Molecular Pharmacology Fast Forward. Published on January 20, 2011 as doi:10.1124/mol.110.069633

MOL #69633

Novel regulation of NF-YB by *miR-485-3p* affects expression of DNA topoisomerase IIα and drug responsiveness

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Molecular Pharmacology Fast Forward. Published on January 20, 2011 as DOI: 10.1124/mol.110.069633
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Running Title

Regulation of NF-YB via *miR-485-3p* affects Top2α expression

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Number of text pages: 32

Number of figures: 5

Number of references: 38

Number of words in the

Abstract: 248

Introduction: 335

Discussion: 794

Molecular Pharmacology Fast Forward. Published on January 20, 2011 as DOI: 10.1124/mol.110.069633
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Abbreviations:

3′-UTR, 3′-untranslated region

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

hsa- miR-485-3p, human microRNA-485-3p

IC_{50}, 50% inhibitory concentration

ICE, inverted CCAAT element

NF-Y, nuclear factor Y

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

PCNA, proliferating cell nuclear antigen

PCR, polymerase chain reaction

RT-PCR, reverse transcription-polymerase chain reaction

Top1, DNA topoisomerase I

Top2, DNA topoisomerase II

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Abstract

NF-YB, a subunit of the transcription factor, nuclear factor Y (NF-Y) complex, binds and activates CCAAT-containing promoters. Our previous work suggested that NF-YB may be a mediator of Top2α, working through the Top2α promoter. DNA topoisomerase II (Top2) is an essential nuclear enzyme and the primary target for several clinically important anticancer drugs. Our teniposide-resistant human lymphoblastic leukemia CEM cells (CEM/VM-1-5) express reduced Top2α protein compared to parental CEM cells. To study the regulation of Top2α during the development of drug resistance, we found that NF-YB protein expression is increased in CEM/VM-1-5 cells compared to parental CEM cells. This further suggests that increased NF-YB may be a negative regulator of Top2α in CEM/VM-1-5 cells. We asked what causes the upregulation of NF-YB in CEM/VM-1-5 cells. We found by microRNA profiling that *hsa-miR-485-3p* is lower in CEM/VM-1-5 cells compared to CEM cells. MicroRNA target prediction programs revealed that the 3'-untranslated region (3'-UTR) of NF-YB harbors a putative *hsa-miR-485-3p* binding site. We thus hypothesized that *hsa-miR-485-3p* mediates drug responsiveness by decreasing NF-YB expression, which in turn negatively regulates Top2α expression. To test this, we overexpressed *miR-485-3p* in CEM/VM-1-5 cells and found that this led to reduced expression of NF-YB, a corresponding upregulation of Top2α and increased sensitivity to the Top2 inhibitors. Results in CEM cells were replicated in drug-sensitive and -resistant human rhabdomyosarcoma Rh30 cells, suggesting that our findings represent a general phenomenon. Ours is the first study to

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show that *miR-485-3p* mediates Top2α downregulation in part by altered regulation of NF-YB.

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Introduction

NF-Y (nuclear factor-Y) is a conserved transcription factor that consists of three different subunits, NF-YA, NF-YB and NF-YC, and binds specifically to the CCAAT elements in promoters (Ronchi et al., 1995). Our previous work suggested that one of these subunits, NF-YB, may mediate Top2α expression, working through inverted CCAAT element 3 (ICE3) in the Top2α promoter (Morgan and Beck, 2001). DNA topoisomerase II (Top2) is an essential nuclear enzyme involved in many cellular processes (Champoux, 1990; Nitiss, 2009a; Wang, 2002) and mammalian cells have two isoforms of the type II enzymes, Top2α (170kDa) (Tsai-Pflugfelder et al., 1988) and Top2β (180kDa) (Chung et al., 1989). Top2-DNA covalent complexes serve as the cytotoxic target for many anticancer drugs such as doxorubicin, etoposide, teniposide and amsacrine (Beck, 1996; Liu, 1989; Pommier et al., 2010). However, tumors frequently become refractory to treatments with Top2 inhibitors due to the emergence of drug resistance (Nitiss, 2009b; Nitiss and Beck, 1996). Our previous work suggested that the transcription factor NF-YB is a mediator of Top2α, working through the Top2α promoter (Morgan et al., 2000). Our current experiments revealed an inverse relation between the expression of Top2α protein and NF-YB protein in drug-sensitive CEM cells compared to the teniposide-resistant CEM/VM-1-5 cells (Danks et al., 1988) and in drug-sensitive human rhabdomyosarcoma Rh30 cells and the etoposide-resistant Rh30/v1 cells (Bhat et al., 1999). We asked in the present study what causes the upregulation of NF-YB in the CEM/VM-1-5 and Rh30/v1 cells that express decreased levels of Top2α.

