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The ROS/SUMO Axis Contributes to the Response of Acute Myeloid Leukemia Cells to Chemotherapeutic Drugs

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SUMMARY

Chemotherapeutic drugs used in the treatment of acute myeloid leukemias (AMLs) are thought to induce cancer cell death through the generation of DNA double-strand breaks. Here, we report that one of their early effects is the loss of conjugation of the ubiquitin-like protein SUMO from its targets via reactive oxygen species (ROS)-dependent inhibition of the SUMO-conjugating enzymes. Desumoylation regulates the expression of specific genes, such as the proapoptotic gene *DDIT3*, and helps induce apoptosis in chemosensitive AMLs. In contrast, chemotherapeutics do not activate the ROS/SUMO axis in chemoresistant cells. However, pro-oxidants or inhibition of the SUMO pathway by anacardic acid restores *DDIT3* expression and apoptosis in chemoresistant cell lines and patient samples, including leukemic stem cells. Finally, inhibition of the SUMO pathway decreases tumor growth in mice xenografted with AML cells. Thus, targeting the ROS/SUMO axis might constitute a therapeutic strategy for AML patients resistant to conventional chemotherapies.

INTRODUCTION

Acute myeloid leukemias (AMLs) are severe hematological malignancies induced by the oncogenic transformation of hematopoietic stem and myeloid progenitor cells. It leads to bone marrow failure and related complications, including infections, anemia, or bleeding. Despite recent progress in the molecular characterization and prognosis refinement of this disease (Cancer Genome Atlas Research Network, 2013), treatments have not significantly changed during the past 30 years. The standard

induction chemotherapy relies on a combination of the nucleoside analog cytarabine (Ara-C) with an anthracyclin, such as daunorubicin (DNR) or idarubicin, sometimes in association with other drugs, such as etoposide (VP16). Although most patients reach the complete remission after initial chemotherapeutic treatment, relapses are frequent, and the global prognosis remains poor with an overall survival of 40% in young patients and much less in old ones (Estey, 2012). Relapses are largely due to the persistence of leukemic stem cells (LSCs), which are refractory to chemotherapeutic drug-induced cell death (Vergez et al., 2011).

Generally, the mechanisms of action of the chemotherapeutic drugs used for AMLs treatment rely on the inhibition of DNA synthesis and the induction of DNA double-strand breaks in highly replicating cancer cells, which in fine lead to their apoptosis. However, these drugs can induce cell death by other mechanisms. In particular, reactive oxygen species (ROS) have been known as critical mediators of genotoxics-induced cell death for long (Mat es et al., 2012). They are also responsible for certain side effects of chemotherapeutic drugs, such as anthracyclin cardiotoxicity (Gewirtz, 1999; Hole et al., 2011). However, their cellular effectors have not been clearly identified (Mat es et al., 2012).

SUMO is a family of three related ubiquitin-like peptidic post-translational modifiers, SUMO-1, -2, or -3, the latter two being almost identical (referred to as SUMO-2/3). SUMO is conjugated to  -amino groups of lysines of numerous target proteins by a heterodimeric SUMO-activating E1 enzyme (AOS1/UBA2), a SUMO-conjugating E2 enzyme UBC9 (encoded by *UBE2I*) and various E3 factors facilitating its transfer from the E2 onto substrates. Most sumoylated proteins go through constant cycles of conjugation/deconjugation due to various desumoylases. Sumoylation changes substrate protein properties, in particular by favoring the recruitment of SUMO-binding partners (Flotho and Melchior, 2013). Sumoylation is sensitive to various stresses that regulate the activity of the SUMO pathway's enzymes. In particular, ROS can inactivate SUMO conjugation by inducing

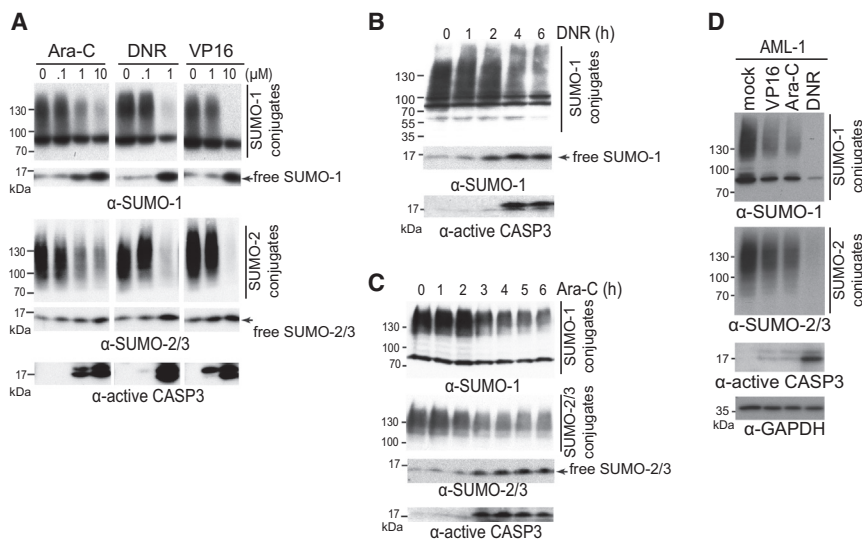


Figure 1. Chemotherapeutic Drugs Induce Desumoylation in AML Cells

(A) HL60 cells were treated with Ara-C, VP16, or DNR for 7 hr and immunoblotted for SUMO-1, SUMO-2, and active caspase-3 (B).

(B and C) HL60 cells were treated with 1 μ M DNR (B) or 2 μ M Ara-C (C) for the indicated times and immunoblotted for SUMO-1, SUMO-2, or active CASPASE-3.

