1B and fig. S9A) (8, 20). Since mutation of D368 (D368A) enhanced the generation of ΔAML1-ETO (Fig. 1B and fig. S9B), we hypothesized that oridonin might exert a protective role on D368 so that ΔAML1-ETO cleavage could be prevented.

In an *in vitro* assay, when purified AML1-ETO was pre-incubated with oridonin, the intensity of ΔAML1-ETO was considerably increased when caspase-3 was added. Pre-incubation of ΔAML1-ETO with oridonin also protected this truncated protein from further attack by caspase-3 (Fig. 2A). Because biotin-oridonin bound to AML1-ETO/ΔAML1-ETO in transfected 293T cells and endogenous AML1-ETO in Kasumi-1 cells (Fig. 2B), we assumed that the binding might occur at cysteine residues which contain -SH groups. Potential binding sites on distinct domains of AML1-ETO were mapped using MALDI-TOF MS. No binding was observed on RHD and NHR3 (fig. S10), whereas NHR1 (also named as TAFH), NHR2 and ZnF were capable of oridonin binding (Fig. 1B and Fig. 2, C, D and E). Our interest was then focused on the three cysteines (C266, C347 and C416) around D368 at NHR1 which might be important for the protective effect of oridonin. Amino acid substitutions were made on these 3 positions and it was observed that in the absence of oridonin ΔAML1-ETO with C347A was slightly more resistant to the action of caspase-3 than C266A or C416A mutants (Fig. 2F), presumably due to a conformation change of AML1-ETO caused by C347A. Notably, unlike C266A and C416A mutants, ΔAML1-ETO with C347A was not protected from caspase-3 cleavage in the presence oridonin (Fig. 2F), suggesting that C347 should be the critical site for oridonin binding and this binding might also lead to conformational change of AML1-ETO which might mask D368 from caspase-3 recognition or affect caspase-3 and NHR1 interaction (fig. S11). These results indicate that oridonin mediates caspase-3 action selectively to D188, instead of D368, and thus stabilizes ΔAML1-ETO.

ΔAML1-ETO antagonizes oncogenic activity of AML1-ETO

Since ΔAML1-ETO persists in t(8;21) cells up to five days after treatment with oridonin (fig. S12) and retains the NHR2 domain forming interface with other proteins, we examined its biological functions in the context of protein-protein interaction. Co-immunoprecipitation (Co-IP) from transfected cells confirmed the interaction between AML1-ETO and ΔAML1-ETO (Fig. 3A (a and b)). Using confocal microscopy, when plasmids expressing AML1-ETO-EGFP and ΔAML1-ETO-DsRed2 were transiently co-expressed in Hela cells, co-localization signal was visible and ΔAML1-ETO shifted the pattern of AML1-ETO from micropunctate to larger speckles (Fig. 3B). Similarly, oridonin could induce a weak generation of ΔAML1-ETO9a in bone marrow (BM) cells of AML1-ETO9a leukemia mice (fig. S13A). ΔAML1-ETO also interacted and co-localized with AML1-ETO9a (fig. S13, B and C). To study the effect of ΔAML1-ETO on AML1-ETO transcription complex, extracts from 293T cells expressing AML1-ETO and/or ΔAML1-ETO were analyzed by size exclusion chromatography (SEC). As shown in Fig. 3C, AML1-ETO complexes showed MWs above 669 KDa (21) while ΔAML1-ETO complexes displayed lower MWs. Co-expression of

AML1-ETO and ΔAML1-ETO shifted the migration of AML1-ETO to lower MW species between 669 KDa and 440 KDa. These data suggest that ΔAML1-ETO may disrupt AML1-ETO complexes via acting on partner proteins.

We subsequently examined DNA binding properties of AML1-ETO complexes by using electrophoretic mobility shift assay (EMSA). The binding of AML1-ETO to a consensus AML1 DNA recognition sequence from *RUNX3* (an AML1 target gene) promoter was diminished by ΔAML1-ETO in a dose-dependent manner (Fig. 4A, line 4-8). ΔAML1-ETO also antagonized AML1-ETO-mediated repression of a luciferase reporter containing *RUNX3* promoter (fig. S14). Moreover, chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis revealed that after treatment of Kasumi-1 cells with oridonin for 24 hrs, the association of AML1-ETO with promoters of known targets including *UBQLN1, CCPG1* and *IL-6R* was diminished (Fig. 4B) and the suppression effect of AML1-ETO on these genes was relieved (Fig. 4C). Thus, ΔAML1-ETO antagonizes the DNA binding of AML1-ETO and accordingly abrogates its trans-regulatory activities.

At the cellular level, co-expression of ΔAML1-ETO with AML1-ETO (Fig. 4D) inhibited the increased cell proliferation and clonogenic ability driven by AML1-ETO in NIH3T3 cells (Fig. 4, E and F). Moreover, nude mice inoculated with AML1-ETO-expressing NIH3T3 cells developed tumors, whereas tumor-inducing ability of cells co-expressing ΔAML1-ETO and AML1-ETO was largely compromised (Fig. 4G). We also tested the effect of ΔAML1-ETO on murine primary BM cells. As previously reported (22), AML1-ETO induced *in vitro* clonogenic activity of murine BM cells in serial replating assay (Fig. 4H). Notably, ΔAML1-ETO inhibited the clonogenic potential driven by AML1-ETO (Fig 4H).

It has been well established that NHR2 mediates the oligomerization of AML1-ETO. Here we showed NHR2 serves as a major surface of AML1-ETO/ΔAML1-ETO interaction (Fig. 3A(c)). ΔAML1-ETO with deletion of NHR2 lost some of its activities at molecular, cellular and organism levels (Fig. 4, A, D and G).

Oridonin inhibits activity of LICs *in vitro* and *in vivo*

Given that ΔAML1-ETO affected the colony-forming ability of AML1-ETO bearing cells, it is reasonable to presume that oridonin may have an effect on LICs. We therefore set out to identify a subset of cells from BM of AML1-ETO9a leukemia mice (8) with the strongest potential to initiate leukemia. As LICs were usually primitive cells, we chose cell surface markers characteristic of normal hematopoietic stem cell (Lin-/Sca-1+/c-Kit+) (23, 24) in phenotype analysis in an attempt to enrich LICs. BM cells were harvested independently from several diseased mice and were analyzed by flow cytometry. Almost all GFP+ leukemia cells were Lin- (99%) and Sca-1- (99.4%) (Fig. 5, A(a) and B), and these cells could be divided into two subsets: Sca-1-/c-Kit- and Sca-1-/c-Kit+ (Fig. 5, A(b) and B). To test which subpopulation represents LICs in this animal model, the two subpopulations were sorted out and analyzed *in vitro* or injected into mice (2,000 cells/mouse). Greater colony-forming capacity of GFP+/Lin-/Sca-1-/c-Kit+ (group A) cells than that of GFP+/Lin-/Sca-1-/c-Kit- (group B) cells was observed (Fig. 5C), and mice receiving group A cells had a shorter survival compared with those receiving group B cells (Fig. 5D,

p<0.001). Leukemia-initiating analysis showed that the LIC frequency of group A (1/167) was higher than that of group B (1/6,159) (table S1). Hence, the group A subpopulation, with a phenotype different from the normal hematopoietic cells, may be representative of LICs in AML1-ETO9a leukemia mice.