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There are recent reports that microRNAs (miRs), a group of non-protein coding, single-stranded RNAs of 20–22 nucleotides, are often aberrantly expressed or mutated in cancer (Calin and Croce, 2006), may play roles as either oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006) and may mediate drug responsiveness (Mishra et al., 2007; To et al., 2008). Accordingly, we asked whether microRNAs might be involved in the regulation of NF-YB which further mediates Top2α expression and drug responsiveness in CEM and CEM/VM-1-5 cells as well as in Rh30 and Rh30/v1 cells. MicroRNAs function through perfect or near perfect base pairing with protein coding mRNA 3'-untranslated regions (3'-UTRs) for mRNA degradation or translational repression (Bartel, 2004). We have found by microRNA profiling that a particular microRNA, *hsa-miR-485-3p*, may target the 3'-UTR of NF-YB to affect expression of Top2α, and our results are presented herein.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions.

The human lymphoblastic leukemia CEM cell line and its teniposide-resistant subline, CEM/VM-1-5, developed in our laboratory (Danks et al., 1988), were cultured in suspension in RPMI-1640. The human rhabdomyosarcoma Rh30 cell line and its etoposide-resistant subline, Rh30/v1 (Bhat et al., 1999) were grown as monolayers and also cultured in RPMI 1640. HEK 293T cells were cultured in DMEM. Both RPMI 1640 and DMEM (BioWhittaker, Inc. Walkersville, MD) were supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), and 2 mM L-glutamine (BioWhittaker, Inc. Walkersville, MD). All cell lines were subcultured twice a week in either RPMI 1640 or DMEM and were incubated at 37°C in a humidified chamber containing 5% CO_2/95% air.

Western Blot Analysis

Nuclear protein extracts were prepared from logarithmically growing cells as described previously (Morgan and Beck, 2001). Protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein samples (~15-30ug) were separated on a 4% to 12% Bis-Tris gradient gel (Invitrogen), electrophoretically transferred onto nitrocellulose, and incubated with either purified mouse anti-Top2α monoclonal antibody (BD, Biosciences, San Jose CA), rabbit anti-NF-YB polyclonal antibody or mouse anti-NF-YA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected using the enhanced chemiluminescence (ECL)

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detection method (Amersham Corp, Arlington Heights, IL) according to the manufacturer's instructions. Chemiluminescence signals were quantified by densitometric scanning using an Imaging Densitometer (Scion Corp., Frederick, MD). Equal loading of nuclear protein was determined by blotting the membrane with anti-PCNA (Proliferating cell nuclear antigen) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of Total RNA and Reverse Transcriptase (RT)-PCR

Total RNAs were extracted from CEM and CEM/VM-1-5 and Rh30 and Rh30/v1 cells with Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Single-stranded cDNA was synthesized by using the ThermoScript RT-PCR System (Invitrogen). The PCR reactions were carried out for each transcript under the following conditions: initial denaturation at 94ºC for five min, 30-40 cycles at 94°C 30s, 50-60°C 60s, and 72°C 90s and 1 cycle of 72°C for 10 min. The 3'-UTR sequences of NF-YB containing the putative *mir-485-3p* binding site were amplified using the primers as follows: NF-YB 3'-UTR (sense: 5'-TCT AGA AAG CAA GTG AAA GGT GCC AT-3'; and antisense: 5'-TCT AGA ATC ATG AAT TAA CCC AGC CG-3'). To delete the putative *mir-485-3p* binding site, we used the primers as follows: (sense: 5'-TCT AGA AAG CAA GTG AAA GGT GCC AT-3'; and antisense: 5'-TCT AGA CCT GAT GCT TGA CTA ATT GAG G -3') and the sequences were defined as NF-YB 3'-UTR-d