(D) Primary AML cells were treated in vitro with VP16 (10 μ M), Ara-C (2 μ M), or DNR (1 μ M) for 24 hr and immunoblotted for SUMO-1, SUMO-2, active-caspase-3, and GAPDH.

the formation of a reversible disulfide bridge between UBA2 and UBC9 catalytic cysteines (Bossis and Melchior, 2006). This disrupts the sumoylation/desumoylation cycle, resulting in protein desumoylation. Such global shifts in the cell sumoylome are thought to play critical roles in the cellular response to these stresses (Tempé et al., 2008). Although sumoylation controls many cellular functions, one well-characterized role is the regulation of transcription via the modification of histones, transcription factors and cofactors, chromatin-modifying enzymes, and basal transcription machinery (Raman et al., 2013). Finally, deregulation of the SUMO pathway has been found in various cancers (Bettermann et al., 2012) and is generally associated with an adverse outcome (Driscoll et al., 2010). Moreover, recent evidence suggests that targeting sumoylation could be beneficial for cancer treatment. In particular, inhibition of sumoylation preferentially induces death of Myc-overexpressing cancer cells (Kessler et al., 2012).

Here, we address the role of the SUMO pathway in AMLs apoptotic response to chemotherapeutic drugs. We show that the genotoxics currently used in the clinic induce rapid ROS-dependent protein desumoylation, which participates both in transcriptome alteration and apoptosis of chemosensitive AML cells. Failure to activate this ROS/SUMO axis is associated with AMLs chemoresistance. However, its induction by different means is sufficient to induce death of chemoresistant AML cell lines, as well as that of AML patient cells, including their leukemic stem cells. Furthermore, inhibition of the SUMO pathway reduces AML cell growth in xenografted mice. Overall, our work identifies the ROS/SUMO axis as a novel player in chemotherapeutic drugs-induced apoptosis and a potential target to overcome chemoresistance in AMLs.

RESULTS

Chemotherapeutic Drugs Induce Massive Desumoylation in Chemosensitive AMLs

A chemosensitive AML model cell line, HL60 (Quillet-Mary et al., 1996), was treated with Ara-C, DNR, and VP16 at doses consis-

tent with plasma concentrations in treated AML patients (Gewirtz, 1999; Krogh-Madsen et al., 2010). This induced a dose-dependent decrease in SUMO-1 and SUMO-2/3 (Figure 1A) conjugate levels and the appearance of free SUMO, which did not result from increased *SUMO-1* or *-2* gene transcription (Figure S1). This suggested that these chemotherapeutic drugs induced SUMO deconjugation from its target proteins. Desumoylation rapidly began after drug addition, as indicated by the increase in the free SUMO pool already after 1 hr of treatment. Desumoylation onset preceded mitochondrial membrane potential loss (Figure S2), caspase-3 activation, and a more global disappearance of SUMO conjugates visible after 3–4 hr (Figures 1B and 1C). Importantly, primary chemosensitive AML cells (Figure 1D), as well as two other chemosensitive AML cell lines (U937 and THP1) (Figure S3), also showed massive drug-induced decrease in SUMO conjugates correlating with caspase-3 activation. These data indicated that one of the early effects of chemotherapeutic drugs currently used to treat AMLs is the induction of protein desumoylation.

Chemotherapeutic Drug-Induced Desumoylation Regulates Gene Expression and Apoptosis

Considering the acknowledged role of desumoylation in the control of gene expression, we asked whether desumoylation could alter specific transcriptional program. To this aim, we profiled and compared the transcriptome of HL60 cells treated with anacardic acid, a natural inhibitor of the SUMO E1 enzyme (Fukuda et al., 2009), with that of mock (DMSO) -treated cells. We found 318 significant differentially expressed (SDE) genes (fold change over 2-fold), 200 being upregulated (71 more than 3-fold), and 118 downregulated (ten more than 3-fold) (Table S1). Gene ontology analyses revealed that upregulated genes are involved in cellular processes such as the response to endoplasmic reticulum (ER) stress, transcription control, nucleosome assembly, cell-cycle arrest, and apoptosis (Figure 2A). No specific process was significantly enriched ($p < 0.01$) for the downregulated genes (data not shown). We confirmed the transcriptional activation of six of the most induced genes by RT-qPCR and showed that the expression of these genes was also strongly activated by Ara-C (Figure 2B), suggesting that chemotherapeutic drug-induced desumoylation is involved in their induction. We further studied the DNA Damage-Induced Transcript 3 (*DDIT3*) gene, as it encodes

the CHOP10/GADD153 protein, an activator of apoptosis involved in the ER stress response. CHOP10 has also been implicated in the apoptotic response of AML cells to chemotherapeutic drugs (Eymin et al., 1997). While *DDIT3* mRNA levels increased upon DNR and Ara-C treatment of HL60 cells, SUMO conjugates rapidly decreased in the gene proximal promoter region (Figures 2C and 2D). Sumoylation of promoter-bound proteins is principally associated with transcriptional repression (Cubéñas-Potts and Matunis, 2013) or limitation of transcriptional activity (Rosonina et al., 2010), including in the case of the *DDIT3* gene (Tempé et al., 2014). Consistent with this idea, counteracting protein desumoylation by overexpressing SUMO-2 significantly reduced *DDIT3* induction by Ara-C (Figure 2E). Moreover, overexpression of SUMO-2 delayed Ara-C-induced apoptosis (Figure 2F). Thus, in chemosensitive AML cells, drug-induced desumoylation stimulates genes, such as *DDIT3*, and facilitates the induction of apoptosis.

ROS Are Involved in Chemotherapeutic Drug-Induced Protein Desumoylation in AMLs

Chemotherapeutic drugs induce the production of ROS (Gewirtz, 1999). As shown in Figure 3A, Ara-C, DNR, and VP-16 led to the formation of the ROS-induced disulfide crosslink between UBA2 and UBC9 catalytic cysteines (Bossis and Melchior, 2006). Importantly, this correlated with a strong decrease in the level of the UBC9~SUMO thioester adduct, the active form of UBC9. Using a mouse retroviral model of AML (Michaud et al., 2010; Moreau-Gachelin, 2006), we showed that the treatment of leukemic animals with Ara-C and, to a lesser extent with DNR, also induced UBC9-UBA2 crosslink in vivo in tumor cells (Figure 3B). Inhibition of NADPH oxidases (NOX), a major source of ROS in cancer cells (Block and Gorin, 2012), by diphenylethionium (DPI) prevented both DNR- and VP16-induced loss of SUMO conjugates, UBC9-UBA2 crosslinking and apoptosis (Figure 3C). Finally, treatments of AML patient cells also led to UBC9-UBA2 crosslinking, the levels of which correlated with cell sensitivity to the different drugs in vitro (Figures 3D and 3E). These data suggest that chemotherapeutic drug-induced protein desumoylation in AMLs is a consequence of ROS production.