We then tested *in vitro* and *in vivo* modulatory activities of oridonin on LICs. The number of colonies formed by group A cells in culture condition was significantly decreased under oridonin (Fig. 5E). In this work, an oridonin formulation suitable for *in vivo* administration was used in animal treatment and a pharmacokinetics analysis of this formulation in rat showed a plasma Cmax of 4.01 μM of oridonin at a dose of 2 mg/kg/day (half maximal inhibitory concentration (IC50) for AML1-ETO9a cells: ~2.5 μM). After AML1-ETO9a mice were treated with the formulation at 20 mg/kg/day (equivalent to previously reported oridonin preparation at 7.5 mg/kg/day (8) in prolonging survival of the mouse model) for three weeks, Western blot showed decreased expression of AML1-ETO9a in spleen cells (fig. S15A), and the colony formation ability of BM group A cells was suppressed (Fig. 5E) though the percentage of group A population in total BM cells was not statistically lowered as compared to controls (fig. S15B). Importantly, after 1,000 group A cells from donor animals thus treated were intravenously injected into recipients and compared to donor animals without oridonin formulation therapy, mice receiving cells from vehicle-treated donors died of disease significantly faster (median survival time (MST): 83.5 days) than those receiving cells from oridonin-treated donors (112.5 days, p=0.0005) (Fig. 5F). Of note, the MST of mice receiving 1,000 group A cells from donors treated with oridonin formulation in combination with all-trans retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF), two differentiation inducers in a sequential manner was significantly increased (168 days, P<0.0001 or p<0.05 as compared to vehicle- or oridonin-treated groups, respectively) (Fig. 5F). A synergistic effect of oridonin/ATRA/G-CSF combination was also confirmed in mice inoculated with bulk leukemia cells, in that oridonin alone, moderately prolonged the survival (MST from 28 days to 31 days, P<0.01) while ATRA/G-CSF showed no improvement (p=0.3057); however, oridonin/ATRA/G-CSF protocol significantly prolonged the survival (MST 45.5 days) as compared to the control and oridonin treatment groups (P<0.0001 in both cases, Fig. 5G).

To evaluate the safety of oridonin, normal C57BL/6 mice were treated with 20 mg/kg/day or 40 mg/kg/day oridonin formulation for 3 weeks. The body weight and blood picture were followed-up and no obvious adverse effects were observed (fig. S16).

DISCUSSION

Previous study demonstrated that oridonin selectively targeted AML1-ETO leukemia cells and triggered caspase-3-dependent cleavage of AML1-ETO at D188 with generation of ΔAML1-ETO. However, the cellular targets of oridonin, the precise mechanism with which oridonin generates ΔAML1-ETO and the possible function of ΔAML1-ETO remained unknown. In the present work, we show that oridonin directly binds to GSH and targets the active sites of TrxR and Trx to inhibit their activities. These effects result in a rapid increase of ROS, which in turn induces apoptosis and caspase-3 activation of t(8;21) leukemia cells. Different from other caspase-3 activating compounds investigated here, oridonin is able to induce

generation of ΔAML1-ETO through directly binding to AML1-ETO at C347, the nearest cysteine residue to the caspase-3 cleavage site D368. A possible consequence of this binding can be a configurational change of AML1-ETO vis-à-vis the effect of caspase-3 to D368, preventing the fusion protein from cleavage at this site and directing action of caspase-3 mainly to D188. Oridonin induced-ΔAML1-ETO fragment can interact with AML1-ETO through the NHR2 domain and act as a tumor suppressor by means of interfering with the activity of parental AML1-ETO. Moreover, we find that oridonin inhibits activity of LICs in AML1-ETO9a leukemia mouse with a characteristic immunophenotype of Lin-/Sca-1-/c-Kit+, and thereby improves the survival of leukemia mice. A synergistic effect among oridonin and the differentiation inducers ATRA and G-CSF has been demonstrated in that the combination therapy exerts statistically more significant effect than oridonin monotherapy in terms of inhibition of the activity of Lin-/Sca-1-/c-Kit+ LICs and prolongation of the survival of leukemia mice. Proposed working mechanisms of oridonin against t(8;21) AML are summarized in Fig. 6.

Oridonin has been shown to have myriad of effects on tumor cells, including increase of ROS (25, 26), inhibition of NF-□B activity, histone hyperacetylation, regulation of cell cycle through p16, p21 and p27, c-myc, as well as modulation of MAPK and p53 signaling pathways in solid tumors (27, 28). Here, we provide evidence that oridonin induces apoptosis and caspase-3 activation of t(8;21) leukemia cells mostly by inducing accumulation of ROS and find the molecular targets of oridonin in rapid increasing of ROS.

Previous work showed that leukemia cells expressing AML1-ETO with D188V mutation were less sensitive to oridonin than those expressing wild-type oncoprotein (8). We find in this work that ΔAML1-ETO acts as a tumor suppressor of AML1-ETO, in support of the notion that ΔAML1-ETO may play a role in the anti-leukemia effect and contribute to the selective action of oridonin. It was previously shown that a fusion peptide NC128 containing the intact NHR2 domain disrupted the stability of the AML1-ETO complexes, restored transcription of certain AML1-ETO target genes, and reverted the differentiation block induced by AML1-ETO in myeloid cells (29). A design of small compounds that can interfere with NHR2 domain interaction could be a good strategy, but the high stability of AML1-ETO tetramer is a big challenge. Since oridonin induces cleavage of AML1-ETO to generate an endogenous fragment that effectively disrupts AML1-ETO oligomerization, it may be an ideal alternative for targeting AML1-ETO.

The remarkable success of selective differentiation/apoptosis induction therapy in APL (30) suggests that targeting leukemia-associated oncoprotein(s) is of crucial importance. Indeed, the compound can inhibit, under both *in vitro* and *in vivo* conditions, leukemia-initiating ability of BM LICs from the AML1-ETO9a leukemia mice. It is worth mentioning that oridonin improves survival of, but does not cure, AML-ETO9a leukemia mice. We showed previously that combination of oridonin and Ara-C had a superior effect than either agent alone (8). Here, oridonin/ATRA/G-CSF combination not only significantly impairs the activity of LICs but also substantially improves the survival of leukemia mice. These results suggest that oridonin is a potential compound for molecular target-based therapy of t(8;21) leukemia and a phase 0 trial is currently being performed in the Shanghai Institute of Hematology. Nevertheless, the pathogenesis of this AML subtype is extremely complex and additional targets such as *c-KIT* mutations should be considered, and optimal therapeutic efficacy may require a combination of multiple agents (6).

