

## Metnase mediates chromosome decatenation in acute leukemia cells

Justin Wray,<sup>1</sup> Elizabeth A. Williamson,<sup>1</sup> Sheema Sheema,<sup>1</sup> Suk-Hee Lee,<sup>2</sup> Edward Libby,<sup>1</sup> Cheryl L. Willman,<sup>3</sup> Jac A. Nickoloff,<sup>4</sup> and Robert Hromas<sup>1</sup>

<sup>1</sup>Division of Hematology-Oncology, Cancer Research and Treatment Center, Department of Medicine, University of New Mexico Health Science Center, Albuquerque;

<sup>2</sup>Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis; <sup>3</sup>Department of Pathology, University of New Mexico Health Science Center, Albuquerque; and <sup>4</sup>Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque

**After DNA replication, sister chromatids must be untangled, or decatenated, before mitosis so that chromatids do not tear during anaphase. Topoisomerase II $\alpha$  (Topo II $\alpha$ ) is the major decatenating enzyme. Topo II $\alpha$  inhibitors prevent decatenation, causing cells to arrest during mitosis. Here we report that acute myeloid leukemia cells fail to arrest at the mitotic decatenation checkpoint, and their progression through this checkpoint is regulated by the DNA repair**

**component Metnase (also termed SETMAR). Metnase contains a SET histone methylase and transposase nuclease domain, and is a component of the nonhomologous end-joining DNA double-strand break repair pathway. Metnase interacts with Topo II $\alpha$  and enhances its decatenation activity. Here we show that multiple types of acute leukemia cells have an attenuated mitotic arrest when decatenation is inhibited and that in an acute myeloid leukemia (AML) cell line this is**

**mediated by Metnase. Of further importance, Metnase permits continued proliferation of these AML cells even in the presence of the clinical Topo II $\alpha$  inhibitor VP-16. In vitro, purified Metnase prevents VP-16 inhibition of Topo II $\alpha$  decatenation of tangled DNA. Thus, Metnase expression levels may predict AML resistance to Topo II $\alpha$  inhibitors, and Metnase is a potential therapeutic target for small molecule interference. (Blood. 2009;114:1852-1858)**

### Introduction

DNA double-strand breaks (DSBs) can result from normal cellular events, such as recombination during immune receptor rearrangement, recovery of stalled replication forks, replication of nicked templates, or failed decatenation.<sup>1</sup> Such DSBs can result in translocations or deletions, and this genomic instability can lead to malignancy. Of the normal cell processes producing DSBs, the least well understood is chromosome decatenation. Sister chromatids become intertwined, or catenated, when chromosomes are replicated during DNA synthesis. When decatenation fails, chromatids can tear during anaphase, producing DSBs. Catenation status is actively monitored at 2 points in the cell cycle. One decatenation checkpoint blocks progression from G<sub>2</sub> to M<sub>2</sub>, and another blocks progression from metaphase to anaphase during mitosis.<sup>2-6</sup> These checkpoints are conserved in most organisms, including yeast.<sup>7,8</sup> Previously, it was reported that bladder and lung cancer cells lack decatenation checkpoints and proceed through mitosis even when decatenation is inhibited.<sup>9,10</sup> It is possible that this failure of arrest at the decatenation checkpoints could be a general feature of malignancy, including acute leukemia. The critical decatenating enzyme is Topoisomerase II $\alpha$  (Topo II $\alpha$ ).<sup>2-6,11</sup> Topo II $\alpha$  inhibitors can trigger decatenation checkpoint arrest in normal cells at either of the 2 decatenation checkpoints. Recently, we identified and characterized a novel DNA repair protein called Metnase (also SETMAR). Metnase has an amino terminal SET histone methylase domain and a carboxy terminal transposase/nuclease domain. We previously reported that Metnase promotes nonhomologous end-joining of DSBs and methylates histone 3 lysine 36.<sup>12</sup> Metnase appears to be localized to DSBs by interaction with Pso4, a poorly characterized DNA repair component.<sup>13</sup> Metnase may promote

DSB repair by interacting with DNA ligase IV, the final component of the nonhomologous end-joining pathway.<sup>14</sup> Metnase is present only in primates and exhibits only partial transposase activity.<sup>12,14-19</sup> We also found that Metnase has endonuclease activity specific for supercoiled DNA and improves Topo II $\alpha$ -mediated decatenation in vitro and in vivo in noncancerous human cells.<sup>16,20</sup> In this study, we hypothesized that Metnase mediates decatenation in acute leukemia cells. If there is a failure of decatenation arrest in acute leukemia cells when Topo II $\alpha$  is inhibited, then perhaps Metnase mediated the continued progression of those cells through the decatenation checkpoints.

We report here that both acute myeloid and acute lymphoblastic leukemia (AML and ALL) cells fail to arrest in mitosis when Topo II $\alpha$  is inhibited. In AML, we show that Metnase levels mediate progression through mitosis by enhancing Topo II $\alpha$  function. Most importantly, we found that Metnase mediates cell proliferation through and protects Topo II $\alpha$  from VP-16, a Topo II $\alpha$  inhibitor that is widely used in cancer treatment, including AML salvage therapy. This implies that Metnase is a critical mediator of leukemia resistance to the cytotoxic effects of Topo II $\alpha$  inhibitors.

### Methods

#### Western analysis of Metnase and Topo II $\alpha$ expression

All leukemia cell lines were grown in RPMI media (HyClone) fully supplemented with 1% antimycotic/antibiotic (Cellgro), and 10% fetal bovine serum (Atlanta Biologicals). HEK-293T cells were cultured in fully supplemented Dulbecco modified Eagle medium (HyClone). Human CD34<sup>+</sup>

Submitted August 22, 2008; accepted April 26, 2009. Prepublished online as *Blood* First Edition paper, May 20, 2009; DOI 10.1182/blood-2008-08-175760.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2009 by The American Society of Hematology

hematopoietic progenitors were purchased from the National Heart, Lung, and Blood Institute Programs of Excellence in Gene Therapy program at the Fred Hutchinson Cancer Center (Seattle, WA), and cultured in StemSpan H3000 media (StemCell Technologies) supplemented with StemSpan CC100 (StemCell Technologies) for 5 days, incubated with 10 ng/mL granulocyte-macrophage colony-stimulating factor (Leukine; Bayer Healthcare Pharmaceuticals) for 24 hours as described previously.<sup>21</sup> Western blot analysis was performed on total protein lysates using antisera specific for Metnase (1:4000; Lampire),<sup>12</sup> Topo II $\alpha$  (1:2000; Topogen),<sup>20</sup> and finally  $\beta$ -actin (1:10 000; Sigma-Aldrich) as the loading control.

