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Abstract

We and others identified a human SET and transposase domain protein termed Metnase (also called SETMAR) that methylates histone H3, and promotes DNA double-strand break repair. We postulated that Metnase could improve decatenation in breast cancer cells. In the funded period, we initially characterized the decatenation activity of Metnase. We showed that Metnase physically interacts and co-localizes with Topoisomerase II α (Topo II α), the key chromosome decatenating enzyme. Metnase promotes progression through decatenation, and increases resistance to the Topo II α inhibitors ICRF-193 and VP-16. Pure Metnase greatly enhanced Topo II α decatenation of kinetoplast DNA to relaxed circular forms. Nuclear extracts containing Metnase decatenated kDNA more rapidly than those without Metnase, and neutralizing anti-sera against Metnase reversed that enhancement of decatenation. Metnase automethylates at K485, and the presence of a methyl donor blocked the enhancement of Topo II α decatenation by Metnase, implying an internal regulatory inhibition. Importantly, we showed that inhibiting Metnase in breast cancer cells did block decatenation. Reducing Metnase levels increased breast cancer cell chemosensitivity to the Topo II α inhibitor VP-16. Thus, Metnase serves as an enhancer of Topo II α decatenation, but can automethylate to repress this enhancement. In addition, these data suggest that cancer cells could subvert Metnase to mediate clinically relevant resistance to Topo II α inhibitors.

Introduction

Chromosomes must be untangled, or decatenated, before cell division, or serious genomic instability results. Failure to decatenate chromosomes is likely a mechanism behind the genomic abnormalities seen in many breast cancers. There is evidence that the human NHEJ DNA repair protein and histone methylase Metnase plays a key role in physically decatenating replicated chromosomes. Therefore, abnormalities in Metnase expression or function could play a role in breast cancer genetic instability, and thereby produce a more aggressive tumor. This project investigates this possibility by studying the decatenation of chromosomes by Metnase.

Body

Metnase Interacts with Topo II α

Since Topo II α is the critical decatenating enzyme, the physical interaction between Metnase and Topo II α was first investigated using co-immunoprecipitation assays. In reciprocal co-immunoprecipitation experiments shown in Fig. 1A, Topo II α was detected in Metnase immunoprecipitates and Metnase was present in Topo II α immunoprecipitates. Co-immunoprecipitation was also observed in the presence of DNase I, indicating that Metnase and Topo II α interact independently of DNA. Alanine substitution mutations were created in Metnase residues known to be essential for histone methylase (amino acids 210-212, 247-249) or nuclease activity (aa 489-491). We previously found that each of these residues are required for Metnase NHEJ activity [9]. These mutations also abrogated the Metnase-Topo II α interaction (Fig. 1B). Purified Metnase also interacted with purified Topo II α (Fig. 1C), indicating that these proteins directly interact, and this interaction is not mediated by any other protein or by DNA.

Next, we examined Metnase and Topo II α intracellular co-localization using immunofluorescence microscopy. This analysis revealed that Metnase and Topo II α frequently co-localize, and colocalization was particularly strong on condensed chromosomes (Fig. 2). When cells were sorted by DNA content, there was a 2-fold greater co-localization of Metnase

and Topo II α in G2/M cells than in G1 or S phase cells (Fig. 2). When cells were treated with a high concentration of the Topo II α inhibitor ICRF-193, which inhibits Topo II α without cleaving DNA, co-localization was blocked (Fig. 2). Co-localization was also blocked by high concentrations of VP-16 (Fig. 2), which inhibits Topo II α after it cleaves DNA.

Metnase Mediates Progression Through the Metaphase Decatenation Checkpoint

The decatenation checkpoints can be triggered by exposure to ICRF-193, which blocks Topo II α function without damaging DNA, and therefore does not activate the G2/M DNA damage checkpoint [14-21]. Progression through the metaphase decatenation checkpoint, the last point at which chromatids can decatenate before aberrant separation, was analyzed by measuring the percentage of cells arrested in metaphase by ICRF-193. Increases in the percentage of ICRF-193-induced metaphase cells reflect arrest at the metaphase phase decatenation checkpoint. We examined HEK-293 cells expressing normal, 3-fold increased, or 5-fold decreased levels of Metnase (Fig. 3A), after treatment with ICRF-193, VP-16, or vehicle control (Fig. 3B, C). HEK-293 cells express moderate levels of both Topo II α and Metnase, and typically display an attenuated metaphase phase decatenation checkpoint. These cells were chosen because they provide enhanced sensitivity for detecting increased decatenation checkpoint arrest with Metnase under-expression. Most cell types express Metnase [9], but HEK-293T cells do not express Metnase at detectable levels, perhaps due to their transformation with T Antigen [48]. They have an intact metaphase decatenation checkpoint, which allowed us to analyze the decatenation checkpoint in matched cells that either express or do not express Metnase.