MATERIALS AND METHODS

Cell Culture, reagents and plasmids

Cells were cultured using standard protocols. For information about reagents and plasmids, see Supplementary Material for details.

Western blot analysis, immunoprecipitation (IP), Co-IP, immunofluorescence and EMSA

Western blot, IP, Co-IP and immunofluorescence and EMSA assays were performed using standard protocols and antibodies indicated. See Supplementary Material for details.

Assays for ROS production, TrxR and Trx activities, *in vitro* proteolysis of AML1-ETO protein and SEC

ROS production determination was performed as previously described (31). TrxR and Trx activities were performed with an endpoint insulin assay (32). *In vitro* proteolysis of AML1-ETO protein (20) and SEC assay (21) were performed as described. See Supplementary Material for details.

MALDI-TOF MS assay

His-tagged RHD, NHR1, NHR2, NHR3 and ZnF protein domains (Fig. 1B) were expressed in bacteria BL21 (DE3) and purified by affinity chromatography on Ni-NTA-Agarose (Qiagen). Recombinant purified proteins or Trx were incubated with oridoinin (recombinant protein:oridonin=1:1) for 1 hr at room temperature. The MWs of proteins and adducts were recorded with MALDI-TOF MS (Shanghai Applied Protein Technology Co.Ltd.).

Streptavidin agarose affinity assay

293T cells transiently expressing Flag-AML1-ETO or Flag-ΔAML1-ETO and Kasumi-1 cells were treated with 200 μM biotin-oridonin for 1 hr and lysed with 8 M urea buffer. Cell lysates were incubated with streptavidin agarose beads overnight at 4 ℃. After washing with 6 M urea buffer, streptavidin agarose beads were resuspended in SDS-PAGE loading buffer and analyzed by Western blot. For oridonin blocking, cells were pretreated with 100 μM oridonin for 4 hrs, followed by 200 μM biotin-oridonin treatment for 1 hr.

Proliferation, transformation and *in vivo* tumor formation assay of NIH3T3 cells

Bosc23 cells were transfected with MigR1-X (X: AML1-ETO-IRES-GFP, GFP, ΔAML1-ETO-IRES-YFP, ΔAML1-ETO(ΔNHR2)-IRES-YFP, YFP) plasmids and Ecopac plasmids. Viral supernatants were collected to infect NIH3T3 cells and the GFP and YFP double positive cells were sorted out with MoFlo High-speed Cell Sorter (DakoCytomation) for assays of proliferation, transformation and tumor formation. See Supplementary Material for details.

Murine model establishment and drug treatments

Experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals. For murine model establishment, C57BL/6 mice housed in specific pathogen-free conditions were irradiated

and injected with 3×10^6 leukemia cells as described (7, 8). An oridonin formulation suitable for *in vivo* administration provided by Jiangsu Hengrui Medicine Company was used to treat animals. Three days after leukemia cell transplantation, the mice were subjected to treatment protocols as follows: 5% glucose (vehicle); oridonin formulation given at 20 mg/kg/day for three weeks (oridonin); vehicle (4 days) followed by G-CSF (50 μg/kg/day) and ATRA (10 mg/kg/day) (3 days) for three weeks (G-CSF/ATRA); oridonin formulation (4 days) followed by G-CSF+ATRA (3 days) for three weeks (oridonin/ATRA/G-CSF).

Colony-forming ability and serial replating assay of mice BM cells

2×10^4 indicated mouse cells were used for colony-forming assay with MethoCult M3434 (StemCell Technologies) according to manufacture's instruction. Serial replating assay of BM cell was performed as previously described (22).

Statistical analysis

Results were expressed as means ± SEM, Differences among groups were tested with the Student's t-test. The survival time of mice was analyzed using the Kaplan-Meier method. $P<0.05$ were considered statistically significant.

REFERENCES AND NOTES

1. K.E. Elagib and A.N. Goldfarb, *Oncogenic pathways of AML1-ETO in acute myeloid leukemia: multifaceted manipulation of marrow maturation.* Cancer Lett. **251**, 179-186 (2007)
2. M. Higuchi, D. O'Brien, P. Kumaravelu, N. Lenny, E.J. Yeoh, and J.R. Downing, *Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia.* Cancer Cell. **1**, 63-74 (2002)
3. T. Okuda, Z. Cai, S. Yang, N. Lenny, C. Lyu, J. van Deursen, H. Harada, and J. Downing, *Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors.* Blood. **91**, 3134 (1998)
4. T.S. Fenske, G. Pengue, V. Mathews, P.T. Hanson, S.E. Hamm, N. Riaz, and T.A. Graubert, *Stem cell expression of the AML1/ETO fusion protein induces a myeloproliferative disorder in mice.* Proc Natl Acad Sci U S A. **101**, 15184-15189 (2004)
5. Y. Yuan, L. Zhou, T. Miyamoto, H. Iwasaki, N. Harakawa, C.J. Hetherington, S.A. Burel, E. Lagasse, I.L. Weissman, K. Akashi, and D.E. Zhang, *AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations.* Proc Natl Acad Sci U S A. **98**, 10398-10403 (2001)
6. Y.Y. Wang, L.J. Zhao, C.F. Wu, P. Liu, L. Shi, Y. Liang, S.M. Xiong, J.Q. Mi, Z. Chen, R. Ren, and S.J. Chen, *C-KIT mutation cooperates with full-length AML1-ETO to induce acute myeloid leukemia in mice.* Proc Natl Acad Sci U S A. **108**, 2450-2455 (2011)
7. M. Yan, E. Kanbe, L.F. Peterson, A. Boyapati, Y. Miao, Y. Wang, I.M. Chen, Z. Chen, J.D. Rowley, C.L. Willman, and D.E. Zhang, *A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis.* Nat Med. **12**, 945-949 (2006)
8. G.B. Zhou, H. Kang, L. Wang, L. Gao, P. Liu, J. Xie, F.X. Zhang, X.Q. Weng, Z.X. Shen, J. Chen, L.J. Gu, M. Yan, D.E. Zhang, S.J. Chen, Z.Y. Wang, and Z. Chen, *Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t(8;21) leukemia in vitro and in vivo.* Blood. **109**, 3441-3450 (2007)
9. S. Liu, R.B. Klisovic, T. Vukosavljevic, J. Yu, P. Paschka, L. Huynh, J. Pang, P. Neviani, Z. Liu, W. Blum, K.K. Chan, D. Perrotti, and G. Marcucci, *Targeting AML1/ETO-histone deacetylase repressor complex: a novel mechanism for valproic acid-mediated gene expression and cellular differentiation in AML1/ETO-positive acute myeloid leukemia cells.* J Pharmacol Exp Ther. **321**, 953-960 (2007)
10. D. Grimwade, H. Walker, F. Oliver, K. Wheatley, C. Harrison, G. Harrison, J. Rees, I. Hann, R. Stevens, A. Burnett, and A. Goldstone, *The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties.* Blood. **92**, 2322-2333 (1998)
11. R. Billstrom, B. Johansson, T. Fioretos, S. Garwicz, C. Malm, O. Zettervall, and F. Mitelman, *Poor survival in t(8;21) (q22;q22)-associated acute myeloid leukaemia with leukocytosis.* Eur J Haematol. **59**, 47-52 (1997)
12. J.M. Rowe and M.S. Tallman, *How I treat acute myeloid leukemia.* Blood. **116**, 3147-3156 (2010)