### Manipulating Metnase levels

THP-1 cells were stably transfected with a control shRNA plasmid, pRS-shGFP, which expressed an shRNA against green fluorescent protein, or a combination of 3 shRNAs against Metnase, targeting nucleotides 1198 to 1226, 1270 to 1298, and 1800 to 1828 (from NM 006515) expressed from pRS (Origene). Stable transfectants were selected with 1  $\mu$ g/mL puromycin and then cloned by limiting dilution. Metnase levels were assessed using reverse-transcribed polymerase chain reaction (RT-PCR) and Western blotting as we described.<sup>12,22</sup> Each experiment using shRNA repressed Metnase was performed in at least 2 independent clones, with nearly identical results.

### Metnase/Topo II $\alpha$ coimmunoprecipitation

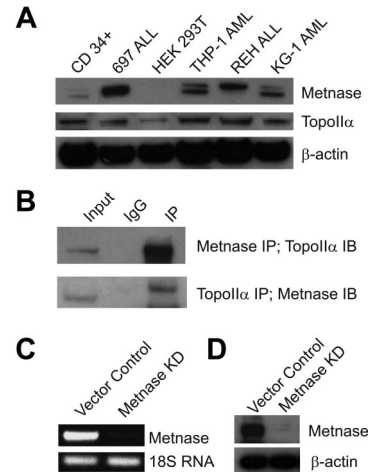
Coimmunoprecipitation of Metnase and Topo II $\alpha$  (anti-Topo II $\alpha$  from Topogen) was performed as follows: cells were lysed with radio immunoprecipitation assay (RIPA) buffer, and protein extracts precleared using 40  $\mu$ L protein A/G beads. A total of 2 mg protein extract was used for each immunoprecipitation; 2  $\mu$ L anti-V5 (Invitrogen), 2  $\mu$ L anti-Metnase, or 2.5  $\mu$ L anti-Topo II $\alpha$  (Topogen) antibodies was used in a total volume of 500  $\mu$ L. Lysates were then incubated overnight in 500  $\mu$ L RIPA buffer with appropriate antibody at 4°C in the presence of 1U DNase I. A total of 30  $\mu$ L of protein A/G beads was then added and incubated for 2 hours at 4°C. The beads were washed 3 times with RIPA buffer and twice with phosphate-buffered saline. The beads were then boiled, samples were run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and analyzed by Western blot using appropriate antisera. The input represents 1% of the total lysate for all experiments.

### Mitotic arrest analysis

ICRF-193 inhibits Topo II $\alpha$  without producing DNA damage, producing cell cycle arrest because of failed decatenation, and not from DNA damage. Three techniques were used to measure mitotic arrest after ICRF-193 exposure. Each of these techniques is complementary to the others. Immunofluorescence of tubulin condensation into a mitotic spindle and 4'-6-diamidino-2-phenylindole (DAPI)-stained chromosome alignment in a metaphase plate measured cells in metaphase as we described.<sup>20</sup> Immunofluorescence of Mitotic Protein Monoclonal 2 (MPM-2), a mitotic marker,<sup>23</sup> measured cells in mitosis. Flow cytometry using propidium iodide measured cells in G<sub>2</sub>/M phase of the cell cycle as we described.<sup>24</sup> Briefly, unsynchronized cells were treated with 10, 25, or 100  $\mu$ M ICRF-193 (MP Biomedicals) or equivalent volume of dimethyl sulfoxide (DMSO; vehicle control) for the required time points, harvested, cytospun, fixed with ice-cold methanol, and stained with anti-tubulin antibody conjugated to fluorescein isothiocyanate (FITC; Abcam) and/or anti-MPM-2 conjugated to Texas Red (Upstate Biotechnology) for 2 hours at room temperature. Cells were counterstained with DAPI to visualize nuclear morphology. Interphase and metaphase cells were counted by immunofluorescence microscopy using a Zeiss Confocal fluorescence microscope with 63 $\times$  objectives equipped with laser and filter settings for DAPI, FITC, and Texas Red. The increase in cells in mitosis with ICRF-193 over the vehicle controls reflects mitotic decatenation arrest.<sup>3-7</sup> Each immunofluorescence experiment was performed in triplicate, and more than 350 cells were counted per condition. Statistical analyses were performed using Student *t* tests, and *P* values are as indicated.

### Analysis of apoptosis

Apoptosis was analyzed by flow cytometric measurement of annexin-V expression in vector control or shRNA Metnase-transduced THP-1 cells



**Figure 1. Topo II $\alpha$  and Metnase are expressed and interact in blood cells.** (A) Western blot of multiple acute leukemia cell lines and normal CD34<sup>+</sup> hematopoietic progenitors to show expression of Metnase and Topo II $\alpha$ . HEK-293T cells do not express Metnase by virtue of their transformation by T antigen. They serve as a control for these studies. (B) Metnase and Topo II $\alpha$  coimmunoprecipitated with each other from THP-1 AML cells in the presence of DNase I, indicating that their interaction is independent of DNA. (C) Metnase shRNA reduces Metnase mRNA in THP-1 AML cells as shown by RT-PCR. (D) Metnase shRNA reduces the expression of Metnase protein in THP-1 cells as shown by Western analysis.

treated with 0.5  $\mu$ M VP-16 for 24 hours. The annexin V-FITC Apoptosis Detection Kit I (BD Biosciences PharMingen) was used.

### Kinetoplast DNA decatenation

Purified recombinant Topo II $\alpha$  (GE Healthcare) and catenated kinetoplast DNA (kDNA; Topogen) were used according to the manufacturer's instructions. Recombinant Metnase was purified as we described.<sup>12</sup> kDNA (200 ng/ $\mu$ L) was incubated in the manufacturer's buffer with increasing concentrations of Metnase and/or Topo II $\alpha$  for 1 hour at 37°C per the manufacturer's instructions.<sup>20</sup> VP-16 or its buffer was added to reactions at a final concentration of 0.5  $\mu$ M. kDNA decatenation was then visualized by 1% agarose gel electrophoresis in ethidium bromide and quantified by densitometry using ImageJ software (<http://rsbweb.nih.gov/ij/>).

## Results

### Metnase and Topo II $\alpha$ interact in acute leukemia cells

We first examined whether the DNA repair component Metnase was expressed in 4 human acute leukemia cells and normal human CD34<sup>+</sup> hematopoietic progenitors using Western blot analysis. We also measured the presence of Topo II $\alpha$  in these lines. Normal CD34<sup>+</sup> hematopoietic progenitors and the leukemia lines expressed both Metnase and Topo II $\alpha$  at various levels (Figure 1A). Metnase can have 2 bands on Western analysis as it can be phosphorylated, as seen in the leukemia cell lines. HEK-293T cells express no Metnase, based on their transformation by SV-40 T antigen, and thus serve as a control.