When cells were treated with ICRF-193, the percentage of cells arrested in metaphase was inversely proportional to Metnase levels. Thus, Metnase expression in HEK-293T cells resulted in a 3-fold decrease in metaphase cells compared to vector controls after a 4 hr ICRF-193 treatment, and a 2.5-fold decrease after an 18 hr treatment. Reducing Metnase expression in HEK-293 cells resulted in a 12-fold increase in metaphase cells after ICRF-193 exposure compared to control cells, and increasing Metnase expression in HEK-293 cells resulted in a 5-fold decrease in the percentage of metaphase cells. We also observed that ICRF-193 treatment yields a significantly higher percentage of metaphase cells in untransduced HEK-293T, which lack Metnase, compared to untransduced HEK-293 cells which express moderate levels of Metnase. Thus, resistance to ICRF-193-induced metaphase arrest correlates with Metnase expression level. Interestingly, increased Metnase also reduced metaphase arrest induced by VP-16 (Fig. 3B,C). Because VP-16 blocks Topo II α after DNA cleavage, this arrest could be due to activation of the DNA damage G2/M checkpoint and/or the decatenation checkpoint. Nonetheless, high levels of Metnase allowed cells to continue to proliferate in the presence of toxic concentrations of VP-16 (Fig. 3D). These effects could result from Metnase potentiation of Topo II α decatenation and/or DSB repair by NHEJ. Alternatively, it is also possible that Metnase blocks VP-16 (and ICRF-193) access to Topo II α . Regardless, these results suggest that Metnase is an important mediator of resistance to VP-16, which is commonly used in treating human malignancies.

We confirmed the effects of Metnase expression on progression through the decatenation checkpoints by analyzing cell cycle profiles in the presence or absence of ICRF-193 using flow cytometry. This measures both the G2/M and metaphase decatenation checkpoints [19]. When HEK-293T cells were treated for 4 hours with 10 μ M ICRF-193, the increase in the G2/M fraction was 13.5-fold higher in control cells compared to cells transfected with a Metnase expression vector (Fig. 2E). After an 18 hour treatment with ICRF-193, the difference between control and Metnase expressing cells was smaller, but there were still significantly less cells arrested in G2/M with increased Metnase levels (Fig. 2E). Together, these results indicate that

Metnase promotes progression through decatenation checkpoint arrest induced by Topo II α inhibitors.

Based on these in vivo data, we propose that increased expression of Metnase enhances Topo II α activity, increasing the efficiency of chromosome decatenation and bypassing decatenation checkpoint arrest. Cancer cells also show attenuated decatenation checkpoints when Topo II α is inhibited by ICRF-193 [23,24]. It is possible that the resistance to ICRF-193 in these cells is partially mediated by robust Metnase levels.

Metnase Enhances Topo II α Decatenation Activity

To understand the molecular mechanism of the promotion of progression through the metaphase decatenation checkpoint by Metnase, we characterized the activity of purified Metnase on supercoiled and catenated DNA in vitro. Metnase converts supercoiled plasmid to nicked, relaxed and linear forms, but does not further degrade DNA (Fig. 4A), as we showed previously [13]. We next examined the activity of Metnase on catenated kinetoplast DNA (kDNA), which is composed of inter-locked DNA mini-circles [28,29]. As shown in Fig. 3B Metnase converts catenated kDNA into mostly linear DNA and a smaller amount of free, nicked open circular DNA. In contrast, Topo II α converts kDNA into fully decatenated nicked circular and relaxed, covalently closed circular DNA (Fig. 4C).

Since Metnase both interacts with Topo II α and possesses catenated DNA cleavage activity, we next tested whether Metnase and Topo II α might function together in decatenating kDNA. At concentrations of Metnase and Topo II α where neither protein had appreciable activity on kDNA, together they synergistically decatenated kDNA into nicked and relaxed fully decatenated circular forms (Fig. 4D, E). To rule out the possibility that the enhanced decatenation is due to non-specific Metnase nuclease activity, we analyzed kDNA decatenation by Topo II α in the presence or absence of DNase I. In contrast to Metnase, DNase I did not enhance Topo II α decatenation to circular forms, but it did produce linear DNA which over time was degraded (Fig. 4F). Indeed, the presence of DNase I inhibited Topo II α decatenation activity. The enhancement of decatenation by Metnase is likely due to more than just its nuclease activity, since Metnase can still enhance Topo II α decatenation in buffers and at levels where there is little nuclease function. This implies that Metnase perhaps functions as a structural co-factor to increase Topo II α activity. This model is more consistent with the known mechanism of Topo II α , as DNA cleavage, strand passage, and religation are catalyzed by Topo II α in a coordinated set of covalent reactions [14].