13. J. Jin, D.Z. Jiang, W.Y. Mai, H.T. Meng, W.B. Qian, H.Y. Tong, J. Huang, L.P. Mao, Y. Tong, L. Wang, Z.M. Chen, and W.L. Xu, *Homoharringtonine in combination with cytarabine and aclarubicin resulted in high complete remission rate after the first induction therapy in patients with de novo acute myeloid leukemia.* Leukemia. **20**, 1361-1367 (2006)
14. Y.Y. Wang, G.B. Zhou, T. Yin, B. Chen, J.Y. Shi, W.X. Liang, X.L. Jin, J.H. You, G. Yang, Z.X. Shen, J. Chen, S.M. Xiong, G.Q. Chen, F. Xu, Y.W. Liu, Z. Chen, and S.J. Chen, *AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec.* Proc Natl Acad Sci U S A. **102**, 1104-1109 (2005)
15. C.C. Chen, J.P. Gau, Y.B. Yu, C.H. Lu, K.D. Lee, and J.Y. You, *Prognosis and treatment outcome in patients with acute myeloid leukemia with t(8;21)(q22;q22).* Adv Ther. **24**, 907-920 (2007)
16. B. Jiao, C.F. Wu, Y. Liang, H.M. Chen, S.M. Xiong, B. Chen, J.Y. Shi, Y.Y. Wang, J.H. Wang, Y. Chen, J.M. Li, L.J. Gu, J.Y. Tang, Z.X. Shen, B.W. Gu, W.L. Zhao, Z. Chen, and S.J. Chen, *AML1-ETO9a is correlated with C-KIT overexpression/mutations and indicates poor disease outcome in t(8;21) acute myeloid leukemia-M2.* Leukemia. (2009)
17. M.L. Circu and T.Y. Aw, *Reactive oxygen species, cellular redox systems, and apoptosis.* Free Radic Biol Med. **48**, 749-762 (2010)
18. J.E. Biaglow and R.A. Miller, *The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy.* Cancer Biol Ther. **4**, 6-13 (2005)
19. K.F. Tonissen and G. Di Trapani, *Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy.* Mol Nutr Food Res. **53**, 87-103 (2009)
20. Y. Lu, Z. Peng, T. Yuan, Q. Yin, L. Xia, and G. Chen, *Multi-sites cleavage of leukemogenic AML1-ETO fusion protein by caspase-3 and its contribution to increased apoptotic sensitivity.* Leukemia. **22**, 378-386 (2007)
21. C. Kwok, B.B. Zeisig, J. Qiu, S. Dong, and C.W. So, *Transforming activity of AML1-ETO is independent of CBFbeta and ETO interaction but requires formation of homo-oligomeric complexes.* Proc Natl Acad Sci U S A. **106**, 2853-2858 (2009)
22. Y. Liu, M.D. Cheney, J.J. Gaudet, M. Chruszcz, S.M. Lukasik, D. Sugiyama, J. Lary, J. Cole, Z. Dauter, W. Minor, N.A. Speck, and J.H. Bushweller, *The tetramer structure of the Nervy homology two domain, NHR2, is critical for AML1/ETO's activity.* Cancer Cell. **9**, 249-260 (2006)
23. G.J. Spangrude, S. Heimfeld, and I.L. Weissman, *Purification and characterization of mouse hematopoietic stem cells.* Science. **241**, 58-62 (1988)
24. C.L. Li and G.R. Johnson, *Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization.* Blood. **85**, 1472-1479 (1995)
25. Y. Cheng, F. Qiu, and T. Ikejima, *Molecular mechanisms of oridonin-induced apoptosis and autophagy in murine fibrosarcoma L929 cells.* Autophagy. **5**, 430-431 (2009)
26. F. Huang, C. Nie, Y. Yang, W. Yue, Y. Ren, Y. Shang, X. Wang, H. Jin, C. Xu, and Q. Chen, *Selenite induces redox-dependent Bax activation and apoptosis in colorectal cancer cells.* Free Radic Biol Med. **46**, 1186-1196 (2009)
27. C.H. Leung, S.P. Grill, W. Lam, Q.B. Han, H.D. Sun, and Y.C. Cheng, *Novel mechanism of inhibition of*

nuclear factor-kappa B DNA-binding activity by diterpenoids isolated from Isodon rubescens. Mol Pharmacol. **68**, 286-297 (2005)

28. F.H. Gao, X.H. Hu, W. Li, H. Liu, Y.J. Zhang, Z.Y. Guo, M.H. Xu, S.T. Wang, B. Jiang, F. Liu, Y.Z. Zhao, Y. Fang, F.Y. Chen, and Y.L. Wu, *Oridonin induces apoptosis and senescence in colorectal cancer cells by increasing histone hyperacetylation and regulation of p16, p21, p27 and c-myc.* BMC Cancer. **10**, 610 (2010)
29. C. Wichmann, L. Chen, M. Heinrich, D. Baus, E. Pfitzner, M. Zornig, O.G. Ottmann, and M. Grez, *Targeting the oligomerization domain of ETO interferes with RUNX1/ETO oncogenic activity in t(8;21)-positive leukemic cells.* Cancer Res. **67**, 2280-2289 (2007)
30. Z.Y. Wang and Z. Chen, *Acute promyelocytic leukemia: from highly fatal to highly curable.* Blood. **111**, 2505-2515 (2008)
31. L. Wang, W.L. Zhao, J.S. Yan, P. Liu, H.P. Sun, G.B. Zhou, Z.Y. Weng, W.L. Wu, X.Q. Weng, X.J. Sun, Z. Chen, H.D. Sun, and S.J. Chen, *Eriocalyxin B induces apoptosis of t(8;21) leukemia cells through NF-kappaB and MAPK signaling pathways and triggers degradation of AML1-ETO oncoprotein in a caspase-3-dependent manner.* Cell Death Differ. **14**, 306-317 (2007)
32. M. Luthman and A. Holmgren, *Rat liver thioredoxin and thioredoxin reductase: purification and characterization.* Biochemistry. **21**, 6628-6633 (1982)