We next tested whether Metnase and Topo II $\alpha$  could interact in the AML cell line THP-1 by coimmunoprecipitation. We found that Metnase was present in immunoprecipitates of Topo II $\alpha$  and that Topo II $\alpha$  was present in Metnase immunoprecipitates (Figure 1B). The interaction of Metnase with Topo II $\alpha$  was not dependent on DNA, as the inclusion of DNase in the immunoprecipitate buffer did not affect the results.

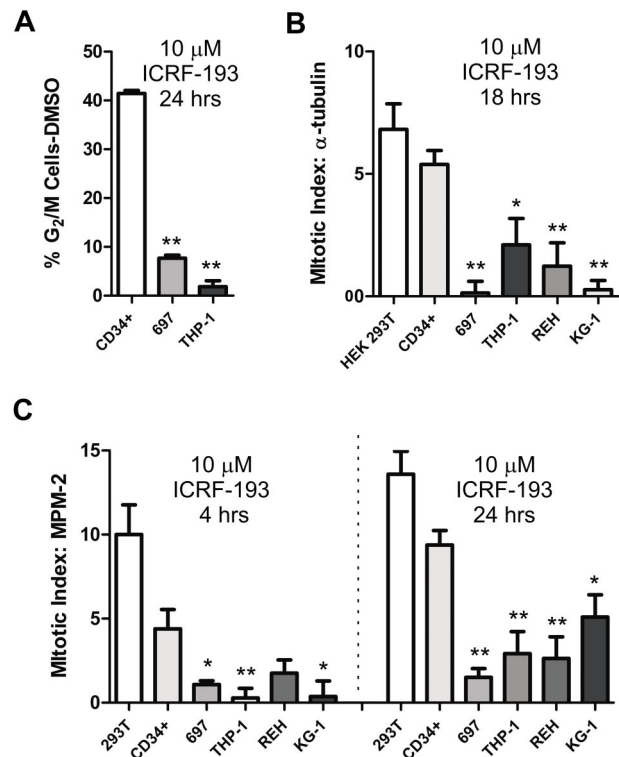
### Defective mitotic decatenation arrest in acute leukemia cells

The bisdioxopiperazines, including ICRF-193, inhibit Topo II $\alpha$  after DNA religation and therefore do not produce significant DNA DSBs. The obscuring effects of DNA damage on the cell cycle are minimized, and the mitotic arrest induced by these drugs primarily results from the failure of chromosomal decatenation.<sup>2-6</sup> Thus, ICRF-193 can be used to assess whether cells progress through mitosis despite the inhibition of decatenation. Because bladder and lung cancer cells do not demonstrate decatenation arrest after ICRF-193 exposure,<sup>9,10</sup> we hypothesized that acute leukemia cells would also fail to arrest in mitosis when Topo II $\alpha$  is inhibited with ICRF-193. We tested this by exposing several acute leukemia cell lines to ICRF-193 and measuring the increase in the fraction of cells in mitosis by 3 complementary methods. We used flow cytometry to measure the increase in the fraction of cells in the G<sub>2</sub>/M phase of the cell cycle. We used immunofluorescence assessment of tubulin condensation and DAPI nuclear morphology to measure cells in metaphase because mitotic spindle formation and chromosome alignment can be seen with this technique. We also used immunofluorescence to assess the presence of MPM-2, which measures total mitotic cells. The increase in the fraction of cells in mitosis in the presence of ICRF-193 with vehicle controls subtracted indicates the fraction of cells arrested because of decatenation failure.<sup>3-6</sup> We examined 2 AML lines, THP-1 and KG-1, and 2 ALL lines 697 and REH, and compared these with normal human CD34<sup>+</sup> hematopoietic progenitors and HEK-293T cells. The HEK-293T cells arrest appropriately at mitosis when Topo II $\alpha$  is inhibited with ICRF-193 and served as a control.<sup>20</sup>

We first analyzed the ability of ICRF-193 to arrest CD34<sup>+</sup> cells, 697 ALL and THP-1 AML cells in G<sub>2</sub>/M using flow cytometry (Figure 2A). We found that ICRF-193 increased the percentage of CD34<sup>+</sup> cells in G<sub>2</sub>/M by 41%, whereas it increased the percentage of 697 cells by only 7.7% and THP-1 cells by 1.8%. Thus, there is a 5-fold increase in CD34<sup>+</sup> cells in G<sub>2</sub>/M compared with 697 cells, and 22-fold increase compared with THP-1 cells, indicating that CD34<sup>+</sup> cells are arrested significantly more efficiently by ICRF-193 inhibition of Topo II $\alpha$  than acute leukemia cells.

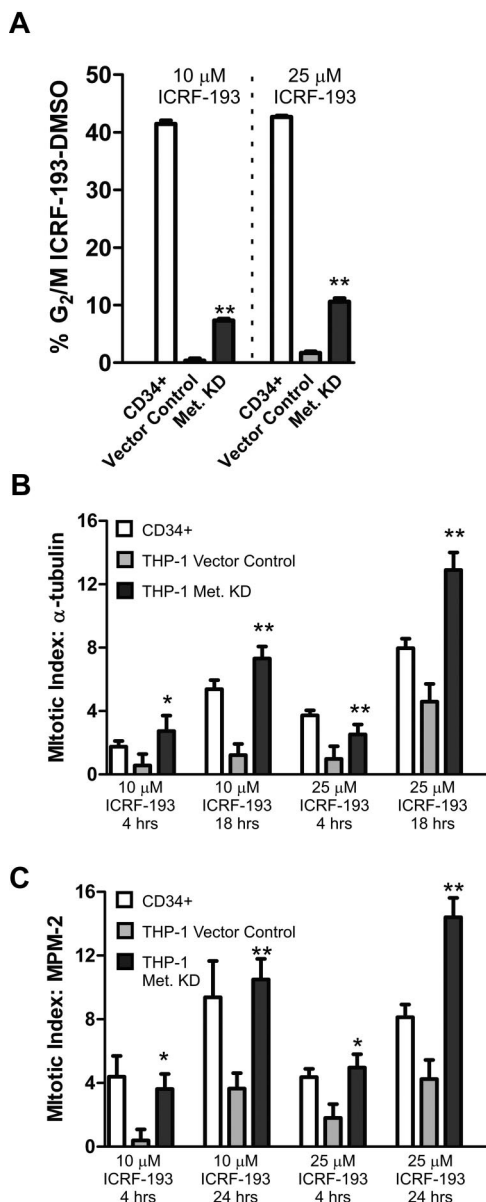
Next, we analyzed the fractional increase of CD34<sup>+</sup> and acute leukemia cells in metaphase after exposure to ICRF-193 using immunofluorescence of the condensation of tubulin into a mitotic spindle and alignment of chromosomes along a metaphase plate (Figures 3-4). After exposure to ICRF-193, there were 5.4% more CD34<sup>+</sup> cells in metaphase, 0.1% more 697 cells, 2.1% more THP-1 cells, 1.2% more REH cells, and 0.3% more KG-1 cells (Figure 2B). It is not surprising that there is a lower percentage increase of cells using this method, as metaphase cells are only a small fraction of G<sub>2</sub>/M cells. Using this method, there were 2.6-fold more CD34<sup>+</sup> cells in metaphase compared with THP-1 cells, 4-fold more compared with REH cells, 20-fold more compared with KG-1 cells, and 38-fold more compared with 697 cells. Each of these differences is statistically significant using the Student *t* test as indicated in Figure 2B. Thus, ICRF-193 arrested significantly more CD34<sup>+</sup> cells in metaphase compared with all 4 acute leukemia lines. The fractional increase of CD34<sup>+</sup> cells in metaphase after ICRF-193 is similar, although slightly lower, to that seen in HEK-293T cells, which have an intact mitotic decatenation arrest.<sup>20</sup>