Construction of Expression Vectors

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The *miR-Crtl* (pCDH-empty vector) and *miR-485-3p* expression vectors (pCDH-*miR-485-3p*) were packaged into lentiviruses by cotransfection of HEK293T cells with three plasmids: a lentiviral expression vector plus pMD2.G and psPAX2. (The latter two plasmids were generous gifts of Dr. Didier Trono, Department of Genetics and Microbiology, University of Geneva, Geneva, Switzerland). CEM/VM-1-5 cells transduced with *miR-Crtl* or *miR-485-3p* expression vectors are defined as *CEM/VM-1-5 miR-Crtl* or *CEM/VM-1-5 miR-485-3p* respectively (Same as Rh30/v1 cells). The NF-YB 3'-UTR containing the putative *mir-485-3p* binding site and NF-YB 3'-UTR-d without the putative *mir-485-3p* binding site were amplified by PCR and then cloned into the pGL3-TK vector (generous gift of Dr. Hyun-Young Jeong, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, Illinois). The luciferase reporters containing the NF-YB 3'-UTR with or without the putative *mir-485-3p* binding site are defined as pGL3-NF-YB-3'-UTR or the pGL3-NF-YB-3'-UTR-d, respectively.

Cytotoxicity Assay.

Drug-induced cytotoxicity was measured by the MTT assay (Mosmann, 1983). Exponentially growing cells were plated at 4000-5000 cells/well in 96-well microtiter plates (100 µl/well) in triplicate. Etoposide (Sigma), doxorubicin (Sigma) or vinblastine (Sigma) was added to the cells at various concentrations in a final volume of 200 µl/well, and the cells were incubated at 37°C for 72 h. After drug exposure, 20 µl of MTT compound (Sigma; 5 mg/ml in PBS) were added to each well and the cells were incubated at 37°C for 3 h. The plates were centrifuged in a swinging bucket rotor (3000 rpm, 20 min, 4°C), and the medium was removed. After 200 µl of dimethyl sulfoxide

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(DMSO) were added to each well and the plates were incubated for 10 min at 37°C, the metabolic activity of the cells was measured by quantifying the conversion of the yellow MTT to a purple metabolite, MTT-formazan. Absorbance was read at 560 nm using a microplate reader. Experimental samples were measured for each drug concentration and the experiments were replicated thrice at a minimum. The IC_{50} value (the concentration of drug that killed 50% of the cells) was calculated by GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA).

Luciferase Assay

Luciferase assays were carried out in HEK293T cells. Cells were seeded in 24-well plates in triplicate and transfected with appropriate plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h of transfection, cells were lysed, and treated with Dual-Light assay system (Applied Biosystems) according to the manufacturer's protocol. Relative luciferase activities were measured and normalized against β-galactosidase activity. All the experiments were replicated thrice at a minimum.

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Results

Inverse expression of Top2α and NF-YB proteins in CEM and CEM/VM-1-5 cells and Rh30 and Rh30/v1 cells

Previous work from our laboratory suggested that the transcription factor NF-YB is a mediator of Top2α, working through the inverted CCAAT element 3 (ICE3) in the Top2α promoter (Morgan and Beck, 2001). We found that NF-YB protein levels were 3-fold higher in CEM/VM-1-5 cells, compared to CEM cells (Fig. 1A, B) and there is an inverse relation between the protein levels of Top2α and NF-YB in CEM and CEM/VM-1-5 cells by Western blots (Fig. 1A). To determine whether this is a cell-line specific phenomenon, we examined the human rhabdomyosarcoma Rh30 cell line and compared it to its etoposide-resistant subline, Rh30/v1 (Bhat et al., 1999). We observed a similar result: Top2α is downregulated in the etoposide-resistant Rh30/v1 cell line and there is an inverse relation between the protein levels of Top2α and NF-YB (Fig.1D, E). In addition, the expression of NF-YA protein is similar between drug sensitive and –resistant cells (Supplementary Fig. S1). These data suggest that increased NF-YB may be either related to, or the cause of, reduced Top2α in CEM/VM-1-5 and Rh30/v1 cells. Furthermore, we found that NF-YB mRNA levels were similar in CEM, CEM/VM-1-5 and Rh30, Rh30/v1 cells (Fig. 1C, F), indicating that the observed differences in NF-YB protein levels are likely a consequence of regulation at the posttranscriptional level. Posttranscriptional repression is a major mechanism by which microRNAs regulate gene expression (Bartel, 2004; Carrington and Ambros, 2003). Accordingly, we then

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asked whether microRNAs are involved in the regulation of NF-YB protein during the development of drug resistance.