The ROS/SUMO Axis Is Not Activated by Chemotherapeutic Drugs in Chemosensitive AMLs

We next asked whether chemoresistance could be associated with impaired activation of the ROS/SUMO axis. In contrast to the chemosensitive U937 and HL60 cells, which exhibit a strong desumoylation upon DNR, Ara-C, and VP16 treatment, the chemoresistant AML cell lines TF1 and KG1a (Quillet-Mary et al., 1996) were resistant to drug-induced desumoylation. This correlated with the absence of ROS-dependent crosslinking of UBA2 to UBC9 (Figures 4A and 4B). We therefore tested whether chemoresistant AML cells were intrinsically resistant to ROS-dependent protein desumoylation and whether forced activation of the ROS/SUMO axis could lead to their death. First, TF1 or KG1a cells were treated with increasing doses of glucose oxidase, which causes sustained production of ROS from the degradation of extracellular glucose. This led to UBC9-UBA2 crosslinking and protein desumoylation (Figures 4C and S4), which correlated

with strong induction of *DDIT3* mRNA and massive cell death (Figures 4D and S4). Next, we derived TF1 clones expressing inducible control- or SUMO-1/2/3 miRNAs. SUMO-1/2/3 RNA interference was sufficient to induce massive death of these chemoresistant cells (Figure 4E). Thus, the ROS/SUMO axis is inactive in AML cells that are resistant to chemotherapy-induced apoptosis. However, its reactivation restores a cell death program in these cells.

Inhibition of the SUMO Pathway Targets Chemosensitive AML Cells In Vitro and In Vivo

Finally, we tested the effect of pharmacological inhibition of protein sumoylation on AML cells using anacardic acid. It decreased the amount of SUMO conjugates in chemoresistant TF1 cells (Figure 5A, left panel), activated caspase 3 (Figure 5A, right panel) and induced *DDIT3* mRNA (Figure 5B), whereas Ara-C had no effect. Next, we measured anacardic acid IC_{50} in chemosensitive (HL60, U937) and chemoresistant (TF1, KG1a) cells. All were sensitive to comparable concentrations of the drug (Figure 5C). Importantly, anacardic acid had significantly lower effect on peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T lymphocytes from healthy volunteers, as well as on proliferating mouse embryonic fibroblasts (MEFs) than on AML cells (Figure 4C). Similar to AML cell lines, patient samples showed variable sensitivity to Ara-C (IC_{50} ranging from 2 to >500 μ M), but their IC_{50} for anacardic acid was relatively homogeneous with a median concentration of 42 μ M (Figure 5D). For seven of the patient samples, we compared the IC_{50} of LSCs ($CD34^+ CD38^{low/-} CD123^+$) to the bulk of leukemic cells. Although globally less sensitive to Ara-C-induced cell death, LSCs showed similar sensitivity toward anacardic acid than the bulk of leukemic cells (Figure 5E). Interestingly, anacardic acid led to a strong activation of *DDIT3* mRNA in two primary patient samples, either chemosensitive (Figure 5F, left panel, IC_{50} = 10 μ M for Ara-C) or chemoresistant (Figure 5F, right panel, IC_{50} = 250 μ M for Ara-C), whereas Ara-C induced *DDIT3* expression only in the chemosensitive sample. Finally, nude mice xenografted with chemoresistant KG1a cells and peritumorally treated with anacardic acid showed a significant delay in tumor growth (Figures 5G–5I). Anacardic acid did however not alter general biological parameters in the treated mice, as assayed by weight control or blood cell counting (Figure S5). These data suggest that targeting sumoylation might overcome chemoresistance in AMLs.

DISCUSSION

Although targeted therapies have strongly improved the treatment of a subset of cancer patients, the classical chemotherapeutic drugs remain the standard therapy in most cancers. This is especially true for acute myeloid leukemia patients whose front-line treatment is generally a combination of an anthracycline and the nucleoside analog Ara-C. Here, we show that a role of these drugs is the inhibition of the SUMO pathway. They induce a progressive loss of conjugation of SUMO to its targets, gene promoter-bound proteins being among the most rapidly affected. Recent studies reveal that SUMO can be considered as an integral component of chromatin and regulates specific