We next analyzed the fractional increase of CD34<sup>+</sup> and acute leukemia cells in mitosis generally after exposure to ICRF-193 using immunofluorescence of MPM-2 expression (Figure 2C). After exposure to ICRF-193 for 24 hours, there were 9.4% more CD34<sup>+</sup> cells in mitosis, 1.5% more 697 cells, 2.9% more THP-1



**Figure 2. Leukemia cells show reduced mitotic arrest when decatenation is inhibited.** (A) Normal human CD34<sup>+</sup> hematopoietic progenitors, 697 ALL, and THP-1 AML cells were treated with 10  $\mu$ M ICRF-193 for 24 hours and then evaluated for the increase in G<sub>2</sub>/M cell-cycle fraction by propidium iodide staining for DNA content. (B) Mitotic arrest was induced by 10  $\mu$ M ICRF-193 for 18 hours in CD34<sup>+</sup> cells, 697 ALL and REH ALL cells, and THP-1 and KG-1 AML cells. HEK-293T cells, which do not express Metnase, served as a positive control for mitotic arrest after Topo II $\alpha$  decatenation inhibition. Metaphase cells were imaged by tubulin immunofluorescence and DAPI nuclear morphology and quantified as a percentage of the total cell population with DMSO control subtracted. (C) Mitotic arrest induced by 10  $\mu$ M ICRF-193 for 4 or 24 hours in CD34<sup>+</sup> cells, 697 ALL and REH ALL cells, and THP-1 and KG-1 AML cells. HEK-293T cells, which do not express Metnase, served as a positive control for mitotic arrest after Topo II $\alpha$  decatenation inhibition. Cells in mitosis were imaged by MPM-2 immunofluorescence and quantified as a percentage of the total population with DMSO control subtracted. The acute leukemia cell lines all have decreased mitotic arrest after Topo II $\alpha$  inhibition with ICRF-193 compared with CD34<sup>+</sup> cells. All experiments represent the average of at least 3 independent experiments,  $\pm$  SEM. \*Student *t* test ( $P < .05$ ); \*\*Student *t* test ( $P < .01$ ).

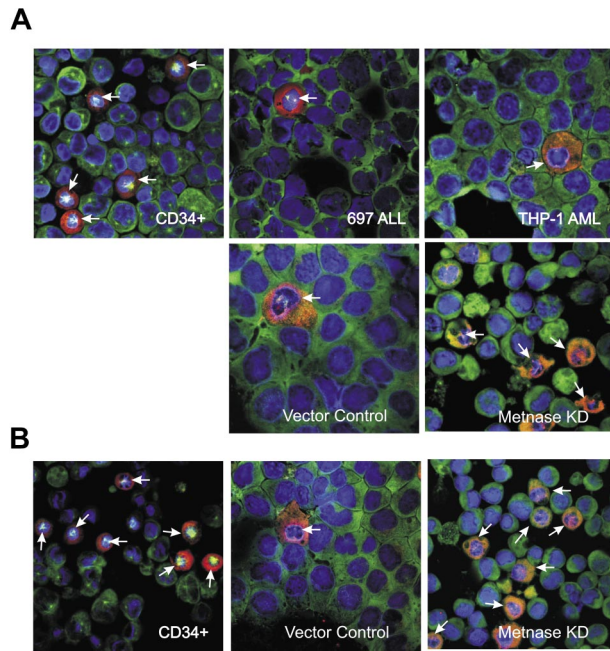
cells, 2.6% more REH cells, and 5.1% more KG-1 cells. In this assay, there were 1.8-fold more CD34<sup>+</sup> cells in mitosis compared with KG-1 cells, 3.2-fold compared with THP-1 cells, 3.2-fold compared with REH cells, and 6.2-fold compared with 697 cells. Each of these differences is statistically significant using the Student *t* test as indicated in Figure 2C. The CD34<sup>+</sup> cells arrest in mitosis in response to ICRF-193 somewhat less efficiently than the decatenation control HEK-293T cells. It should be noted that there was a progressive increase in mitotic arrest, ruling out checkpoint bypass, as measured by MPM-2 after ICRF-193 at 2, 4, 8, and 24 hours (supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). In addition, increasing ICRF-193 concentrations to very high levels did not quantitatively alter the decreased mitotic arrest of the acute leukemia cells compared with the CD34<sup>+</sup> cells (supplemental Figure 2). Thus, acute leukemia cells do not become sensitive to ICRF-193 with increasing time or concentration. Representative photomicrographs are shown in Figure 4. In summary, each of these 3 assays demonstrated that, after Topo II $\alpha$  inhibition with ICRF-193, human acute leukemia cells arrested in mitosis significantly less than normal CD34<sup>+</sup> progenitors.



**Figure 3. The reduction of Metnase restores mitotic arrest in THP-1 cells.** (A) CD34<sup>+</sup> progenitors and THP-1 cells stably transduced with a vector control or the Metnase shRNA vector were treated with 10 or 25 μM ICRF-193 for 24 hours and then evaluated for the increase in G<sub>2</sub>/M cell-cycle fraction by propidium iodide DNA staining. (B) Mitotic arrest was induced by 10 or 25 μM ICRF-193 for 4 or 18 hours in CD34<sup>+</sup> cells, and THP-1 cells stably transduced with a vector control or the Metnase shRNA vector. Metaphase cells were imaged by tubulin immunofluorescence and DAPI nuclear morphology, and quantified as a percentage of the total cell population. (C) Mitotic arrest induced by 10 or 25 μM ICRF-193 for 4 or 24 hours in CD34<sup>+</sup> cells, and THP-1 cells stably transduced with a vector control or the Metnase shRNA vector. Cells in mitosis were imaged by MPM-2 immunofluorescence and quantified as a percentage of the total population. Repressing Metnase increased the mitotic arrest of the THP-1 cells after ICRF-193 exposure so that they approximated the arrest of CD34<sup>+</sup> cells. All experiments represent the average of at least 3 independent experiments, ± SEM. \*Student *t* test (*P* < .05); \*\*Student *t* test (*P* < .01).