To further investigate the potentiation of Topo II α decatenation by Metnase we tested the effect of Metnase on kDNA decatenation with nuclear extracts from HEK-293T cells which do not express Metnase and a derivative stably transfected with a Metnase expression vector (Fig. 5A). As shown in Fig. 4A, nuclear extracts from cells expressing Metnase were significantly more effective at decatenating kDNA than HEK-293T extracts. This effect was Metnase-specific, because the decatenation activity of Metnase-containing extracts was reduced by anti-Metnase antibodies. Thus, Metnase levels in nuclear extracts correlated with the ability of these extracts to decatenate kDNA. We then examined the rate of decatenation of kDNA by nuclear extracts with and without Metnase. Metnase-containing extracts decatenated kDNA at a 3-fold higher rate than HEK-293T control extracts (Fig. 5B and C). These data indicate that although Topo II α is capable of decatenating DNA in the absence of Metnase, Metnase significantly increases the reaction rate.

Metnase Automethylation Prevents Potentiation of Topo II α -Mediated Decatenation

SET domain proteins were originally defined as histone methylases but they also methylate other proteins, including themselves and transcription factors [30-32]. We therefore investigated whether decatenation could be regulated by the histone methylase activity of Metnase. Metnase could methylate itself or Topo II α , modifying their synergistic effect on decatenation. S-adenosyl methionine (SAM) is the primary cellular methyl donor, and we tested whether SAM influenced Metnase potentiation of Topo II α -mediated decatenation. Interestingly, SAM fully abrogated the effect of Metnase on decatenation (Fig. 6A, B). SAM had no effect on decatenation by Topo II α alone, indicating that the abrogation of Metnase potentiation of Topo II α activity is Metnase-dependent. S-adenosyl homocysteine (SAH) is a pseudo-methyl donor that binds to methyltransferase active sites but cannot donate a methyl group. SAH had no effect on the Metnase potentiation of Topo II α decatenation activity (nor did it affect Topo II α activity alone) (Fig. 6A, B), indicating that the SAM abrogation of decatenation with Metnase depends on methyl transfer. When SAM and SAH were both present, decatenation by Metnase and Topo II α partially reduced, indicating that SAH competes with SAM to prevent methylation. However, SAM did not alter the nuclease activity of Metnase (Fig. 6C).

The reduction of decatenation by SAM could reflect Metnase automethylation, or methylation of Topo II α . Therefore, we performed MALDI ToF-ToF mass spectroscopy on Metnase and Topo II α individually and together in the presence or absence of SAM. We found no evidence of Topo II α methylation (data not shown), but in the presence of SAM Metnase was automethylated at several positions. The strongest signal was mono-methylation of K485, with an average of 86% of lysine residues methylated at this position (Fig. 7). Methylation of three other arginine and lysine residues was also apparent (R223, R469, and K122), but <25% of these residues were methylated (data not shown). These results suggest that Metnase automethylation regulates its ability to potentiate Topo II α -dependent decatenation. This represents a novel function for a SET domain protein, and suggests a new regulatory mechanism for this important class of protein post-translational modification.

The data here have general significance for several reasons. First, the precise biochemical process whereby replicated chromosomes are untangled is not well understood, but it is clear that Metnase enhances this process, perhaps at an early, rate-limiting step of decatenation. Second, the automethylation of Metnase is a unique feedback regulatory mechanism to slow decatenation. It also shows that SET domain protein automethylation can affect other functions besides histone methyltransferase activity, as seen with G9a [31,32]. Third, the ability of cells expressing Metnase to continue to proliferate in the presence of lethal concentrations of the clinically relevant Topo II α inhibitor VP-16 (Fig. 2D) suggests a combination chemotherapy strategy in which an inhibitor of Metnase would potentiate the lethal effects of topoisomerase inhibitors on human cancer. Finally, since transposase activity would be deleterious to humans, the benefit of a transposase domain protein in humans has not been defined [3,10-13]. However, these data demonstrate such a benefit, and perhaps shed light on how Metnase was selected for in primates.