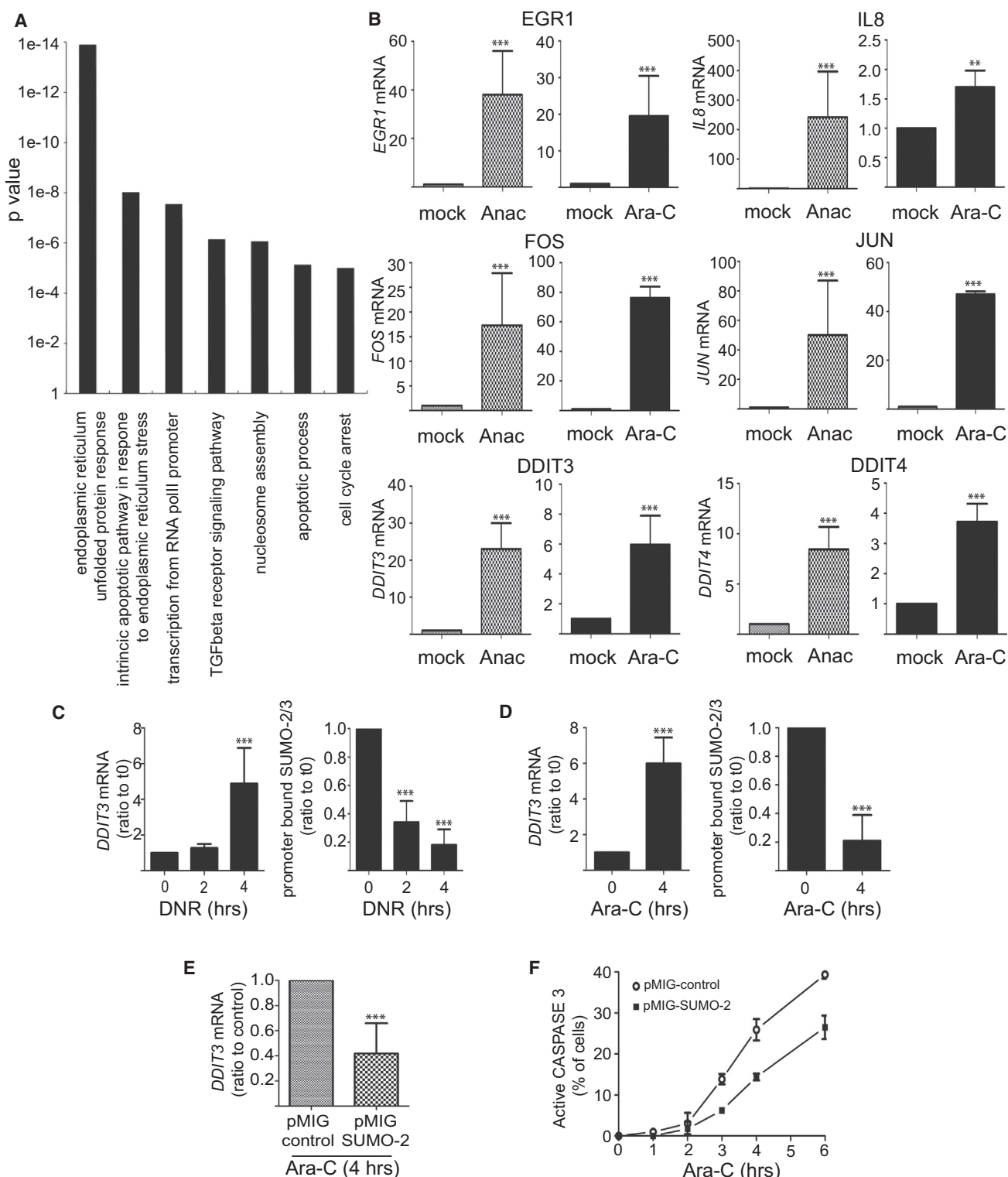


Figure 2. Desumoylation Regulates Specific Transcriptional Programs and Participates in the Induction of Apoptosis

(A) Top categories identified by gene ontologies of genes upregulated (more than 2-fold) in HL60 cells treated with anacardic acid (100 μ M) for 5 hr compared to mock (DMSO)-treated cells.

(B) HL60 cells were treated with 100 μ M anacardic acid (5 hr) or 2 μ M Ara-C (3 hr) or control vehicle and mRNA for the indicated genes were monitored by RT-qPCR (n = 3).

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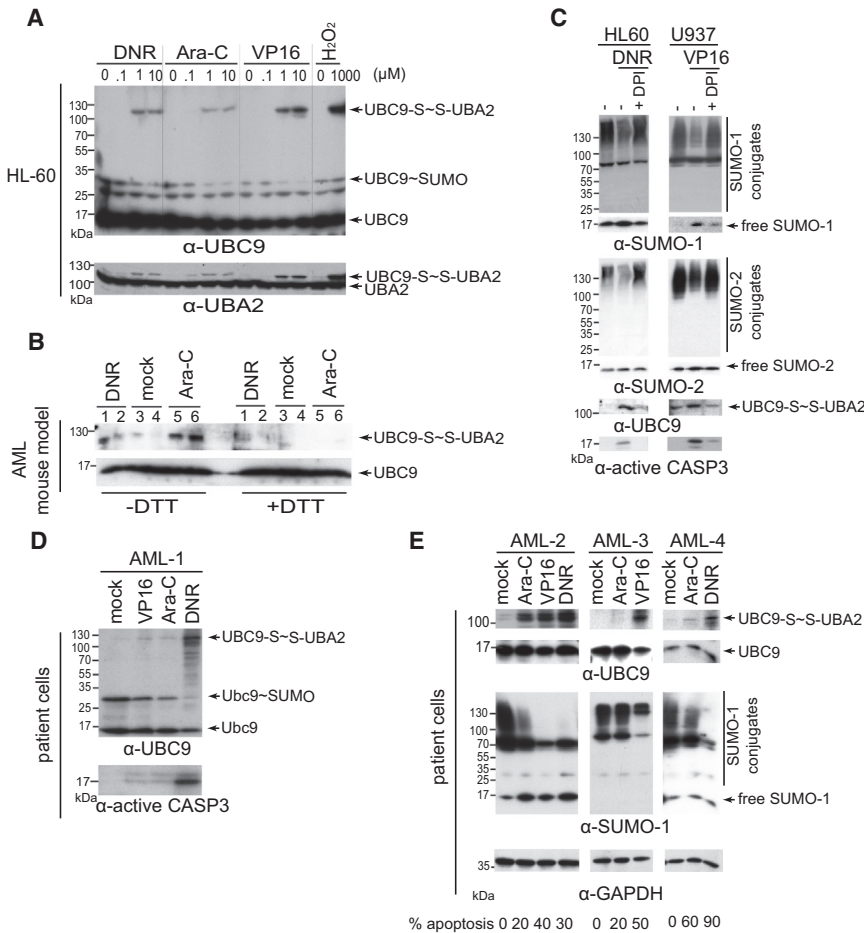


Figure 3. Chemotherapeutic Drug-Induced ROS Inhibit SUMO-E1 and E2 Enzymes

(A) HL60 cells were treated with Ara-C, VP16, or DNR for 7 hr or H₂O₂ (15 min), lysed in a non-reducing sample buffer and immunoblotted for UBC9 and UBA2.
 (B) Leukemic FrCasE-infected mice were treated with DNR (10 mg/kg) or Ara-C (50 mg/kg) every 2 days for 2 weeks and sacrificed 4 hr after the last injection. Spleen extracts (in non-reducing or reducing conditions) were immunoblotted for UBC9.
 (C) HL60 and U937 cells were treated with DNR (1 μM) or VP16 (10 μM) for 7 hr ± DPI (10 μM) and immunoblotted for SUMO-1, UBC9 (non-reducing gel), or active caspase-3.
 (D) Primary leukemic cells (same patient as in Figure 1D) were treated in vitro with VP16 (10 μM), Ara-C (2 μM), or DNR (1 μM) for 24 hr and immunoblotted for UBC9 (non-reducing gel) and active-CASPASE-3.
 (E) Same as (C) with three other patient samples immunoblotted for UBC9 (non-reducing gel), SUMO-1, and GAPDH. Viability was assessed and compared to mock-treated cells.