**Reducing Metnase levels restores mitotic decatenation arrest in the AML line THP-1**

In Figure 2, we found that acute leukemia cells did not arrest in mitosis when Topo IIα was inhibited. We had previously found that Metnase enhanced Topo IIα activity.<sup>20</sup> Therefore, we next investigated whether reducing Metnase protein levels in the AML cell line THP-1 would affect this progression through mitosis despite Topo IIα inhibition. Metnase protein levels were reduced by at least 90%

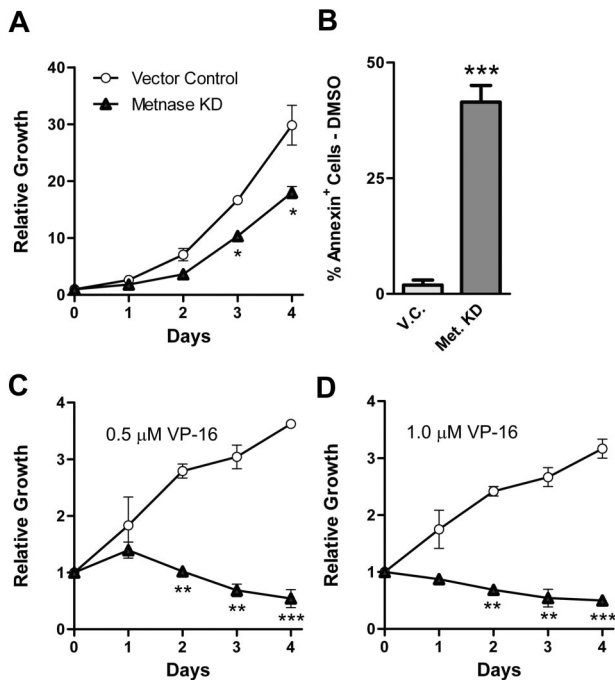


**Figure 4. Photomicrographs showing that leukemia cells show a reduced mitotic arrest after ICRF-193 and restoration of mitotic arrest with reduced Metnase.** (A) Images showing both tubulin (green) and MPM-2 (red) staining, as well as counterstaining with DAPI, after 10 μM ICRF treatment for 24 hours. There are more cells arrested in mitosis after ICRF-193 in the CD34<sup>+</sup> cells than in the THP-1 AML cells. When Metnase is repressed in the THP-1 cells, the fraction of cells in mitosis after ICRF-193 exposure becomes similar to that seen with CD34<sup>+</sup> cells. (B) Similar to panel A, but treated with 25 μM ICRF for 24 hours.

in THP-1 cells stably expressing Metnase shRNAs, as shown by RT-PCR and Western analysis (Figure 1C-D). We then examined these THP-1 cells with reduced Metnase levels for their ability to progress through mitosis when Topo IIα was inhibited using the same 3 assays. Using flow cytometry to assess the fraction of cells in G<sub>2</sub>/M after exposure to ICRF-193, we found that the repression of Metnase decreased progression through mitosis, but not to the same extent as seen in CD34<sup>+</sup> cells. Vector control-transduced THP-1 cells (with normal Metnase levels) had 1.7% cells in G<sub>2</sub>/M, whereas CD34<sup>+</sup> cells had 42% cells in G<sub>2</sub>/M. Repressing Metnase levels significantly increased THP-1 cells in G<sub>2</sub>/M by 6-fold, to 10.6% (Figure 3A). Next, using immunofluorescence to measure condensed tubulin to assess cells in metaphase after ICRF-193 exposure for 18 hours, we found that repressing Metnase increased arrested cells from 1.2% in the vector control to 7.3%, a 6-fold increase. Of note, there were 5.4% CD34<sup>+</sup> cells arrested in metaphase in this assay. Thus, repression of Metnase completely restored metaphase arrest to ICRF-193 (Figure 3B). Finally, we assessed mitotic arrest using MPM-2 immunofluorescence (Figure 3C). We found that repressing Metnase increased mitotic arrest after ICRF-193 exposure for 24 hours from 3.6% to 10.4% in vector controls, compared with 9.4% in CD34<sup>+</sup> cells. Representative photomicrographs are shown in Figure 4. In summary, these data demonstrated that Metnase is required for progression through mitosis when decatenation is inhibited by ICRF-193.

**Metnase promotes THP-1 cell proliferation in the presence of the Topo IIα inhibitor VP-16**

Because Metnase mediated resistance to the Topo IIα inhibitor ICRF-193, we asked whether it also mediated resistance to the clinically relevant Topo IIα inhibitor VP-16.<sup>25</sup> Therefore, we examined whether THP-1 clones with reduced levels of Metnase

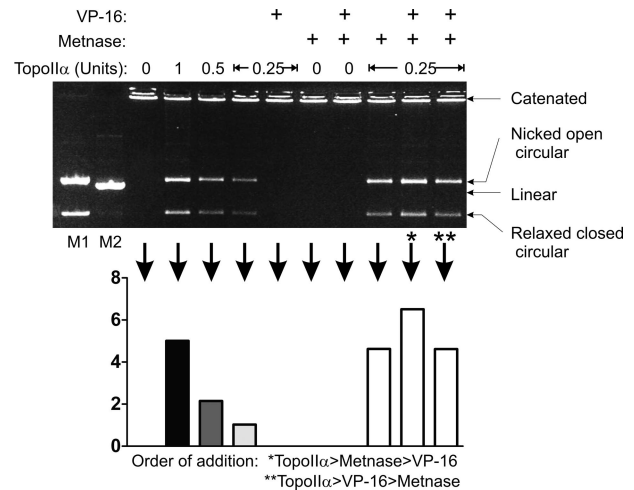


**Figure 5. Metnase protects cells from the cytotoxic effects of the clinically relevant Topo II $\alpha$  inhibitor VP-16.** (A) Proliferation was measured in THP-1 cells expressing Metnase shRNA or vector control using growth curves. (B) THP-1 cells expressing Metnase shRNA or a vector control were subjected to 24 hours of 0.5  $\mu$ M VP-16 and then analyzed for apoptosis using annexin-V expression. (C-D) THP-1 cells transfected with control vector proliferate in the presence of 0.5  $\mu$ M (C) or 1.0  $\mu$ M (D) VP-16, but THP-1 cells expressing Metnase shRNA do not. Data are averages ( $\pm$  SEM) of triplicate experiments performed twice. \*Student *t* test ( $P < .05$ ); \*\*Student *t* test ( $P < .01$ ); \*\*\*Student *t* test ( $P < .005$ ).

were more sensitive to the antiproliferative effects of VP-16. THP-1 cells with repressed Metnase proliferated at a slightly decreased rate than vector control cells (Figure 5A), perhaps because of decreased endogenous decatenation rates. We next examined the proliferation of THP-1 cells with repressed Metnase in the presence of VP-16, compared with vector control cells. We found that these AML cells showed a markedly decreased ability to proliferate in the presence of VP-16 (Figure 5C-D); but when Metnase was decreased in these cells, they did not proliferate at all. Indeed, they were actively killed as indicated by the negative slope in Figure 5C and D. Finally, we tested whether repressing Metnase increased apoptosis in the THP-1 cells using flow cytometric analysis of the expression of annexin (Figure 5D). We found that repressing Metnase increased apoptosis in the THP-1 cells by 22-fold. In summary, these data indicate that Metnase not only mediates resistance to the non-DNA-damaging Topo II $\alpha$  inhibitor ICRF-193 but also mediates resistance to the DNA damaging, clinically relevant Topo II $\alpha$  inhibitor VP-16.