Metnase levels influence breast cancer decatenation

We also found that breast cancer cell lines had an attenuated but not completely abrogated decatenation checkpoint. Whereas HEK-293T cell had an average of 6.1% of cells arrested in metaphase after 10 μ M ICRF-193 for 18 hrs, MB321 breast cancer cells had an average of 4.8% (Fig. 5). Interestingly, when Metnase levels are repressed using siRNA, there is an increase in metaphase arrest seen with ICRF-193 exposure (Fig. 5). This implies that lowering Metnase levels decreases Topo II α -mediated decatenation in MBA321 breast cancer cells. Thus, Metnase likely enhances Topo II α function in breast cancer as well as HEK-293 cells. Next, we

tested whether reducing Metnase levels would increase sensitivity to the Topo II α inhibitor VP-16. We found that siRNA repression of levels Metnase increased sensitivity to VP-16 by 8-fold (Fig. 5). This demonstrates that Metnase not only enhances Topo II α decatenation function, but also mediates resistance to a clinically relevant Topo II α inhibitor.

Figure Legends

Figure 1. Physical interaction between Metnase and Topo II α . (A) V5-tagged Metnase immunoprecipitated (IP) from HEK-293 cells co-precipitates Topo II α , detected by western blotting. pCD- vector control, NS- non-specific antisera. (B) Mutations in Metnase SET or nuclease domains block its co-immunoprecipitation with Topo II α [9]. (C) Purified FLAG-tagged Metnase interacts with purified Topo II α independently of any other protein.

Figure 2. (A) Immunofluorescence co-localization of Metnase (green) with Topo II α (red) during chromosome condensation indicated by orange color in merged photomicrograph. (B) Co-localization is maximal in G2/M cells. (C) Co-localization of Metnase and Topo II α is abrogated by 10 μ M ICRF-193 and by 10 μ M VP-16.

Figure 3. Metnase promotes progression through the metaphase decatenation checkpoint. (A) Manipulated Metnase levels in HEK-293 and HEK-293T cells. β -actin and 18S rRNA served as loading controls for Western blots and RT-PCR, respectively. (B) Decatenation checkpoint arrest induced by ICRF-193 was monitored the increase in metaphase cells using immunofluorescence microscopy of cells stained with anti-tubulin antibodies (green) and DAPI (blue). pCD- vector control, U6- siRNA control. (C) Quantitative analysis of ICRF-193 or VP-16 induces mitotic checkpoint arrest after subtraction of vehicle controls. (D) HEK-293T cells transfected with pCAPP-Metnase or empty vector were seeded in medium with or without 5 μ M VP-16 on day 0 and harvested and counted daily. (E) Flow-cytometric analysis of G2/M fractions in HEK-293T cells transfected with empty or Metnase expression vectors after ICRF-193 treatment.

Figure 4. Metnase cleaves supercoiled plasmid DNA and promotes Topo II α -mediated decatenation of kDNA. (A) Purified recombinant Metnase nicks supercoiled plasmid to relaxed open circular and linearized plasmid, but does not degrade it. (B) Purified Metnase decatenates kDNA, forming linear DNA and a small amount of nicked, open circular (OC) DNA. M1, M2-markers for kDNA forms as indicated. (C) Purified Topo II α decatenates kDNA forming nicked OC and relaxed closed circular (CC) DNA. (D) At concentrations of Metnase and Topo II α where neither alone has an appreciable decatenation activity, they act synergistically to decatenate kDNA to nicked OC and relaxed CC DNA. (E) Plot of densitometric scans of kDNA decatenation shown in panel D. (F) DNase I nicks, linearizes, and ultimately degrades kDNA.

Figure 5. Decatenation of kDNA by nuclear extracts is enhanced by Metnase. (A) kDNA was treated with nuclear extracts from HEK-293T cells transfected with empty vector (control extracts) or derivatives stably transfected with a Metnase expression vector (Metnase extracts). Topo II α levels were similar in the two cell lines. Reactions containing anti-sera against Metnase show significantly reduced decatenation. (B) Time course of kDNA decatenation reactions catalyzed by nuclear extracts as in panel A. (C) Graph of panel B time course.