of the SUMO pathway in chemosensitive AML cell lines and patient samples. This is due to their ability to promote the formation of a disulfide-bond between the catalytic cysteines of the SUMO E1 and E2 enzymes (Bossis and Melchior, 2006). Although only a fraction of both E1 and E2 are crosslinked upon chemotherapeutic drug treatment, this inactivation involves the active fraction of these enzymes.

Given the fact that desumoylases are not inhibited by these ROS concentrations (Felgioni and Nisticò, 2013), this explains the massive protein desumoylation we observed. Importantly, the inhibition of ROS production with an NADPH oxidase inhibitor strongly dampened drug-induced protein desumoylation and delayed entry into apoptosis. This confirms the role of ROS production in drug-induced death of chemosensitive AML cells. An important issue is whether chemotherapeutic drugs can also induce the ROS/SUMO axis in other types of cancer. Our data (data not shown) suggest that this might not always be the case because, even though we could detect the UBC9-UBA2 cross-link in an ALL (acute lymphocytic leukemia) cell line treated with DNR or Ara-C, we could not in epithelial cancer cell lines, such as MCF-7, HEK293, or HeLa. This might reflect differences in antioxidant or ROS production capacities between cancer types.

transcriptional programs (Neyret-Kahn et al., 2013). Consistent with this, our gene expression data suggest that desumoylation triggers the expression of genes associated with the ER stress, apoptosis induction, nucleosome remodeling, and cell-cycle arrest. Considering the various roles of sumoylation, in particular, in the control of genome integrity (Jackson and Durocher, 2013), we do not exclude that drug-induced hyposumoylation might also have other consequences, including impairment of genotoxics-induced DNA damage repair. However, our data suggest that one of its important roles is to regulate the expression of specific genes involved in AML cell response to chemotherapeutic drugs.

ROS can no longer be considered solely as toxic molecules causing random damages to biomolecules. They are also essential second messengers regulating numerous signaling pathways (Pausen and Carroll, 2010). Consistent with this, we show here that they are responsible for drug-induced inhibition

(C and D) HL60 cells were treated with 1 μM DNR (C) or 2 μM Ara-C (D) for the indicated times before analysis of *DDIT3* mRNA (left panels, n = 4 for DNR, n = 6 for Ara-C). SUMO-2/3 on the *DDIT3* promoter was assayed by ChIP (right panels) and normalized to DNA input. SUMO level in nontreated cells was set to 1 (n = 7 for DNR, n = 3 for Ara-C).

(E) HL60 cells infected with pMIG or pMIG-SUMO-2 lentiviral vectors were treated with Ara-C (2 μM) for 4 hr. *DDIT3* mRNA was RT-qPCR assayed (n = 7).

(F) The same cells as in (E) were treated with 2 μM Ara-C for the indicated times and flow cytometry-analyzed for active-CASPASE-3 (n = 3).

Results are expressed as means ± SD.

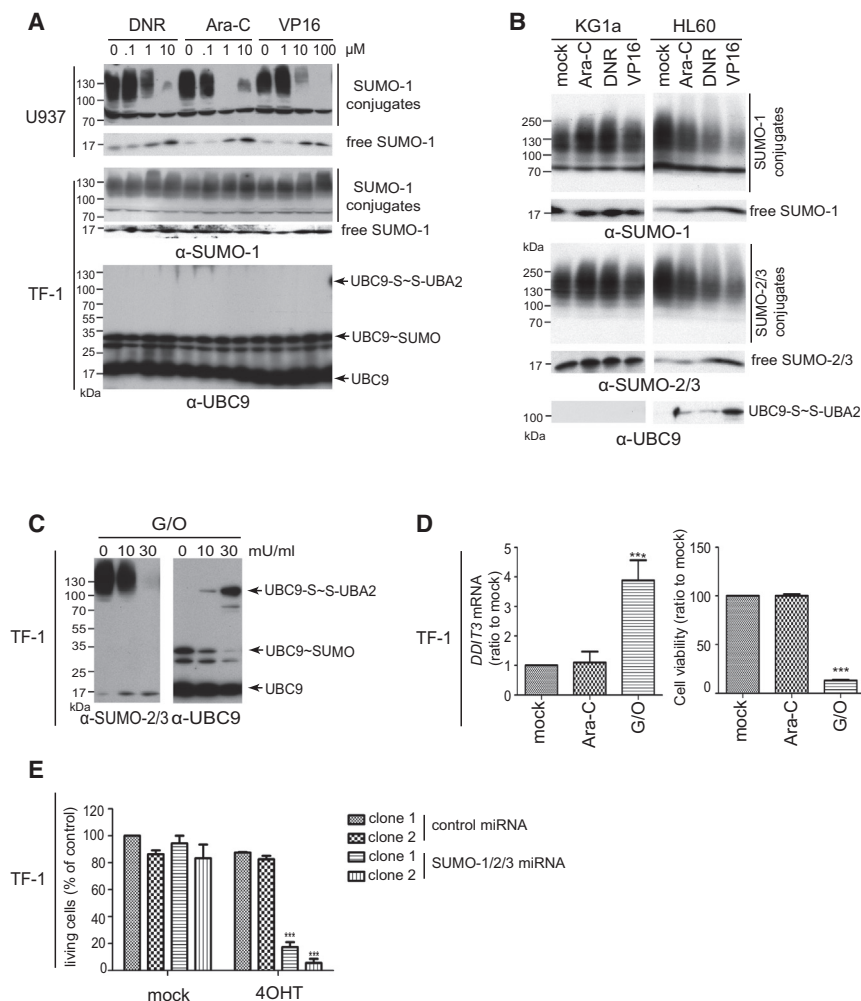


Figure 4. Reactivation of the ROS/SUMO Axis Restores Chemoresistant AML Cells Death