#### Metnase directly mediates Topo II $\alpha$ resistance to inhibitors

In previous studies, we found that Metnase mediated resistance to 2 Topo II $\alpha$  inhibitors, ICRF-193, and VP-16. However, these studies were performed in intact cells. Even though Metnase coimmunoprecipitated Topo II $\alpha$ , it is possible that they are in a complex, and there could be an intermediary factor translating the effect of Metnase to Topo II $\alpha$ . Thus, it was not clear whether the ability of Metnase to enhance Topo II $\alpha$  function was a direct effect or whether Metnase enhanced Topo II $\alpha$  via another nuclear factor. To address this question, we analyzed in vitro decatenation of kDNA-interlocked mini-circles using purified Topo II $\alpha$  and Metnase



**Figure 6. Metnase mediates resistance to the Topo II $\alpha$  inhibitor VP-16 in vitro.** In vitro analysis of Topo II $\alpha$  decatenation of kDNA was performed in the presence of Metnase and/or VP-16. The relaxed closed circle DNA is the fully decatenated species. kDNA decatenation (graph) was quantified by densitometric scans of the gel image. Pure Metnase (200 ng/ $\mu$ L) enhanced pure Topo II $\alpha$  decatenation when added before or after VP-16.

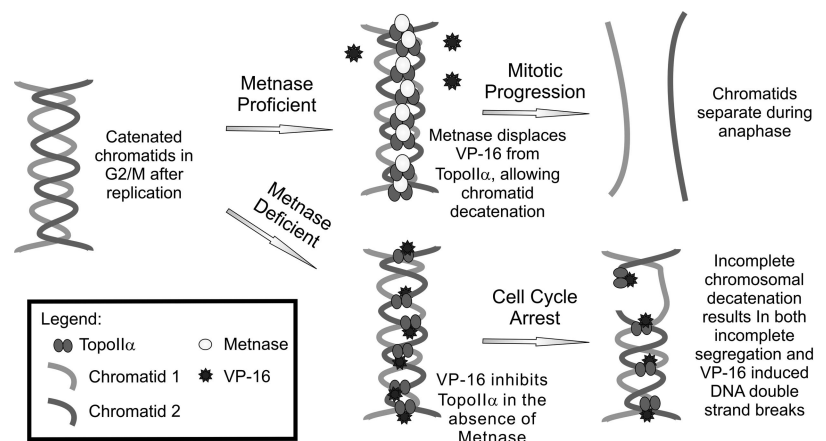
(Figure 6). Purified Metnase enhances the ability of pure Topo II $\alpha$  to decatenate kDNA in vitro by 3-fold as we reported previously.<sup>20</sup> As expected, VP-16 completely blocks the ability of Topo II $\alpha$  to decatenate kDNA. Significantly, Metnase fully restores Topo II $\alpha$  decatenation activity in the presence of VP-16, whether Metnase is added before or after VP-16. These data demonstrate that Metnase directly interacts with Topo II $\alpha$  to enhance decatenation and does not act through an intermediary.

## Discussion

Decatenation is a common yet poorly understood cause of DSBs that can lead to chromosomal translocations.<sup>1,2,8</sup> Topo II $\alpha$  is the key decatenating enzyme, but beyond that little is known about the biochemical mechanism of decatenation. When Topo II $\alpha$  is inhibited by ICRF-193, which does not cause DNA damage and does not activate the DNA damage checkpoints, cells should normally arrest before mitosis, in a decatenation checkpoint.<sup>3-8</sup> However, bladder and lung cancer cells progressed through mitosis, even when Topo II $\alpha$  was inhibited by ICRF-193.<sup>9,10</sup> Here we found that both ALL and AML cells also did not arrest in mitosis when ICRF-193 was present, even with increasing concentrations and increasing exposure times. This progression through mitosis when Topo II $\alpha$  was inhibited was not shared by normal human CD34<sup>+</sup> hematopoietic progenitors. In case the failure of mitotic arrest by the acute leukemia cells was an artifact of the assay, we examined this phenomenon using 3 distinct assays: flow cytometry of G<sub>2</sub>/M accumulation, expression of MPM-2 to define mitosis, and mitotic spindle formation by tubulin with chromosome alignment to define metaphase. Each of these complementary assays gave the same qualitative result.

This progression through mitosis by acute leukemia cells, even when Topo II $\alpha$  is inhibited, has several interesting implications. First, the lack of decatenation arrest seen in a third type of cancer may indicate that this is a general feature of malignancy. The finding that normal human CD34<sup>+</sup> progenitors did not share this characteristic makes it seem more probable that this indeed is a characteristic of transformation, at least in leukemia. If so, it may contribute to the general genomic instability seen in all cancer. Such a decatenation failure could even be an initiating event in

**Figure 7. Model of Metnase mediating resistance to Topo II $\alpha$  inhibitors in AML.** Topo II $\alpha$  inhibitors are common in the clinical management of AML during salvage therapy. Metnase expression has been shown here to mediate resistance to VP-16. When Metnase is present in AML cells, it is able to interact with Topo II $\alpha$ , enhancing its function and results in proper chromosomal decatenation during metaphase. In the case where Metnase is deficient, the presence of VP-16 reduces Topo II $\alpha$  activity and cells are not able to decatenate sister chromatids, which results in metaphase arrest.



malignant transformation, contributing to the chromosomal events that lead to oncogenesis.<sup>1</sup> Or it could be a result of transformation, contributing to unregulated proliferation by allowing malignant cells to more rapidly progress through mitosis.<sup>9,10</sup> This resistance to decatenation checkpoint arrest may also contribute to the clonal evolution of drug resistance; not just resistance to Topo II $\alpha$  inhibitors, but by promoting genomic instability, decatenation failure would increase the evolution of selecting mutations.