Figure 6. SAM blocks the Metnase potentiation of Topo II α decatenation. (A) kDNA decatenation assays performed as in Fig. 4 in the presence or absence of the methyl donor SAM or non-donating SAM analog, SAH (2 mM each, except 1 mM each when both were present). (B) Plot of decatenation with 0.25-1 units of Topo II α , 200 ng Metnase, 2 mM SAM or SAH based on densitometric scans of gels as in panel A. Shown are averages (+SD) for three experiments, with all values normalized to 1 unit of Topo II α (control = 100%). Statistics calculated by t tests. (C) SAM (2 mM) does not affect the Metnase nuclease activity toward the kDNA substrate. Metnase was present at 0, 100, or 200 ng indicated by triangles.

Figure 7. Metnase is auto-methylated at residue K485. (A) MALDI-tof-tof mass spectrum of Metnase alone, showing the unmodified K485 peptide at m/z of 979.4849 as predicted. (B) Mass spectrum of Metnase incubated with SAM in methylase buffer showing methylation of the K485 peptide at m/z of 993.5866.

Figure 8. A- 6-fold repression of Metnase RNA levels by siRNA (M2). GFP siRNA serves as a control. **B-** After subtraction of vehicle control, percent cells arrested in metaphase after ICRF-193 exposure. ***- statistically significant. **C-** Survival curves for Metnase knock-downs (M2) and GFP controls (EGFP) after exposure to increasing concentrations of VP-16 as determined by colony formation assays.

Figure 1.

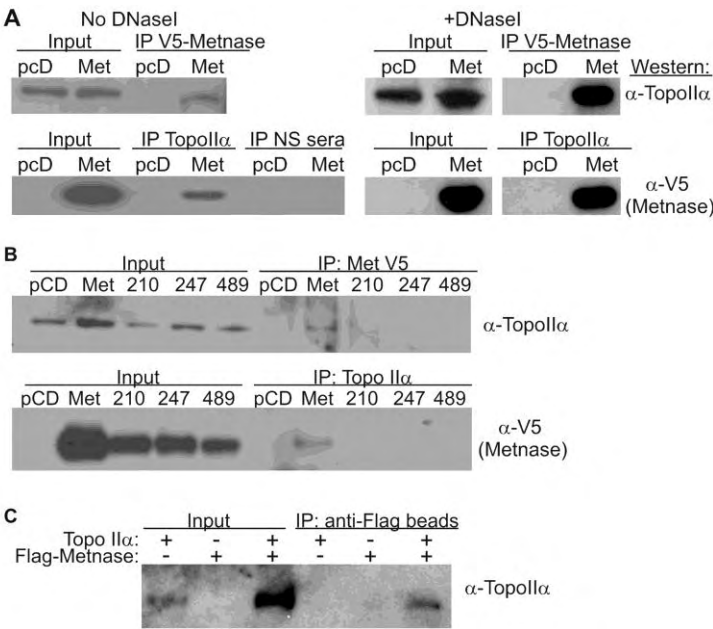


Figure 2.