Expression of microRNAs in human lymphoblastic leukemia CEM and CEM/VM-1-5 cells

We used microRNA profiling by Applied Biosystems real-time PCR array (TaqMan® Human MicroRNA Array v1.0.) to analyze microRNA expression in CEM and CEM/VM-1-5 cells. The array carries unique hairpin-loop RT primer sets that allow for detection of 365 mature human microRNAs in the cells. Compared to CEM cells, microRNA profiling revealed that CEM/VM-1-5 cells with acquired resistance to teniposide demonstrated substantial changes in microRNA expression. Compared to parental controls, thirty-two microRNAs were consistently either up or downregulated in CEM/VM-1-5 cells, as determined by Ct (threshold cycle) values from three separate microRNA profiling experiments (He et al., ms in preparation). Since higher Ct values indicate lower expression levels, one of the microRNAs, *hsa-miR-485-3p*, was found to be consistently substantially lower in CEM/VM-1-5 cells compared to CEM cells after conversion (Wu et al., 2009) to expression level (Fig. 2A, B). Interestingly, the decreased expression of *miR-485-3p* in CEM/VM-1-5 cells is inversely related to the overexpression of NF-YB protein (Fig. 1A, B).

Using miRanda (http://www.microrna.org/microrna/home.do), Target Scan (http://www.targetscan.org/) and MicroCosm (http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl) prediction programs, we found that the 3'-UTR of NF-YB

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harbors a putative *hsa-miR-485-3p* binding site (Fig. 2B), which is conserved in human, rhesus monkeys, chimpanzees and dogs. Moreover, we found by sequencing that there is no mutation in the region of NF-YB 3'-UTR to which *miR-485-3p* binds in either CEM or CEM/VM-1-5 cells (data not shown), suggesting that the binding of microRNAs to the NF-YB 3'-UTRs is unlikely to be altered.

NF-YB is a direct target for *miR-485-3p*

To validate that NF-YB is *miR-485-3p* direct target, we constructed a luciferase reporter (pGL3-TK) carrying the NF-YB 3'-UTR with the putative *miR-485-3p* binding site. We co-transfected *miR-485-3p* expression vector and luciferase reporter into HEK293T cells. Relative luciferase activity of the NF-YB 3'-UTR with *miR-485-3p* binding site, which was co-transfected with the *miR-485-3p* expression vector (pCDH-*miR-485-3p*) was significantly lower (~46%) than that of the *miR-Crtl* (pCDH-empty vector) co-transfected cells (Fig. 3A). Moreover, the relative luciferase activity of the pGL3-NFYB-3'-UTR with the *miR-485-3p* binding site was also lower (~45%) than that of the pGL3-NF-YB-3'-UTR-d (pGL3-NF-YB 3'-UTR without *miR-485-3p* binding site), which was carried out in HEK293T cells co-transfected with the *miR-485-3p* expression vector (Fig. 3B).

***MiR-485-3p* modulates the sensitivity of CEM/VM-1-5 cells to Top2 inhibitors**

To ascertain whether *miR-485-3p* regulates NF-YB, we examined the effects of overexpressing *miR-485-3p* on NF-YB expression in CEM/VM-1-5 cells, which have

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reduced endogenous *miR-485-3p* expression (Fig. 2A). Thus, we transduced CEM/VM-1-5 cells with either the pCDH-*miR-485-3p* or the *miR-Crtl* (pCDH-empty vector) virus particles. We validated *miR-485-3p* expression in *CEM/VM-1-5 miR-485-3p* cells by Taqman Real-time PCR (data not shown). Western blot analysis revealed decreased NF-YB protein levels in pCDH-*miR-485-3p* virus stably-transduced CEM/VM-1-5 cells, compared to the *miR-Crtl* transduced CEM/VM-1-5 cells and no difference in NF-YA protein levels was seen in response to *miR-485-3p* overexpression cells (Fig. 4A).