Although the signaling that regulates decatenation is quickly evolving, the physical mechanism by which decatenation takes place is less well understood.<sup>9,10</sup> We recently found that the DNA repair component Metnase enhances Topo II $\alpha$  decatenation activity.<sup>20</sup> Therefore, we examined whether the enhancement of Topo II $\alpha$  by Metnase might be one mechanism by which acute leukemia cells bypass the mitotic decatenation arrest. We found that repressing Metnase levels using shRNA in the AML cell line THP-1 partially restores the total G<sub>2</sub>/M arrest, yet completely restored the ability of these cells to arrest in mitosis, specifically, when Topo II $\alpha$  is inhibited by ICRF-193. Thus, to some extent, Metnase mediates resistance to ICRF-193; and when Metnase levels are reduced, these cells become sensitive to mitotic arrest in the presence of ICRF-193.

Repressing Metnase levels in the THP-1 cells also restored their sensitivity to the clinically relevant Topo II $\alpha$  inhibitor VP-16. Thus, Metnase appears to mediate resistance to Topo II $\alpha$  inhibitors generally. The restoration of sensitivity to VP-16 by the repression of Metnase raises an intriguing question. THP-1 cells normally proliferate in the presence of low doses of VP-16. VP-16 causes DNA DSBs by binding to and locking Topo II $\alpha$  in the cleavage complex stage, before DNA strand religation and causing subsequent DNA DSBs. The question raised by the data here is whether the THP-1 cells with repressed Metnase died in the presence of VP-16 from catastrophic mitosis after failed decatenation or because of unrepaired DNA DSBs. Metnase is a component of the DNA DSB repair pathway, so it is conceivable that these cells could die of DNA damage-induced apoptosis. The alternative is that the lack of Metnase in these cells allowed free access of VP-16 to Topo II $\alpha$ , and these cells underwent a catastrophic mitotic event. Given the *in vitro* data in Figure 6, where Metnase allows Topo II $\alpha$  to function even in the presence of VP-16, a situation where the rest of the components of the nonhomologous end-joining pathway of DSB repair are not present, the latter hypothesis is more attractive.

The *in vitro* kDNA decatenation experiments showed that pure Metnase improved the decatenation ability of Topo II $\alpha$  without an intermediary, and the coimmunoprecipitation experiments indicate that Metnase and Topo II $\alpha$  interact. These data seem to imply that Metnase directly protects Topo II $\alpha$  from its inhibitor in cells. There

are several possible mechanisms by which it could do this. Metnase could have a higher affinity for Topo II $\alpha$  than its inhibitors and sterically block ICRF-193 and VP-16 from interaction with Topo II $\alpha$ . Metnase protected Topo II $\alpha$ , even after addition of VP-16 in the *in vitro* kDNA assays, which could mean that it displaces VP-16 from Topo II $\alpha$ . Alternatively, it could enhance the function of the small amount of Topo II $\alpha$  that remains free from VP-16. The finding that Metnase directly enhances Topo II $\alpha$  decatenation seems to imply cells proficient for Metnase never engage the decatenation checkpoint-induced cell cycle arrest because Topo II $\alpha$ -mediated decatenation is not inhibited. As such, it functions as a positive regulator of Topo II $\alpha$  and perhaps plays a greater role in the physical decatenation process than in the decatenation checkpoint signaling pathway. Cells may arrest in mitosis when Metnase is repressed not just because the decatenation checkpoint is activated, but also because they physically cannot separate their chromosomes to progress through metaphase. It is also possible that the acute leukemia cells failed to arrest in mitosis when Topo II $\alpha$  inhibitors are present because Metnase protected Topo II $\alpha$  from inhibition, and the checkpoint signaling cascade was never activated.

The data in this study demonstrate that Metnase protection of Topo II $\alpha$  is a novel mechanism of resistance of cancer cells to VP-16. Probably the best investigated mechanism of VP-16 resistance is active efflux of the drug via the ABC transporters.<sup>26-29</sup> Loss of the checkpoint proteins p21 or p53 can also result in VP-16 resistance.<sup>30,31</sup> The role in which the enzymatic DNA repair components play in clinical VP-16 resistance is less well investigated. A rare report showed that increased levels of the recombination repair component RAD51 and the nonhomologous end-joining repair component DNA protein kinase catalytic subunit correlated with VP-16 resistance in lung cancer cells.<sup>32</sup> Interestingly, there are several different tumor types that show intrinsic or acquired resistant to VP-16,<sup>26,33-35</sup> and it is possible that Metnase mediates this resistance and could be used as a clinical marker for treatment outcome.

These data give rise to a model of mitotic progression of leukemia cells when VP-16 is present (Figure 7). Cells with appropriate levels of functional Metnase prevent Topo II $\alpha$  inactivation by VP-16 (or ICRF-193) and can decatenate sister chromatids. Such cells can progress through anaphase because the chromatids are no longer entangled. This model is important for at least 3 reasons. First, acute leukemia cells fail to arrest in mitosis when Topo II $\alpha$  inhibitors are present, compared with normal CD34<sup>+</sup> cells. It is possible that this may be a general characteristic of malignant transformation for all types of cancer. Second, Metnase enhancement of Topo II $\alpha$  decatenation in the presence of VP-16

sheds light on the molecular mechanism of decatenation in the cancer cell. It demonstrates that Metnase is a novel enhancer of Topo II $\alpha$  biochemical function, allowing cancer cells to progress rapidly through mitosis by preventing difficulties with the mechanical separation of sister chromatids. Third, decreasing Metnase levels in AML cells restores their sensitivity to VP-16. Therefore, Metnase levels may predict clinical responsiveness to Topo II $\alpha$  inhibitors. In this context, Metnase is an excellent candidate for small molecule inhibition to enhance the efficacy of Topo II $\alpha$  inhibitors in AML therapy, particularly salvage chemotherapy.<sup>25</sup>

## Acknowledgments

This work was supported by the National Institutes of Health (NIH, Bethesda, MD; R01 CA100862, J.A.N.; an Activities to Promote Research Collaborations supplement to CA100862, J.A.N., R.H.; R01 CA102283, R.H.; and R01 HL075783, R.H.), and the Leukemia & Lymphoma Society (White Plains, NY; SCOR 7388-06, R.H., C.L.W.). Images were generated in the University of New Mexico Cancer Center Fluorescence Microscopy Facility, which received support from NCR1 S10 RR14668, NSF MCB9982161, NCR1 P20 RR11830, NCI P30 CA118100, NCR1 S10 RR19287, NCR1 S10 RR016918, the University of New Mexico Health

Sciences Center, and the University of New Mexico Cancer Center. Data were generated in the Flow Cytometry Shared Resource Center supported by the University of New Mexico Health Sciences Center and the University of New Mexico Cancer Center.