Acknowledgements: We thank Dr. Xiao-Wei Zhang at Shanghai Institute of Hematology and Dr. Arthur Zelent at Samuel Waxman Cancer Research Foundation Joint Translational Research Center, Shanghai Institute of Hematology for helpful discussion and suggestion. Prof Dong-Er Zhang at University of California San Diego, USA for providing AML1-ETO9a cells; Dr. Shuo Dong at Baylor College of Medicine, USA for providing the pGL3-RUNX3 reporter plasmid; Dr. Fa-Yang Qiu at Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China for helping to synthesize biotin-oridonin and Dr. Guo-Yu Meng at Shanghai Institute of Hematology for technical assistance. The authors are indebted to all members of the Shanghai Institute of Hematology for their long-term support. **Funding:** Supported in part by the Mega project of Ministry of Science and Technology (2008ZX09312-026); The Chinese National Key Basic Research Project (973, 2010CB529200); The Grant for Innovation Group and key program of the National Natural Science Foundation of China (30821063; 30830119); National Natural Science Foundation of China (81170506); Samuel Waxman Cancer Research Foundation CoPI Program; And the Key Project of Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX1-YW-R-26 and KSCX2-YW-R-235). **Author contributions:** T.-Z., C.F.-W., P.-L., H.Y.-W., Y.-L., Y.Y.-W., Y.Y.-X., M.M.-H., H.M.-C., W.N.-Z., Y.-L. performed research; K.K.-L., L.M.-C. simulated the structural binding mode; G.B.-Z., J.X.-L., K.P. analyzed data and wrote the paper; S.J.-C., Z.-C. designed research, analyzed data and wrote the paper. **Competing interests:** The authors declare that they have no competing interests.

FIGURES and LEGENDS

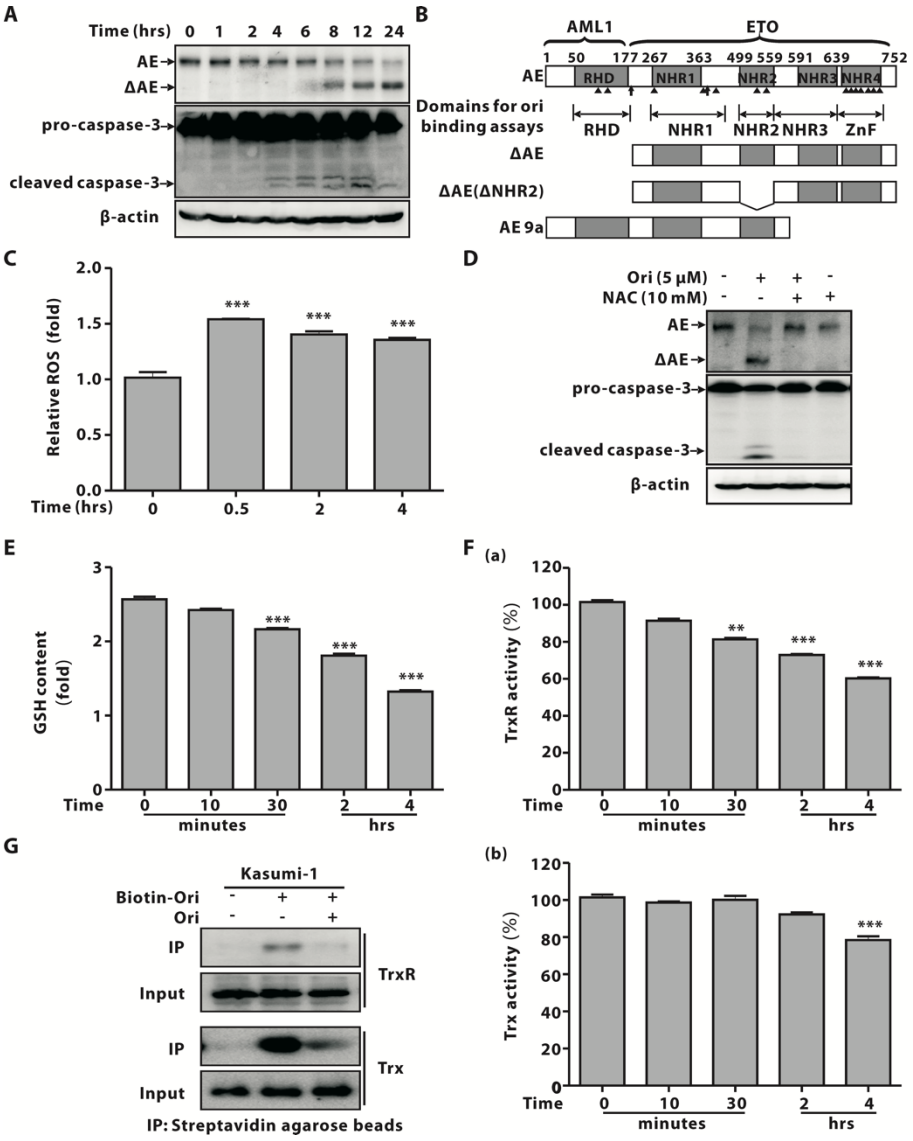

Fig. 1. Oridonin induces caspase-3 activation and AML1-ETO cleavage through increasing intracellular ROS. (A) Cells were treated with oridonin (ori, 5 μM, same for **C**-**F**) and Western blot (WB) was performed to check AML1-ETO (AE) status and caspase-3 activation. ΔAE, ΔAML1-ETO; hrs, hours. β-actin was used as internal control. **(B)** Schematic structures of AML1-ETO-related constructs, including domains for oridonin binding assays. ↑: caspase-3 cleavage site (D188 and D368 respectively), ▲: position of cysteine. **(C)** Intracellular ROS was measured under oridonin for indicated hrs using DCFH-DA (mean ± SEM). **(D)** Cells were pre-incubated with NAC for 1 hr before oridonin treatment for 12 hrs. Caspase-3 activation and generation of ΔAML1-ETO were analyzed by WB. **(E)** GSH content was measured in Kasumi-1 cells treated with oridonin (mean ± SEM) using a total GSH kit (Dojin Laboratories). **(F)** TrxR (a) and Trx (b) activities in Kasumi-1 cells treated with oridonin (mean ± SEM). **(G)** Streptavidin agarose affinity assay was performed to detect oridonin-interacting TrxR and Trx in Kasumi-1 cells. All experiments were independently performed in triplicates. *p<0.05, **p<0.01, ***p<0.001, compared to 0 hr.