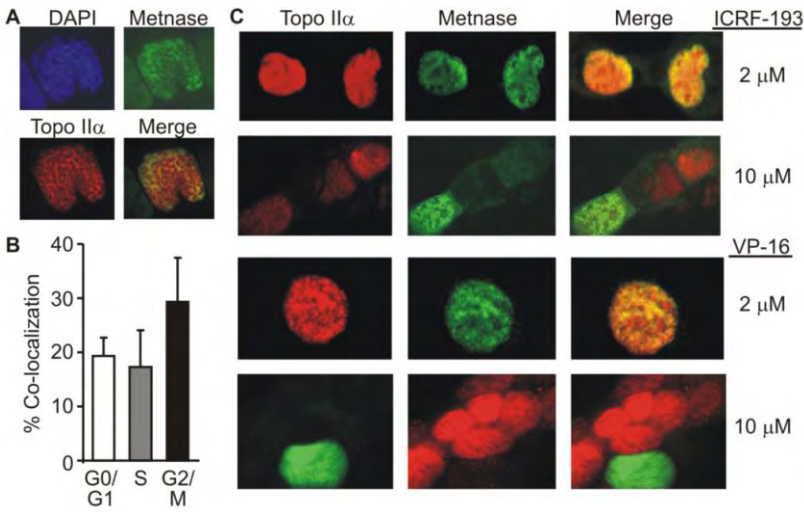


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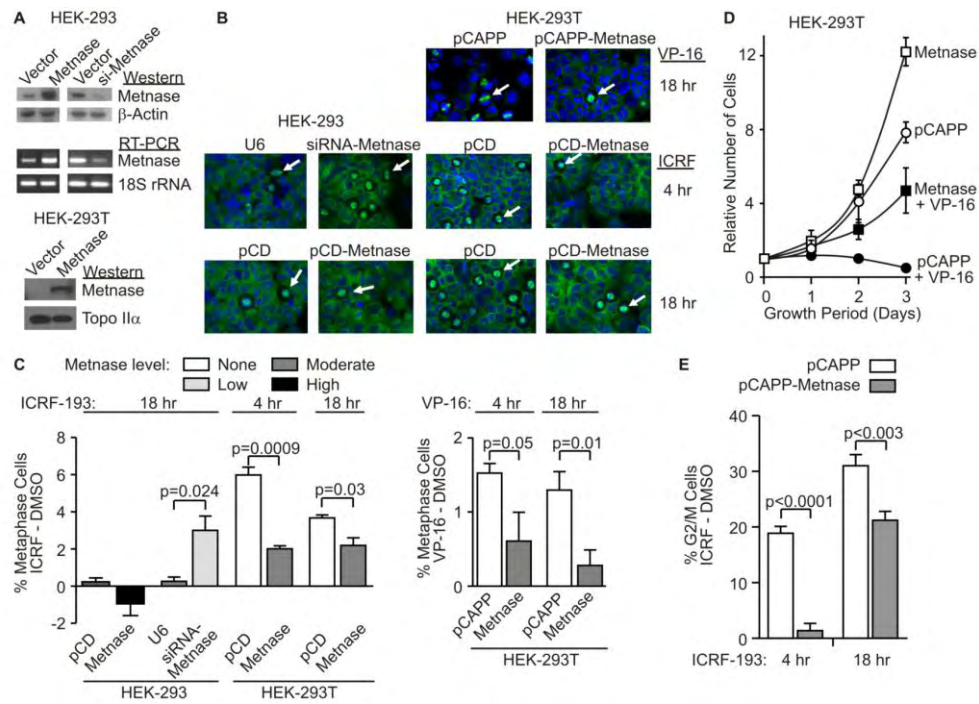


Figure 4.

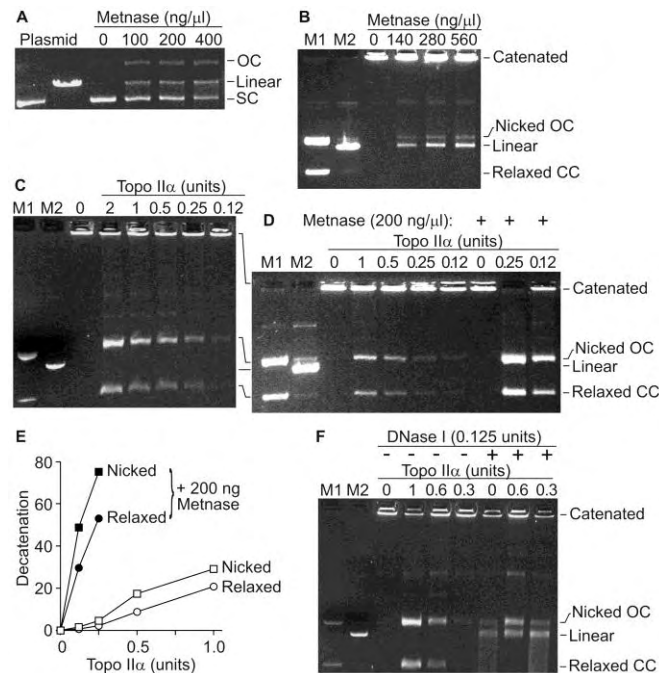


Figure 5.