## Authorship

Contribution: J.W. performed research and wrote the paper; E.A.W. performed research and edited the paper; S.S. performed research; S.-H.L. provided reagents, edited the paper, and analyzed data; E.L. analyzed data; C.L.W. analyzed data and edited the paper; J.A.N. designed experiments, analyzed data, and edited the paper; and R.H. conceived the project, designed experiments, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The present address of Dr Nickoloff is Department of Environmental and Radiological Health Sciences, Colorado State University, Ft Collins, CO.

Correspondence: Robert Hromas, Cancer Research and Treatment Center, University of New Mexico Health Science Center, 900 Camino de Salud, Albuquerque, NM 87131; e-mail: rhromas@salud.unm.edu.

## References

- Nickoloff JA, De Haro LP, Wray J, Hromas R. Mechanisms of leukemia translocations. *Curr Opin Hematol*. 2008;15:338-345.
- Downes CS, Clarke DJ, Mullinger AM, Gimenez-Abian JF, Creighton AM, Johnson RT. A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells. *Nature*. 1994;372:467-470.
- Clarke DJ, Vas AC, Andrews CA, Diaz-Martinez LA, Gimenez-Abian JF. Topoisomerase II checkpoints: universal mechanisms that regulate mitosis. *Cell Cycle*. 2006;5:1925-1928.
- Toyoda Y, Yanagida M. Coordinated requirements of human topoisomerase II and cohesin for metaphase centromere alignment under Mad2-dependent spindle checkpoint surveillance. *Mol Biol Cell*. 2006;17:2287-2302.
- Mikhailov A, Shinohara M, Rieder CL. Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway. *J Cell Biol*. 2004;166:517-526.
- Skoufias DA, Lacroix FB, Andreassen PR, Wilson L, Margolis RL. Inhibition of DNA decatenation, but not DNA damage, arrests cells at metaphase. *Mol Cell*. 2004;15:977-990.
- Damelin M, Bestor TH. The decatenation checkpoint. *Br J Cancer*. 2007;96:201-205.
- Deming PB, Cistulli CA, Zhao H, et al. The human decatenation checkpoint. *Proc Natl Acad Sci U S A*. 2001;98:12044-12049.
- Doherty SC, McKeown SR, McKelvey-Martin V, et al. Cell cycle checkpoint function in bladder cancer. *J Natl Cancer Inst*. 2003;95:1859-1868.
- Nakagawa T, Hayashita Y, Maeno K, et al. Identification of decatenation G2 checkpoint impairment independently of DNA damage G2 checkpoint in human lung cancer cell lines. *Cancer Res*. 2004;64:4826-4832.
- Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol*. 2002;3:430-440.
- Lee SH, Oshige M, Durant ST, et al. The SET domain protein Metnase mediates foreign DNA integration and links integration to nonhomologous end-joining repair. *Proc Natl Acad Sci U S A*. 2005;102:18075-18080.
- Beck BD, Park SJ, Lee YJ, Roman Y, Hromas RA, Lee SH. Human Pso4 is a Metnase (SETMAR)-binding partner that regulates Metnase function in DNA repair. *J Biol Chem*. 2008;283:9023-9030.
- Hromas R, Wray J, Lee SH, et al. The human set and transposase domain protein Metnase interacts with DNA ligase IV and enhances the efficiency and accuracy of non-homologous end-joining. *DNA Repair (Amst)*. 2008;7:1927-1937.
- Plasterk RH, Izsvak Z, Ivics Z. Resident aliens: the Tc1/mariner superfamily of transposable elements. *Trends Genet*. 1999;15:326-332.
- Roman Y, Oshige M, Lee YJ, et al. Biochemical characterization of a SET and transposase fusion protein, Metnase: its DNA binding and DNA cleavage activity. *Biochemistry*. 2007;46:11369-11376.
- Miskey C, Papp B, Mates L, et al. The ancient mariner sails again: transposition of the human Hsmar1 element by a reconstructed transposase and activities of the SETMAR protein on transposon ends. *Mol Cell Biol*. 2007;27:4589-4600.
- Liu D, Bischerour J, Siddique A, Buisine N, Bigot Y, Chalmers R. The human SETMAR protein preserves most of the activities of the ancestral Hsmar1 transposase. *Mol Cell Biol*. 2007;27:1125-1132.
- Cordaux R, Udit S, Batzer MA, Feschotte C. Birth of a chimeric primate gene by capture of the transposase gene from a mobile element. *Proc Natl Acad Sci U S A*. 2006;103:8101-8106.
- Williamson EA, Rasila KK, Corwin LK, et al. The SET and transposase domain protein Metnase enhances chromosome decatenation: regulation by automethylation. *Nucleic Acids Res*. 2008;36:5822-5831.
- O'Rourke JP, Ness SA. Alternative RNA splicing produces multiple forms of c-Myb with unique transcriptional activities. *Mol Cell Biol*. 2008;28:2091-2101.
- Williamson EA, Farrington J, Martinez L, et al. Expression levels of the human DNA repair protein metnase influence lentiviral genomic integration. *Biochimie*. 2008;90:1422-1426.
- Westendorf JM, Rao PN, Gerace L. Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc Natl Acad Sci U S A*. 1994;91:714-718.
- Xu D, Yoder M, Sutton J, Hromas R. Forced expression of Genesis, a winged helix transcriptional repressor isolated from embryonic stem cells, blocks granulocytic differentiation of 32D myeloid cells. *Leukemia*. 1998;12:207-212.
- Bishop JF. Approaches to induction therapy with adult acute myeloid leukaemia. *Acta Haematol*. 1998;99:133-137.
- Dingemans AM, Pinedo HM, Giaccone G. Clinical resistance to topoisomerase-targeted drugs. *Biochim Biophys Acta*. 1998;1400:275-288.
- Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. *Oncologist*. 2003;8:411-424.
- Pérez-Tomás R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr Med Chem*. 2006;13:1859-1876.
- O'Connor R. The pharmacology of cancer resistance. *Anticancer Res*. 2007;27:1267-1272.
- Hodkinson PS, Mackinnon AC, Sethi T. Extracellular matrix regulation of drug resistance in small-cell lung cancer. *Int J Radiat Biol*. 2007;83:733-741.
- Valkov NI, Sullivan DM. Tumor p53 status and response to topoisomerase II inhibitors. *Drug Resist Updat*. 2003;6:27-39.
- Hansen LT, Lundin C, Helleday T, et al. DNA repair rate and etoposide (VP16) resistance of tumor cell subpopulations derived from a single human small cell lung cancer. *Lung Cancer*. 2003;40:157-164.
- Toner GC, Frydenberg M. Poor prognosis germ-cell tumors: an unresolved challenge. *Semin Urol Oncol*. 2002;20:251-261.
- Hande KR. Clinical applications of anticancer drugs targeted to topoisomerase II. *Biochim Biophys Acta*. 1998;1400:173-184.
- Joel S. The clinical pharmacology of etoposide: an update. *Cancer Treat Rev*. 1996;22:179-221.