(A) U937 and TF1 cells were treated with Ara-C, VP16 or DNR for 7 hr and immunoblotted for SUMO-1 or UBC9 (nonreducing gel). (B) HL60 and KG1a cells were treated with Ara-C (2 μM), DNR (1 μM), or VP16 (10 μM) for 7 hr and processed as in (A). (C) TF1 cells were treated with glucose oxidase (G/O) for 6 hr and immunoblotted for SUMO-2 or UBC9 (nonreducing gel). (D) TF1 cells were treated with Ara-C (2 μM) or glucose oxidase (10 mU/ml). *DDIT3* mRNA was analyzed after 6 hr of treatment using mock-treated cells as a reference (left panel, n = 3), and cell viability was assessed at 24 hr (right panel, n = 3). (E) TF1 clones expressing a control or SUMO-1/2/3 miRNAs under the control of 4-OHT-inducible promoter were treated with 4OHT (20 nM) for 5 days and viability was assessed by MTS (n = 3). Results are expressed as means ± SD.

cell lines in vitro and in vivo as well as that of patient leukemic cells, including LSCs. Moreover, the absence of overt toxicity of anacardic acid on nontransformed cells and in living mice (except local sensitization when injected subcutaneously; data not shown) suggests that inhibiting the SUMO pathway may have less severe side effects than pro-oxidant therapies. Chemical engineering of anacardic acid to improve its solubility and bioavailability or developing novel SUMO pathway inhibitors might therefore offer an avenue to improve the outcome of

Chemotherapeutic drugs do not activate the ROS/SUMO axis in chemoresistant AML cells. The absence of ROS-induced UBC9-UBA2 disulfide-crosslinking upon treatment suggests that this might be due to lower ROS production and/or higher antioxidant capacity of chemoresistant AML cells. Along this line, LSCs, which are highly resistant to chemotherapeutic drugs and thought to be responsible for relapses, produce less ROS than the bulk of leukemic cells (Lagadinou et al., 2013). At least two lines of evidence suggest that increasing ROS concentration could be of therapeutic value for treating AMLs: (1) the inhibition of antioxidant systems induces primitive CD34⁺ AML cell death (Pei et al., 2013) and (2) pro-oxidants induce the regression of acute promyelocytic leukemia (a subtype of AMLs characterized by a chromosome translocation fusing the *PML* and *RARA* genes) in mouse models (Jeanne et al., 2010). However, the clinical usefulness of pro-oxidant therapies might be limited by their toxicity (Hole et al., 2011; Matés et al., 2012). An alternative strategy to activate the ROS/SUMO axis in chemoresistant cells may therefore consist of targeting the SUMO pathway. In support of this idea, anacardic acid, a natural molecule of the Chinese pharmacopeia known to trigger apoptosis of various cancer cell lines in vitro (Tan et al., 2012) induced death of chemoresistant AML

AMLs patients by targeting leukemic cells, including LSCs resistant to conventional chemotherapies.

EXPERIMENTAL PROCEDURES

Pharmacological Inhibitors, Reagents, and Antibodies

Cytosine-β-D-arabofuranoside (Ara-C), daunorubicin-hydrochloride (DNR), etoposide (VP-16), glucose-oxidase, and hydrogen-peroxide were from Sigma. Anacardic acid from Merck Millipore. SUMO-1 (21C7) and SUMO-2 (8A2) hybridomas were from the Developmental Studies Hybridoma Bank. Goat anti-SUMO-2 (used for chromatin immunoprecipitation [ChIP]) and anti-UBA2 were previously described (Bossis and Melchior, 2006). Anti-UBC9 (sc-10759) and GAPDH (sc-25778) were from Santa Cruz Biotechnology; anti-cleaved CASPASE-3 (D175) were from Cell Signaling Technology. Antibodies and gating strategies used to phenotype patient samples were described previously (Vergez et al., 2011).

Cell Lines and Clinical Samples

U937, HL60, THP1, KG1a, and TF1 cells (DSMZ, Germany) were cultured in RPMI or Iscove modifier Dulbecco's medium (IMDM) (for KG1a) with 10% fetal bovine serum (FBS). TF1 were cultured with addition of 2 ng/ml GM-CSF (PeproTech). Mouse embryonic fibroblasts were a kind gift from M. Bialic and were cultured in DMEM with 10% FBS. For treatments, cells were seeded at 0.3×10^6 cells/ml the day before the experiment, and fresh medium was added together with the drugs. PBMC and CD4⁺ lymphocytes were purified