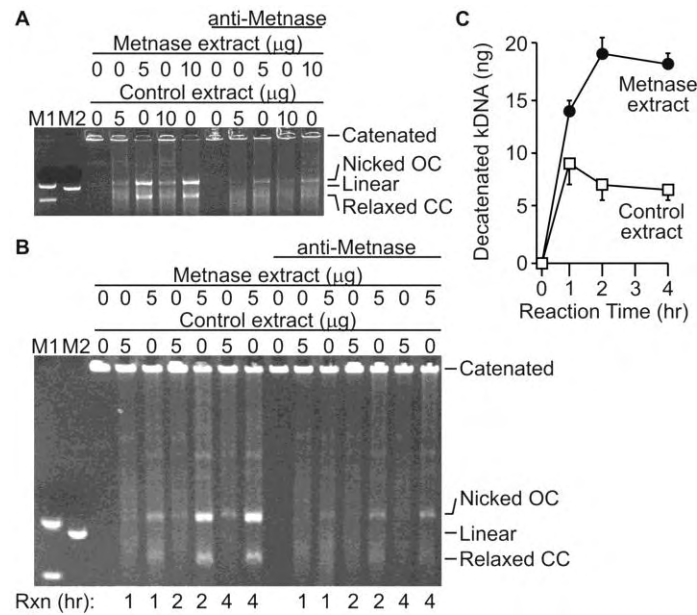


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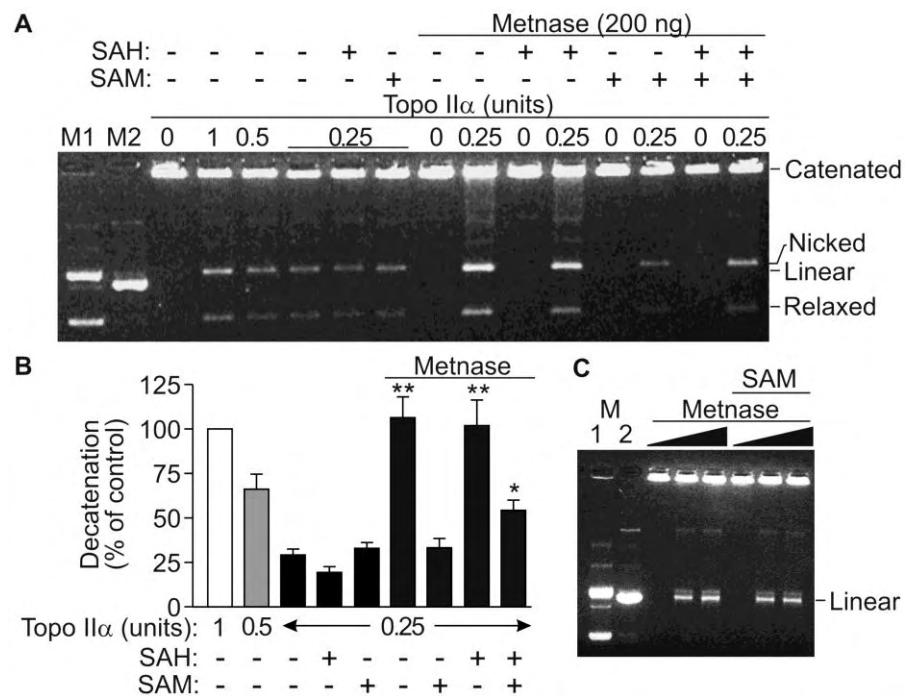


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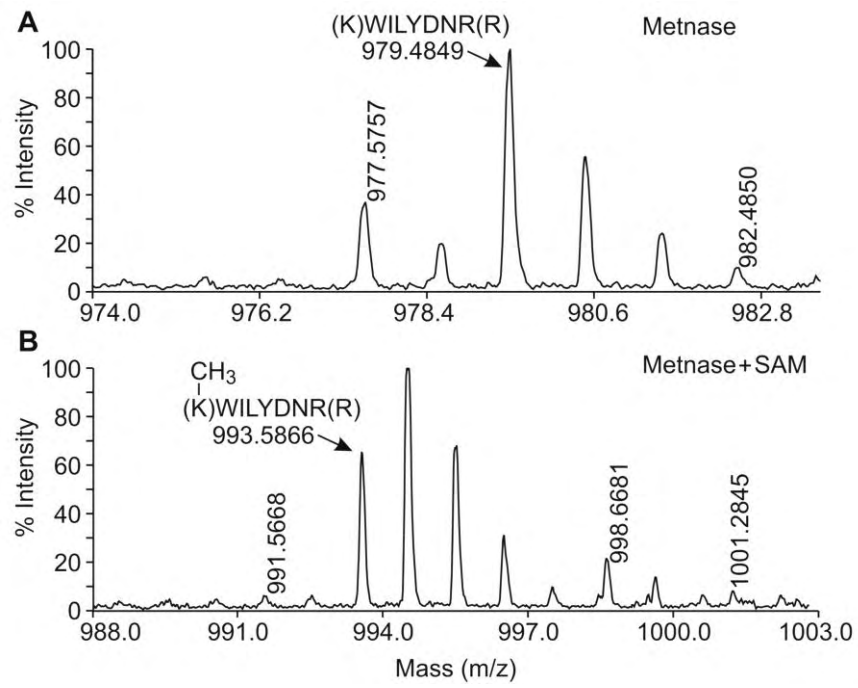
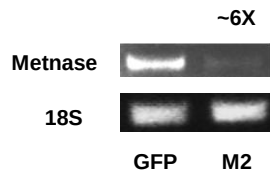
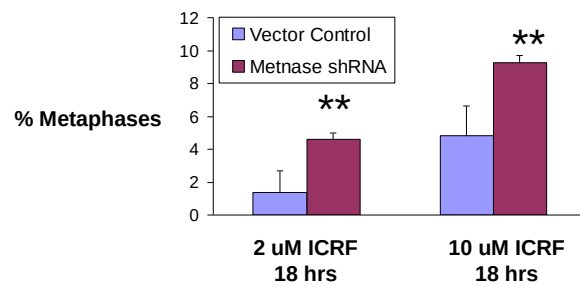