To determine whether this was a cell line-specific phenomenon, we examined the etoposide-resistant human rhabdomyosarcoma Rh30/v1 cells. Introduction of *miR-485-3p* into these cells also decreased their NF-YB protein levels in Rh30/v1 cells (Fig. 4B) indicating that the *miR-485-3p*-mediated regulation of NF-YB is not cell line-specific.

Because *miR-485-3p* targets NF-YB and NF-YB may negatively regulate the expression of Top2α, the above findings suggested an interesting relationship between NF-YB, *miR-485-3p*, and Top2α. Accordingly, we analyzed the levels of Top2α in *CEM/VM-1-5 miR-485-3p* cells (Fig. 4A). Interestingly, we found that the level of Top2α protein is upregulated, whereas the level of NF-YB is downregulated compared to *CEM/VM-1-5 miR-Crtl* cells, suggesting that NF-YB regulates Top2α expression via *miR-485-3p*. A similar effect was observed in the etoposide-resistant Rh30 subline, Rh30/v1 (Fig. 4B). We thus hypothesized that *hsa-miR-485-3p* may regulate drug responsiveness by increasing NF-YB expression, which in turn negatively regulates Top2α expression. To test this, we performed the following experiments.

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We examined the effects of *miR-485-3p* on the of drug sensitivity of CEM/VM-1-5 cells. MTT assays revealed that *CEM/VM-1-5 miR-485-3p* cells exhibited enhanced sensitivity, 2-fold to etoposide, compared to *CEM/VM-1-5 miR-Crtl* cells, as indicated by decreased IC_{50} values (Fig. 4C). Similar results were seen in doxorubicin treated cells (data not shown). However, no significant difference in responsiveness to vinblastine, a microtubule inhibitor, was found in *miR-485-3p* overespression CEM/VM-1-5 cells (Fig. 4D). Taken together, *miR-485-3p*-mediated down-regulation of NF-YB in CEM/VM-1-5 cells was accompanied by increased sensitivity of CEM/VM-1-5 cells to Top2 inhibitors.

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Discussion

In this study, we confirmed our previous observation that Top2α was downregulated in drug-resistant CEM/VM-1-5 and Rh30/v1 cells. Moreover, we found that Top2α downregulation was accompanied by upregulation of transcription factor NF-YB in resistant cells, compared to parental drug sensitive cells. Recent studies indicate that microRNAs are involved in mediating drug sensitivity and resistance. Therefore, based on microRNA profiling results and computer based microRNA target prediction programs, we studied the role of *miR-485-3p* that is differentially expressed in drug-sensitive and –resistant cells and has a putative target to the 3'-UTR of NF-YB. We first examined the repression effect of *miR-485-3p* to its target, NF-YB, by overexpressing *miR-485-3p* in the cells. We also confirmed, by luciferase assay, the binding of *miR-485-3p* to NF-YB 3'-UTR. Overexpressing *miR-485-3p* in CEM/VM-1-5 cells led to reduced expression of NF-YB, a corresponding upregulation of Top2α and increased sensitivity to the Top2 inhibitors.

Cell cycle-dependent regulation of Top2α expression is mediated in part by the transcription factor, nuclear factor-Y (NF-Y) (Magan et al., 2003), a conserved transcription factor that consists of three different subunits, NF-YA, NF-YB and NF-YC, and binds specifically to the CCAAT elements in the promoter (Ronchi et al., 1995). NF-Y has been reported to regulate the expression of several key cell cycle regulators, such as cyclin B1/B2 and cdc25 C (Manni et al., 2001). Some studies show that knocking-out of NF-YB causes early mouse embryo lethality (Bhattacharya et al., 2003)