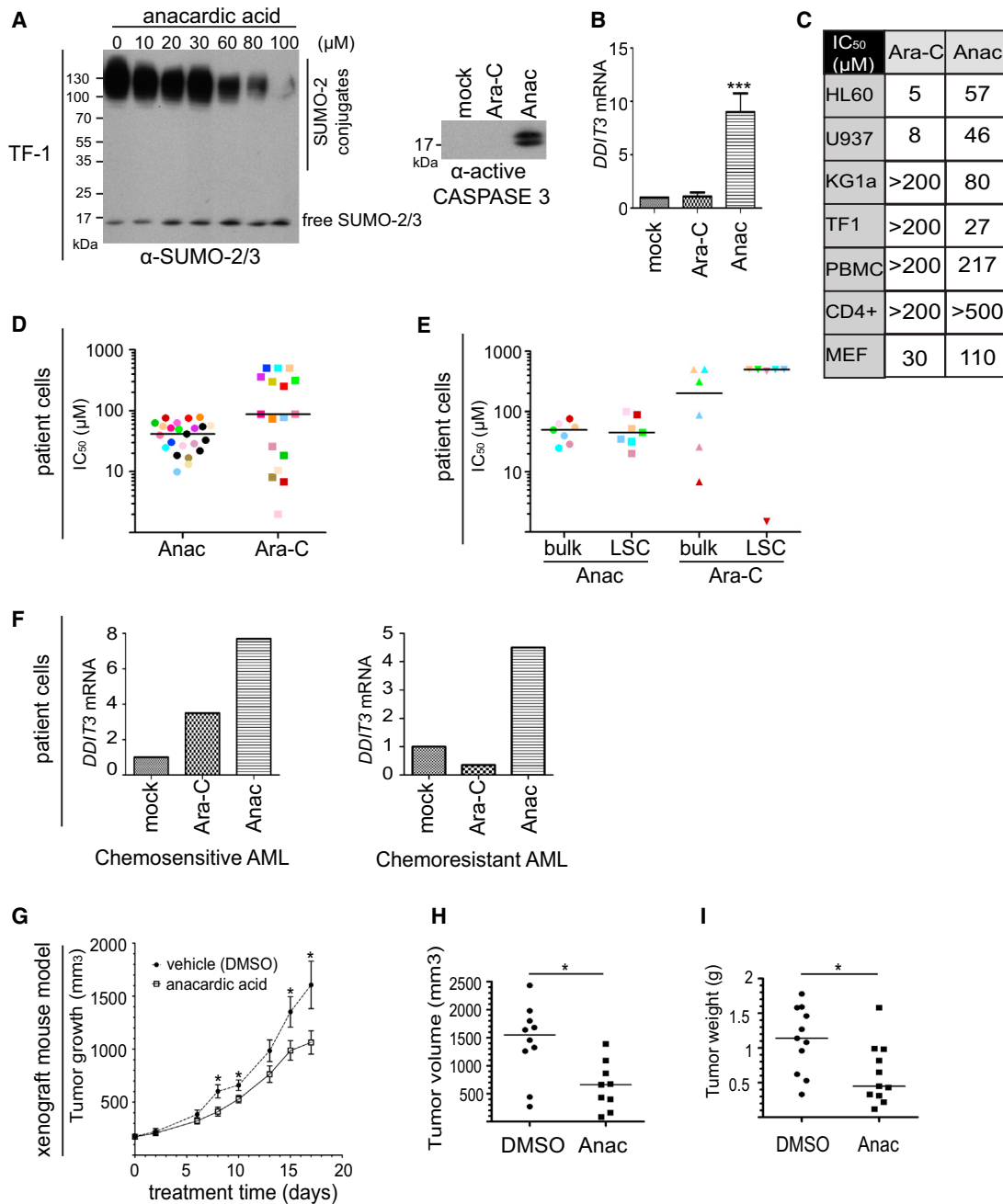


Figure 5. Inhibition of Sumoylation with Anacardic Acid Induces Chemoresistant Cells Death and Reduces Tumor Growth In Vivo

(A) TF1 cells were treated with anacardic acid for 8 hr and immunoblotted for SUMO-2 or active-CASPASE-3.

(B) TF1 cells were treated with Ara-C (2 μM) or anacardic acid (Anac, 100 μM) for 4 hr before *DDIT3* mRNA RT-qPCR assay (n = 3).

(C) HL60, U937, TF1, KG1a, PBMC, CD4⁺ T lymphocytes, and MEF cells were treated with increasing doses of anacardic acid or Ara-C for 24 hr before viability assay using MTS (n = 3).

(D and E) Primary AML cells IC₅₀ of anacardic acid (n = 23) and Ara-C (n = 17) was measured on the bulk of leukemic cells (CD45/SSC gating) at 24 hr (D). For some of the samples (n = 7), IC₅₀ of the bulk of leukemic cells was compared to that of LSCs (CD34⁺CD38^{low}/CD123⁺) (E). IC₅₀ >500 μM could not be calculated precisely and were set to 500 μM. The same color is used for data coming from the same patient sample.

(F) AML cells were treated with 50 μM anacardic acid or 10 μM Ara-C for 24 hr before *DDIT3* mRNA RT-qPCR assay.

(G–I) Mice xenografted with KG1a were treated with anacardic acid or the vehicle (DMSO), and tumor growth was measured for 17 days (G). Mice were then sacrificed and tumor volume (H) as well as tumor weight (I) were measured. Results are expressed as means ± SD.

from peripheral blood. Bone marrow aspirates containing leukemic blasts from patients diagnosed with AMLs were obtained as previously described (Vergez et al., 2011) after informed consent and stored at the HIMIP collection (DC-2008-307-collection1). A transfer agreement was obtained (AC-2008-129) after approbation by the "Comité de Protection des Personnes Sud-Ouest et Outremer II" (Ethical Committee). For some experiments, fresh leukemic blasts recovered at diagnosis were immediately treated with the drugs or inhibitors. In most cases, frozen cells were thawed in IMDM with 20% FBS and immediately processed.

Lentiviral and Retroviral Infections

Retroviral constructs expressing SUMO-2 were constructed by inserting His-tagged human SUMO-2 cDNA into the pMIG retroviral vector. The 4-hydroxy-tamoxifen(4-OHT)-inducible control and SUMO-1/2/3 miRNA (miR-SUMO-1/2/3) lentivirus were a kind gift from Dr. W. Paschen (Yang et al., 2013). Viruses were produced in HEK293T cells by transfection using Lipofectamine-2000 (Invitrogen) of viral constructs together with gag-pol (lentiviral or retroviral) and env (VSVG) expression vectors. Viral supernatants were collected 48 hr after transfection, 0.45 μ M filtered and used to infect AML cell lines. For pMIG-infected cells, only GFP-positive cells were considered in the flow cytometry analysis. For the miR-control and miR-SUMO-1/2/3, clones resistant to hygromycin and puromycin were selected and tested for inhibition of SUMO-1/2/3 expression.

Microarray-Based Whole-Transcript Expression Analysis and Profiling

Total RNA was extracted using the GenElute Mammalian Total RNA kit (Sigma) and treated with DNase I according to the manufacturer's specifications. For each condition, three independent batches of RNA were prepared and controlled for purity and integrity using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kits (Agilent Technologies). Only RNA with no sign of contamination or degradation (RIN >9) were further processed to generate amplified and biotinylated sense-strand cDNA targets using the GeneChip WT PLUS Reagent kit from Affymetrix according to the manufacturer's specifications. After fragmentation, cDNA targets were used to probe Affymetrix GeneChip Human Gene 2.0 ST arrays, which were then washed, stained, and scanned according to Affymetrix instructions (user manual P/N 702731 Rev. 3).