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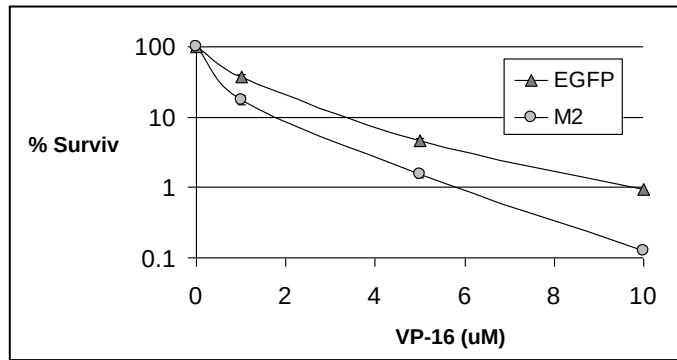
A.



B.



C.



Key Research Accomplishments

- The function of transposase domain proteins in humans is not well understood. Transposases move DNA segments from one location in the genome to another, and such movement would be deleterious in humans. However, the enhancement of decatenation by Metnase provides a functional benefit to human cells, where the activities of a transposase are modified to assist in stable cell division.
- While decatenation can take place with Topo II α alone, the rate of such decatenation is significantly improved by Metnase. Yet automethylation of Metnase abrogates this improvement. Metnase therefore functions as an autoregulated enhancer of decatenation.
- Topo II α decatenates DNA in a multi-component complex called the toposome. Besides Topo II α , physical mediators of decatenation are not well described. This work defines another such mediator. This lends insight into the composition of the toposome, and how it functions to decatenate DNA. Components of the toposome could enhance or slow decatenation depending on the integrity of the DNA to be decatenated. Thus, Metnase could be a key regulatory component of the toposome.
- The finding that an NHEJ repair component assists in decatenation links DNA repair to decatenation. It is possible that automethylation of Metnase slows decatenation when there is DNA damage present, and this would allow the cell to repair that damage before decatenation and mitosis.

Conclusions

Metnase was found to decrease sensitivity to the Topo II α inhibitor VP-16. Metnase could mediate resistance to Topo II α inhibitors during cancer therapy. Despite major advances, perplexing problems remain in the therapy of breast cancer. Once breast cancer recurs it is ultimately fatal. One of the most important therapeutic modalities for treating breast cancer, in neo-adjuvant, adjuvant or a metastatic setting, is Topo II α inhibition with anthracyclines such as doxorubicin. Despite the general sensitivity of breast cancer cells to anthracyclines, there is a fraction of patients whose tumors are resistant to these agents. These patients undergo therapy with these agents but recur despite appropriate duration and level of treatment. Predicting which patients would fail these therapies before they are given would save needless toxicity and time. Such a prediction would allow diversion of patients likely to have primary failure to more beneficial treatment. Thus, Metnase levels in breast cancer specimens could be used to predict

who will not respond to Topo II α inhibition. If true, then these patients could be given other therapy, saving time, tumor progression, and toxicity without benefit. In addition, if true, then inhibiting Metnase with a small molecule might improve the efficacy of Topo II α inhibition in the treatment of breast cancer. Thus, Metnase might be an important target for breast cancer therapy.

Reportable Outcomes

The DNA repair component Metnase plays an important role in enhancing the ability of Topo II α in untangling chromosomes. Since Topo II α is a key target of many chemotherapy drugs, targeting Metnase for inhibition using small molecules may markedly enhance the activity of anti-Topo II α chemotherapy. Since over-expression of Metnase in tumors may enhance Topo II α activity, it may mediate chemotherapy resistance. Thus, it could be a prognostic marker defining response to anti-Topo II α chemotherapy.

Manuscript Submitted

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Presentation

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