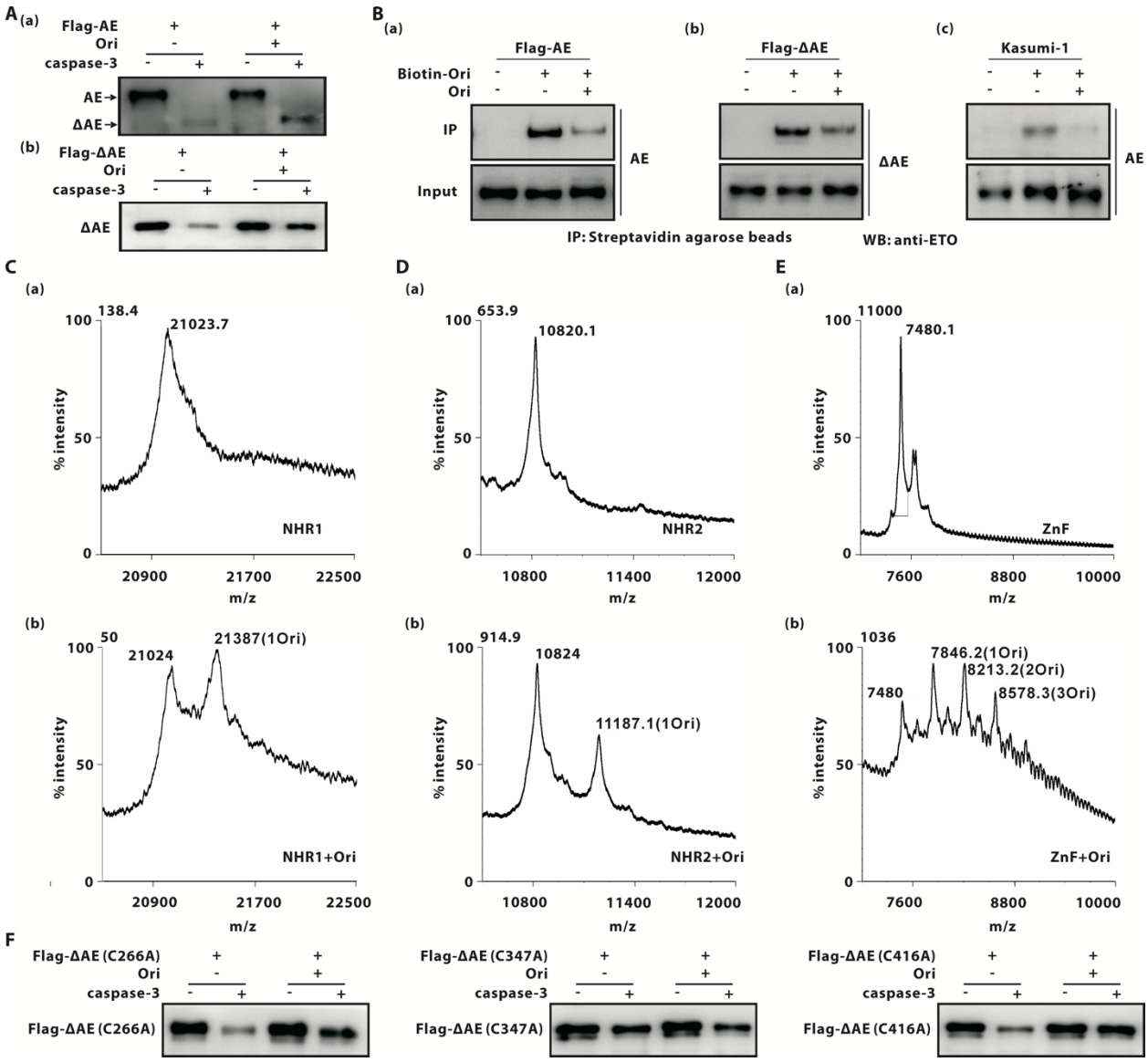

Fig. 2. Oridonin directly binds to AML1-ETO and stabilizes ΔAML1-ETO. (A) Immunoprecipitated Flag-AML1-ETO (a) or Flag-ΔAML1-ETO (b) was incubated with or without 0.2 mM oridonin for 1 hr, washed to eliminate oridonin, and then incubated with caspase-3 (0.2 μg) for 1 hr. WB was performed to detect the fragments of AML1-ETO or ΔAML1-ETO. **(B)** Streptavidin agarose affinity assay was performed to detect oridonin-interacting AML1-ETO or ΔAML1-ETO in 293T cells transiently expressing Flag-AML1-ETO (a) or Flag-ΔAML1-ETO (b) and Kasumi-1 cells (c). **(C-E)** Mass spectrum analysis of NHR1 (C), NHR2 (D) and ZnF (E) domains, as illustrated in Fig. 1B, in the absence (a) or presence (b) of oridonin. **(F)** Immunoprecipitated Flag-ΔAML1-ETO mutant proteins were incubated with or without 0.2 mM oridonin for 1 hrs, washed and then incubated with caspase-3 (0.2 μg) for 1 hr. WB was performed to detect ΔAML1-ETO.

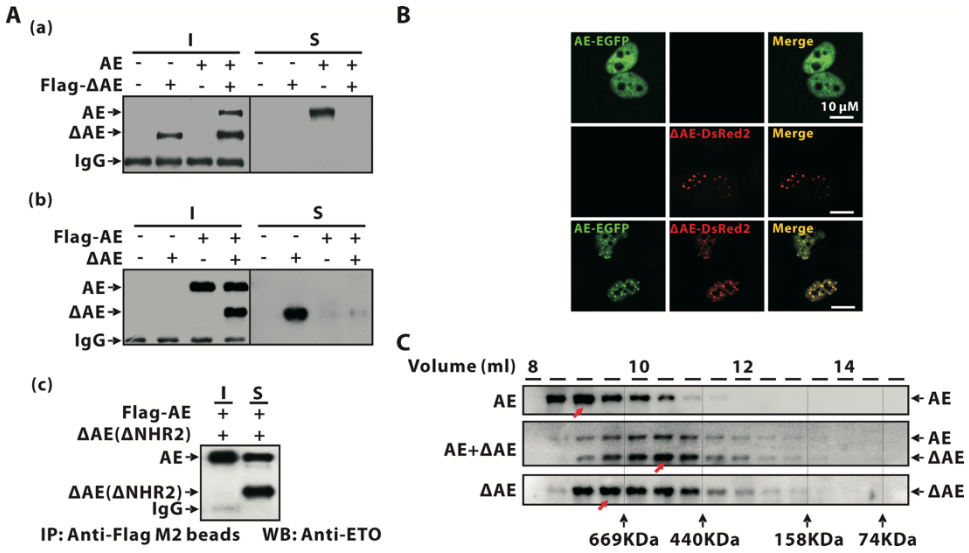

Fig. 3. ΔAML1-ETO interacts with AML1-ETO through NHR2 domain. (A) Co-IP analysis of nuclear extracts from transfected 293T cells to check interaction between AML1-ETO and **Δ**AML1-ETO (a and b), and between AML1-ETO and ΔAML1-ETO with deletion of NHR2 [ΔAML1-ETO(ΔNHR2)] (c). I: immunoprecipitate; S: supernatant. **(B)** Direct immunofluorescence staining in Hela cells transfected with indicated plasmids to reveal possible co-localization of AML1-ETO and **Δ**AML1-ETO. **(C)** SEC examination for apparent molecular weight distribution of AML1-ETO in the presence of ΔAML1-ETO. MW markers are shown at the bottom. ↗: position of peak intensity.