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and overexpressing a dominant-negative mutant of NF-YA, which contains amino-acid substitutions within the DNA-binding domain, also inhibits cell proliferation and growth (Hu and Maity, 2000). Since we showed earlier (Morgan and Beck, 2001) that NF-YB may be a mediator of Top2α at inverted CCAAT element 3 (ICE3) in the Top2α promoter, and other studies also suggested the regulatory role for NF-Y in the Top2α promoter (Magan et al., 2003), we further examined the possibility that NF-YB causes downregulation of Top2α in the drug-resistant cells. We have now shown in the present study that the NF-YB protein is upregulated in the CEM/VM-1-5 cells that have decreased levels of Top2α compared to the drug sensitive CEM cells. We observed a similar result in the human rhabdomyosarcoma Rh30 cell line, and its etoposide-resistant subline, Rh30/v1, which also has downregulated Top2α protein compared to the drug sensitive parents, strongly suggesting that NF-YB is a negative regulator of Top2α. However, examination of Top2α and NF-YB gene expression levels in the National Cancer Institute (NCI)-60 panel of human tumor cancer cell lines (http://discover.nci.nih.gov/cellminer/) (Shankavaram et al., 2009) suggests that there is no significant overall correlation, negative or positive, between Top2α and NF-YB at the mRNA level (Fig.5). Some cell lines show a positive relationship between the two genes, others show a negative relationship, and yet others show an inverse relationship, as we have demonstrated here. The CEM cell line in this figure shows that both genes are overexpressed. One explanation for this apparent contradiction with our present results is that CEM cell line in the NCI-60 panel is diploid (Roschke et al., 2003) instead of near tetraploid as are our CEM and CEM/VM-1-5 cell lines (Kusumoto et al., 1996). By contrast, some of the cell lines showing an inverse relationship between Top2α and NF-

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YB are derived from solid tumors, and, of possible relevance to our results, one is in the drug resistant NCI/ADR-RES cell line. Moreover, examination of the genes of cell lines where both genes are inversely expressed (Fig. 5 middle group), the ratio of Top2α to NF-YB and the differential (opposite) expression of both genes tends to be higher than in the rest of the cells (i.e. the upper and lower groups in Fig. 5 in which genes are expressed in the same direction). We can speculate that the inverse relationship that we have observed herein and in those NCI-60 cells with inverse expression of these genes may reflect aspects of the biology of drug resistance. Clearly, examination of expression of these genes in more pairs of drug-sensitive and drug-resistant cell lines is warranted.

The mechanism underlying the differential expression of *miR-485-3p* in drug-resistant and –sensitive cells is not clear. Some studies suggest that epigenetic alterations (Grady et al., 2008), deregulation of miRNA processing factors (Melo et al., 2009) and chromosomal abnormalities (Zhang et al., 2006) can contribute to downregulation or upregulation of microRNAs. *MiR-485-3p* resides on chromosome 14q32.31, a region that includes *miR-127* (Saito et al., 2006), and *miR-370* (Meng et al., 2008) which has been suggested to be epigenetically regulated. Furthermore, chromosome 14q32.31 is a region where allelic deletions (Suzuki et al., 1989) and translocations (Avet-Loiseau et al., 2002) are frequently identified. Since microRNAs are expressed differently in drug sensitive versus drug resistant cells, and they have been shown to mediate drug response (Bourguignon et al., 2009; Mishra et al., 2007; To et al., 2008), studying the regulation of microRNAs during the emergence of drug resistance may reveal new therapeutic opportunities.

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In conclusion, we believe that ours is the first study to demonstrate that *miR-485-3p* expression can mediate etoposide sensitivity indirectly by fine-tuning Top2α expression through modification of NF-YB expression. Accordingly, *miR-485-3p* can be a putative therapeutic target to modulate etoposide resistance in tumor cells.

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Acknowledgements

We thank Ms. Martina Vaskova for outstanding administrative support. The abstract for the 2009 AACR conference (Abstract number 5514: He X *et al*., MicroRNA profiling in drug-resistant leukemic cells) provides details of the microRNA profiling experiments.