Microarrays, Data Analysis, and Gene Ontology

CEL files generated after array scanning were imported into the Partek Genomics Suite 6.6 (Partek) for preprocessing consisting of estimating transcript cluster expression levels from raw probe signal intensities. Analyses were performed using default Partek settings. Resulting expression data were then imported into R (<http://www.R-project.org/>) for further analysis. First nonspecific filtering was applied to remove transcript clusters with no specified chromosome location. Then, box plots, density plots, relative log expressions (RLEs), and sample pairwise correlations were generated to assess the quality of the data. They revealed no outlier within the series of hybridizations. Principal component analysis (PCA) was also applied to the data set. The first two components of the PCA were able to separate samples according to the treatment. Thus, the treatment was considered as the unique source of variability. Finally, the LIMMA package (Smyth, 2005) (R/Bioconductor) was used to detect differentially expressed genes (DEGs) between treated and nontreated samples. A linear model with treatment as unique factor was fitted to the data before applying eBayes function to calculate the significance of the difference in gene expression between the two groups. *p* values were adjusted by Benjamin and Hochberg's false discovery rate (FDR) (Benjamini and Hochberg, 1995) and genes with FDR less than 0.05 and absolute linear fold change (FC) greater or equal to 2 were considered as DEG. Gene Ontologies associated with the DEG were obtained with BINGO (Maere et al., 2005).

Chromatin Immunoprecipitation and RT-qPCR

ChIPs were performed as previously described (Tempé et al., 2014). The immunoprecipitated DNA and inputs taken from samples before immunoprecipitation were analyzed using the Roche LightCycler 480 with primers specific for the proximal promoter *DDIT3* gene (forward: 5'-atgactcaccacacctctccgtg-3'; reverse: 5'-ccccgtcgtccctctcgtcta-3'). Total RNA was purified using the

GenElute Mammalian Total RNA kit (Sigma). After DNase I treatment, 1 μ g of total RNA was used for cDNA synthesis with the Maxima First Strand cDNA (Thermo Scientific) and used for qPCR with primers specific for the *DDIT3* mRNA (forward: 5'-gtcacaagcactcccagagccc-3'; reverse: 5'-tctgttccggttcctgttctccc-3'). Data were normalized to *GAPDH* or *TBP* mRNA levels.

Caspase 3 Activity Assay

Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with digitonin-containing buffer (eBioscience) for 15 min before addition of anti-cleaved CASPASE-3 antibody. After 2 hr, cells were washed and incubated with an anti-rabbit Alexa 647 antibody (Molecular Probes) for 1 hr, washed, and analyzed by flow cytometry.

Viability Assays

Cells were treated with increasing doses of drugs. After 24 hr, MTS assay (Promega) was used to assess the percentage of metabolically active cells according to manufacturer protocol. For primary AMLs, cells were stained with CD45-V450, CD34-PE-Cy7, CD38-APC, CD123-PE, AnnexinV-FITC, and 7-AAD as previously described (Vergez et al., 2011), and viability of the bulk of leukemic cells (CD45/SSC gating) or of LSCs (CD34⁺CD38^{low/-}CD123⁺) was determined by flow cytometry as the percentage of AnnexinV⁻/7-AAD⁻ cells within each population. IC₅₀ were calculated with Prism 4 software (GraphPad).

In Vivo Treatment with Chemotherapeutic Drugs

The mouse AML model used in this study was the erythroleukemia induced by the FrCasE Murine Leukemia Virus (Michaud et al., 2010). Eight-day-old 129S7/SvEvBrdBkl-Hprt^{b-m2} mice (H-2D^b haplotype) were infected intraperitoneally with 100 μ l of a FrCasE virus suspension containing 5 \times 10⁵ ffu/ml. Mice were examined at regular intervals for clinical signs of erythroleukemia (spleen swelling and reduction in hematocrits). Two-month-old leukemic mice were subjected to intraperitoneal administration of DNR (10 mg/kg) or Ara-C (50 mg/kg) every 2 days for 2 weeks and euthanized 4 hr after the last injection. Their spleens, as well as those of mock-treated leukemic mice of the same age, were lysed in 20 mM Na₂HPO₄ pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, 1 μ g/ml of a aprotinin/pepstatin/leupeptin mix, 10 mM N-Ethyl-Maleimide using a Dounce homogenizer. Homogenates were cleared by centrifugation (20,000 \times g for 10 min), and supernatants were used for immunoblotting analysis after protein concentration normalization.

Tumor Xenografts

Xenograft tumors were generated by injecting 2 \times 10⁶ KG1a cells (in 100 μ l of PBS) subcutaneously on both flanks of NU/NU Nude mice (adult male and females, 25 g, Charles River Laboratories). Mice were given peritumoral injections of anacardic acid (2 mg/kg/day in 30 μ l) or vehicle (DMSO). Tumor dimensions were measured with a caliper and volumes calculated using the formula: $v = \pi/6 \times A \times B^2$, where A is the larger diameter and B is the smaller diameter. At the end of the experiment, tumors were dissected, measured and weighed. Animal experiments were approved by the Ethical Committee from the UMS006 (approval number 13-U1037-JES-08).

Statistical Analyses

Results are expressed as means \pm SD. Statistical analyses were performed by Student's *t* test with Prism 4 software. Differences were considered as significant for *p* values of <0.05. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

ACCESSION NUMBERS

The ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) accession number for the microarray expression data reported in this paper is E-MATB-2382.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.016>.

AUTHOR CONTRIBUTIONS

G.B. and M.P. designed the study. G.B., J.E.S., C.K., M.R., E.S., F.V., T.S., H.B., H.B., and F.B. performed the experiments. T.K. and L.V. performed the transcriptomic analysis. J.E.S., S.M. and C.R. brought essential advice and resources. G.B. and M.P. wrote the manuscript.

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