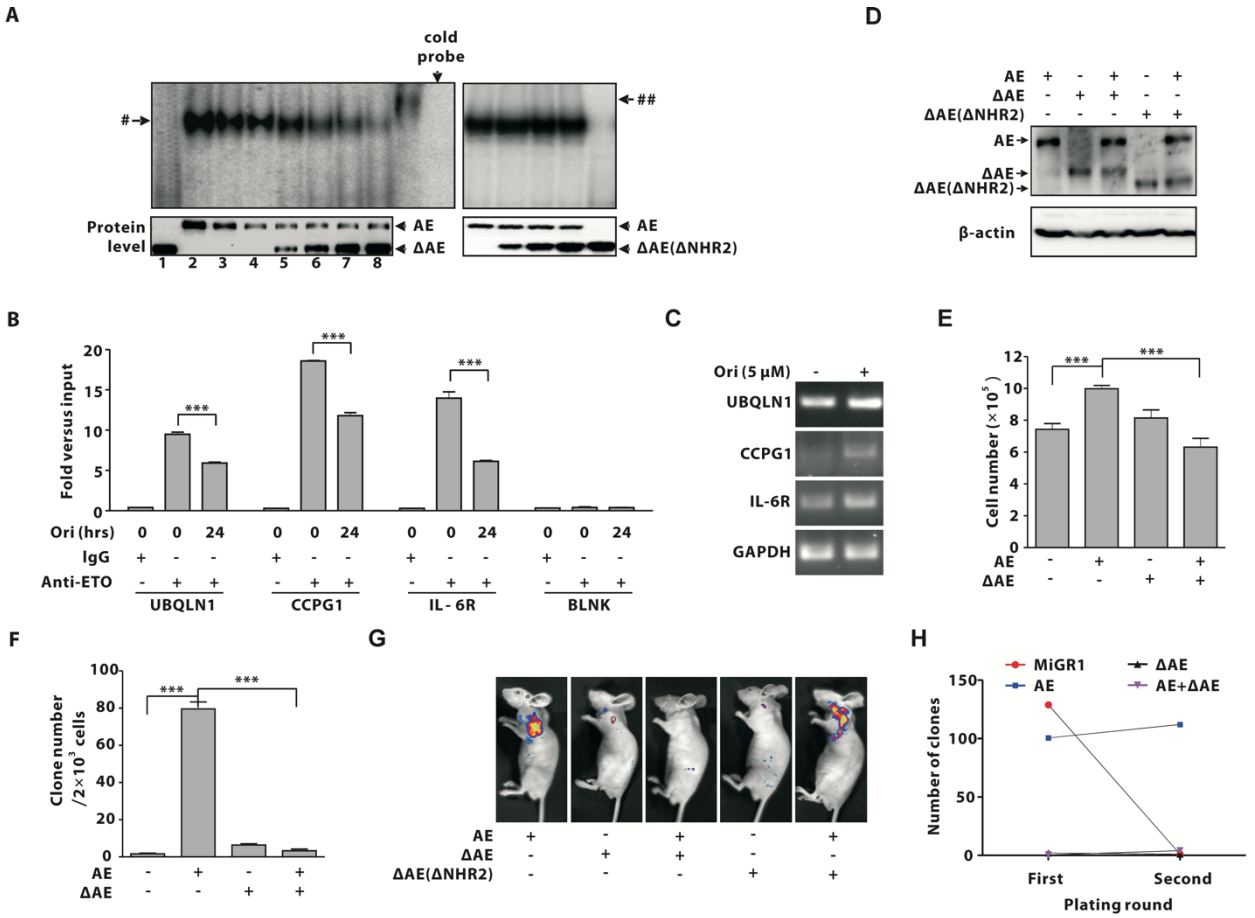

Fig. 4. ΔAML1-ETO antagonizes the activities of AML1-ETO. (A) EMSA assay. AML1-ETO purified from 293T cells were mixed with different amounts of ΔAML1-ETO, and relative amounts of AML1-ETO and/or ΔAML1-ETO or ΔAML1-ETO(ΔNHR2) used are shown at the bottom. #: shift; ##: supershift with anti-ETO antibody. **(B)** ChIP-qPCR was performed in oridonin (5 μM)-treated Kasumi-1 cells to detect binding of AML1-ETO to target gene promoters. *BLNK* was used as a negative control (mean ± SEM). **(C)** Semi-quantitative PCR was performed to detect the mRNA expression of *UBQLN1*, *CCPG1* and *IL-6R* after Kasumi-1 cells were treated with oridonin for 24 hrs. **(D-G)** NIH3T3 cells were infected with different retroviral constructs as indicated. GFP+/YFP+ NIH3T3 cells of each group were isolated. Expression of AML1-ETO, ΔAML1-ETO or ΔAML1-ETO(ΔNHR2) was confirmed by WB (D). Proliferation ability (E), colony-forming capacity (F) and tumorigenicity in nude mice (G) of these cells were examined and data represented as mean ± SEM. **(H)** Serial replating assay was performed in mice BM cells to exam the effect of ΔAML1-ETO on AML1/ETO's clonogenic activity *in vitro*. Numbers are averaged from two experiments, each containing duplicate samples. ***p<0.001.