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Authorship Contribution

Participated in research design: Chen, He, Beck

Conducted experiments: Chen

Contributed new reagents or analytic tools: Mo

Performed data analysis: Chen, He, Mo, Arslan, Reinhold, Pommier, Beck

Wrote or contributed to the writing of the manuscript: Chen, Beck

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Molecular Pharmacology Fast Forward. Published on January 20, 2011 as DOI: 10.1124/mol.110.069633
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Footnotes

This work was supported in part by National Institutes of Health [Grant CA40570] and in part by University of Illinois at Chicago, and was conducted in a facility constructed with support from [Grant C06RR15482] from the National Center for Research Resources, National Institutes of Health.

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Legends for figures

Fig.1 Inverse relation between the protein levels of Top2α and NF-YB in CEM and CEM/VM-1-5 cells and Rh30 and Rh30/v1 cells. Western blots of nuclear Top2α and NF-YB expression in CEM and CEM/VM-1-5 cells (A) and Rh30 and Rh30/v1 cells (D). Proliferating cell nuclear antigen (PCNA) served as loading control for nuclear proteins. Average of Top2α and NF-YB levels from three independent experiments ± SD determined by densitometric scanning on Western blots, and normalized to PCNA signal; either CEM (B) or Rh30 (E) was assigned a value of 1 for comparison. ***p*<0.005, ****p*<0.0001. (C, F) NF-YB mRNA expression was analyzed by semi-quantitative RT-PCR in CEM and CEM/VM-1-5 cells (C) and Rh30 and Rh30/v1 cells (F).

Fig.2 NF-YB is a putative target for *hsa-miR-485-3p.* Expression of *hsa-miR-485-3p* in CEM and CEM/VM-1-5 cells was determined by Applied Biosystems real-time PCR array (TaqMan® Human MicroRNA Array v1.0.) according to the manufacturer's instructions. Threshold cycle (Ct) values (A) or relative expression levels of *miR-485-3p* (B) are means of three pairs of microRNA profile results from CEM and CEM/VM-1-5 cells ± SD. **P*<0.05, ***P*<0.01. (C) Sequence alignment of putative *has-miR-485-3p* binding sites in 3′-UTR of NF-YB gene of four species. The base pairing nucleotides are in bold.

Fig.3 *MiR-485-3p* targets NF-YB 3'-UTR. (A) Luciferase reporter containing a putative *miR-485-3p* binding site, NF-YB-3'-UTR (pGL3-NF-YB-3'-UTR), was co-transfected with

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either *miR-Crtl* (pCDH-empty vector) or the *miR-485-3p* expression vector (pCDH-*miR-485-3p*) with schematic diagram on the left. (B) *MiR-485-3p* expression vector (pCDH-*miR-485-3p*) was co-transfected with either luciferase reporter containing NF-YB-3'-UTR with or without putative *miR-485-3p* binding site which was defined as pGL3-NF-YB-3'-UTR or pGL3-NF-YB-3'-UTR-d respectively with schematic diagram on the left. Relative luciferase activities were measured and normalized against β-galactosidase activity. Values are average of three separate experiments done in triplicate ± SE. **p*<0.05

Fig.4 *MiR-485-3p* inhibition on NF-YB and mediation on drug responsiveness. Western blots of nuclear Top2α, NF-YB, and NF-YA expression in CEM/VM-1-5 (A) and Rh30/v1 cells (B). CEM/VM-1-5 and Rh30/v1 cells were transduced with either *miR-485-3p* expression virus or control virus (*miR-Crtl*). Proliferating cell nuclear antigen (PCNA) served as loading control for nuclear protein. IC_{50} (50% inhibitory concentration) values of cells exposed to etoposide (C) or vinblastine (D) at various concentrations were calculated from the percentage of viable cells after exposure to treatment obtained from MTT assay. Values are average of three independent experiments done in triplicate ± SE. **p*<0.05

Fig.5 NF-YB and Top2α mRNA expression levels in the NCI-60 panel of tumor cell lines. Shown is a graphical summary of NF-YB and Top2α mRNA levels in the form of a mean

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graph of the NCI-60 cells. Expression above the mean levels is drawn to the right of the centerline and below the mean is drawn to the left.