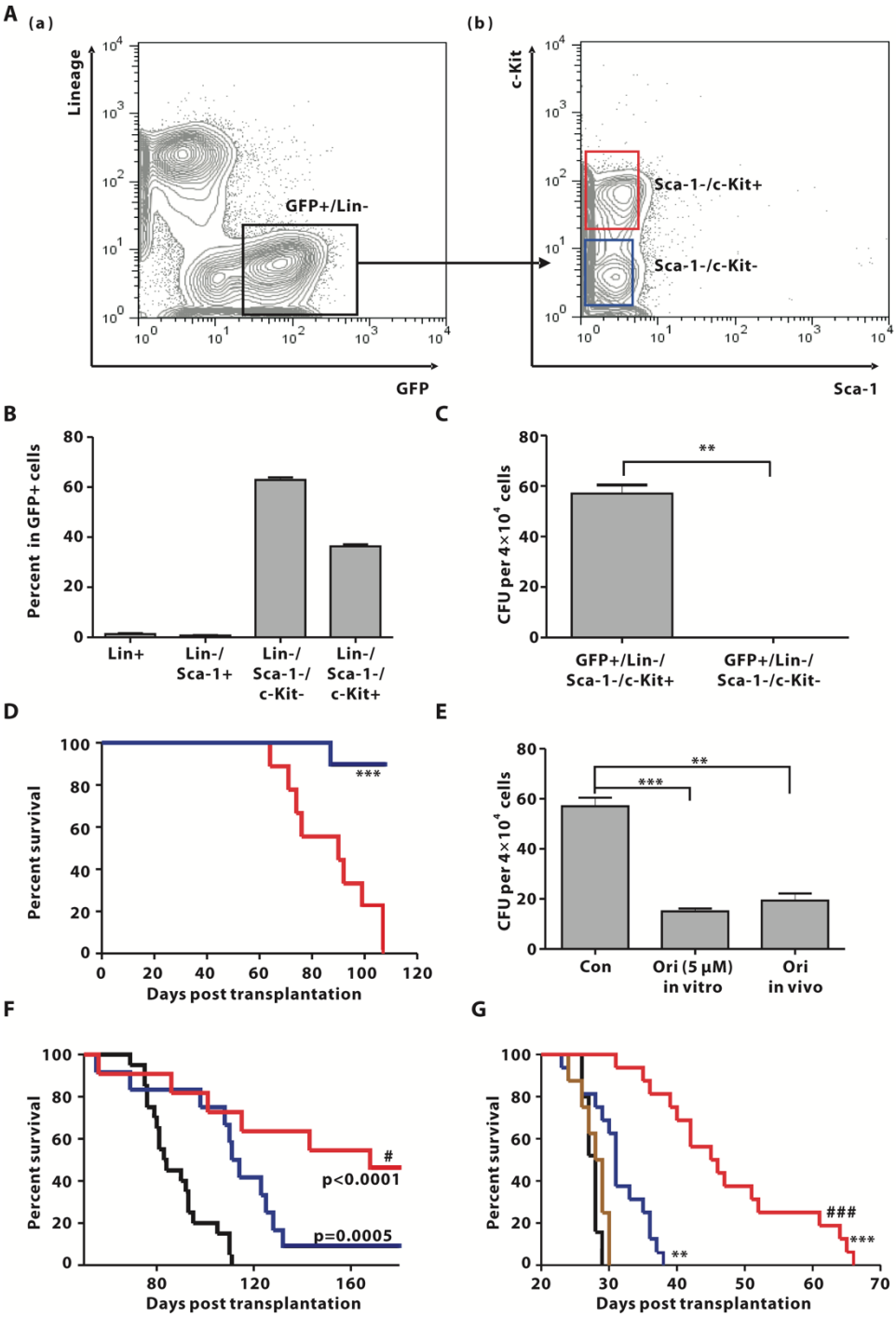

Fig. 5. Oridonin inhibits activity of LICs. (A-B) Flow cytometry assay of BM cells from AML1-ETO9a leukemia mice with indicated markers. **(A)** GFP+/Lin- cells (a) were further analyzed using c-Kit and Sca-1 markers (b). Data are representative of three mice. **(B)** Percentages of Lin+, Lin-/Sca-1+, Sca-1-/c-Kit- and Sca-1-/c-Kit+ in GFP+ cells. **(C)** Colony-forming ability of 4×10^4 GFP+/Lin-/Sca-1-/c-Kit+ and GFP+/Lin-/Sca-1-/c-Kit- cells. **(D)** Kaplan-Meier estimates of cumulative survival of mice inoculated with 2,000 GFP+/Lin-/Sca-1-/c-Kit+ (red) or GFP+/Lin-/Sca-1-/c-Kit- (blue) cells (mean ± SEM). **(E)** Effect of oridonin on colony-forming ability of GFP+/Lin-/Sca-1-/c-Kit+ cells *in vitro* (Ori 5 M) and in vivo when AML1-ETO9a mice were treated with oridonin (Ori) (mean ± SEM). **(F-G)** AML1-ETO9a mice were treated with different protocols: vehicle (black); oridonin (blue); ATRA/G-CSF (brown); oridonin/ATRA/G-CSF (red). **(F)** Kaplan-

Meier estimates of cumulative survival of mice inoculated with 1,000 GFP+/Lin-/Sca-1-/c-Kit+ cells from BM of different donor groups. **(G)** Kaplan-Meier estimates of cumulative survival of mice transplanted with bulk leukemia cells treated with different protocols. For (B), (C) and (E), all experiments were independently performed in triplicates. ***p<0.001, **p<0.01 when compared with vehicle or as indicated; #p<0.05, ###p<0.001 when compared with oridonin.

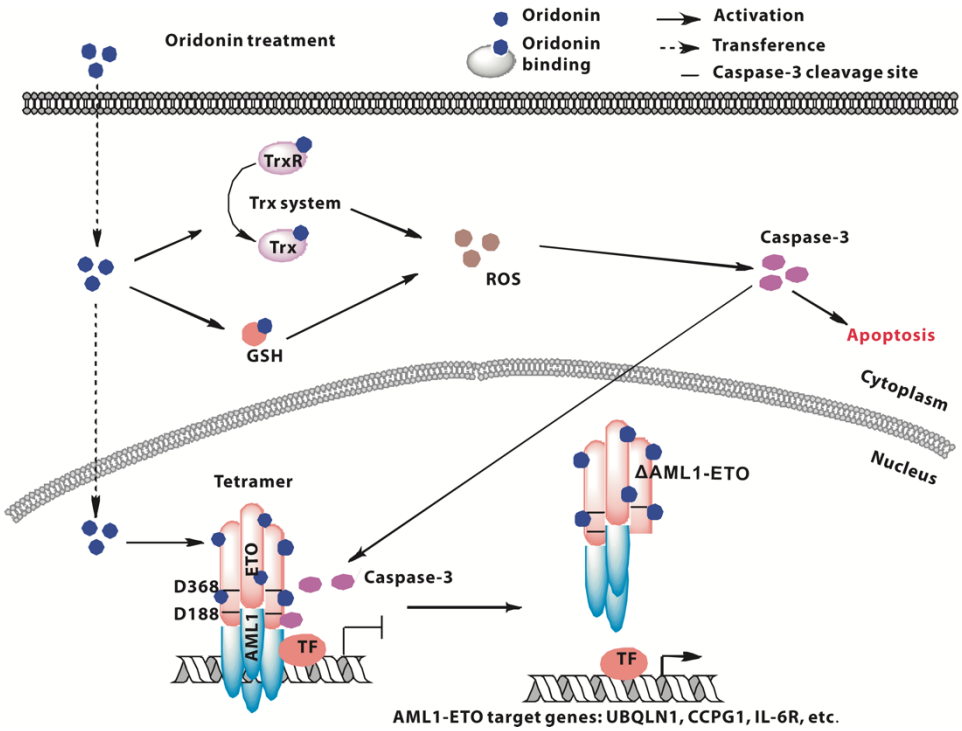

Fig. 6. A summary diagram outlines the working mechanisms of oridonin in the treatment of AML1-ETO positive leukemia. Oridonin interacts with GSH and Trx/TrxR to increase ROS, which in turn activates caspase-3. Moreover, oridonin binds AML1-ETO, directing the enzymatic cleavage at D188 via caspase-3 to generate ΔAML1-ETO, and preventing the protein from further proteolysis. ΔAML1-ETO interacts with AML1-ETO and interferes with the trans-regulatory functions of remaining AML1-ETO oncoprotein.