Fig.1

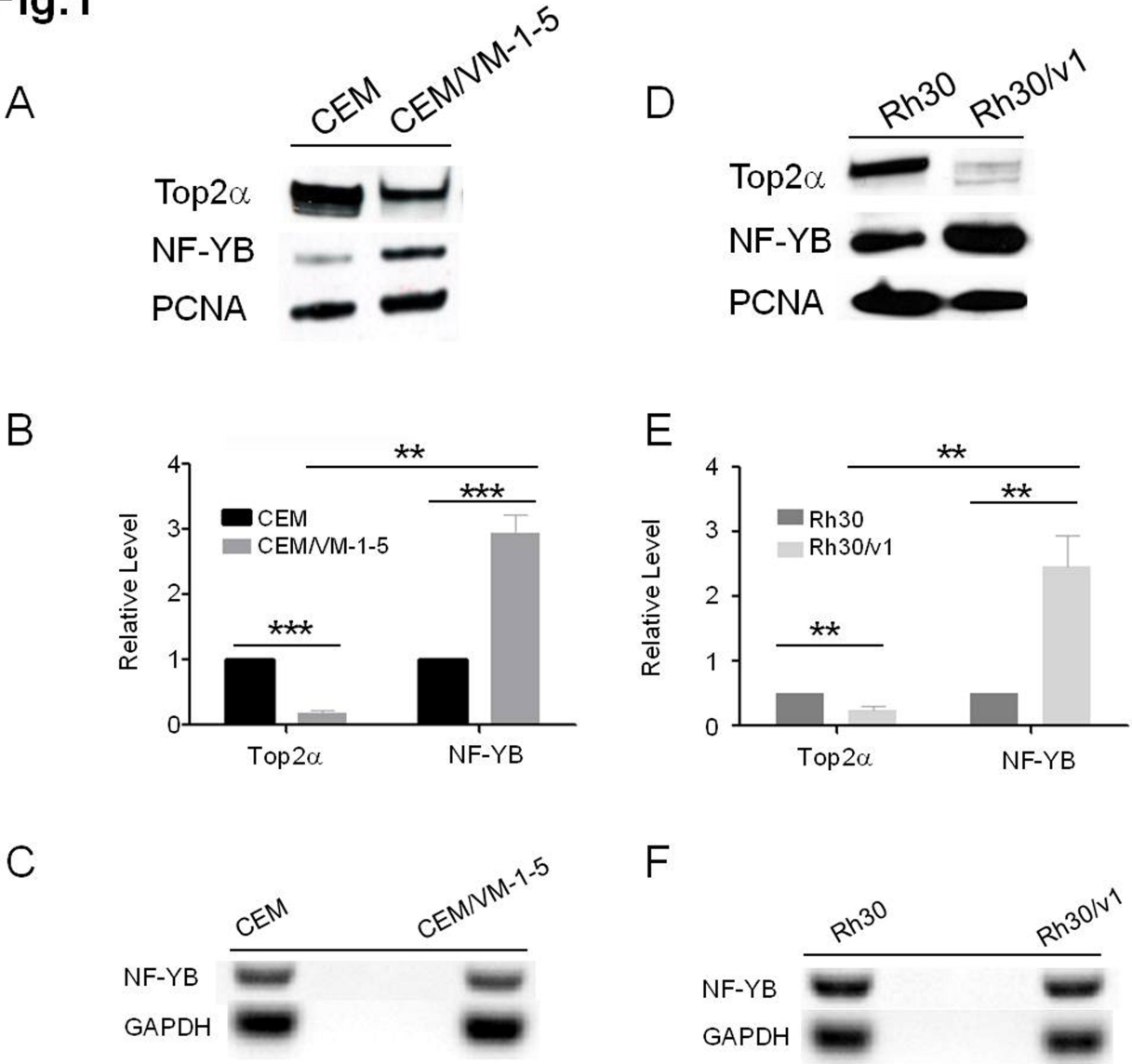
A
CEM
CEM/VM-1-5
Top2α
NF-YB
PCNA
D
Rh30
Rh30/v1
Top2α
NF-YB
PCNA
B
Relative Level
CEM
CEM/VM-1-5
**
***
***
Top2α
NF-YB
E
Relative Level
Rh30
Rh30/v1
**
**
**
Top2α
NF-YB
C
CEM
CEM/VM-1-5
NF-YB
GAPDH
F
Rh30
Rh30/v1
NF-YB
GAPDH

Fig.2

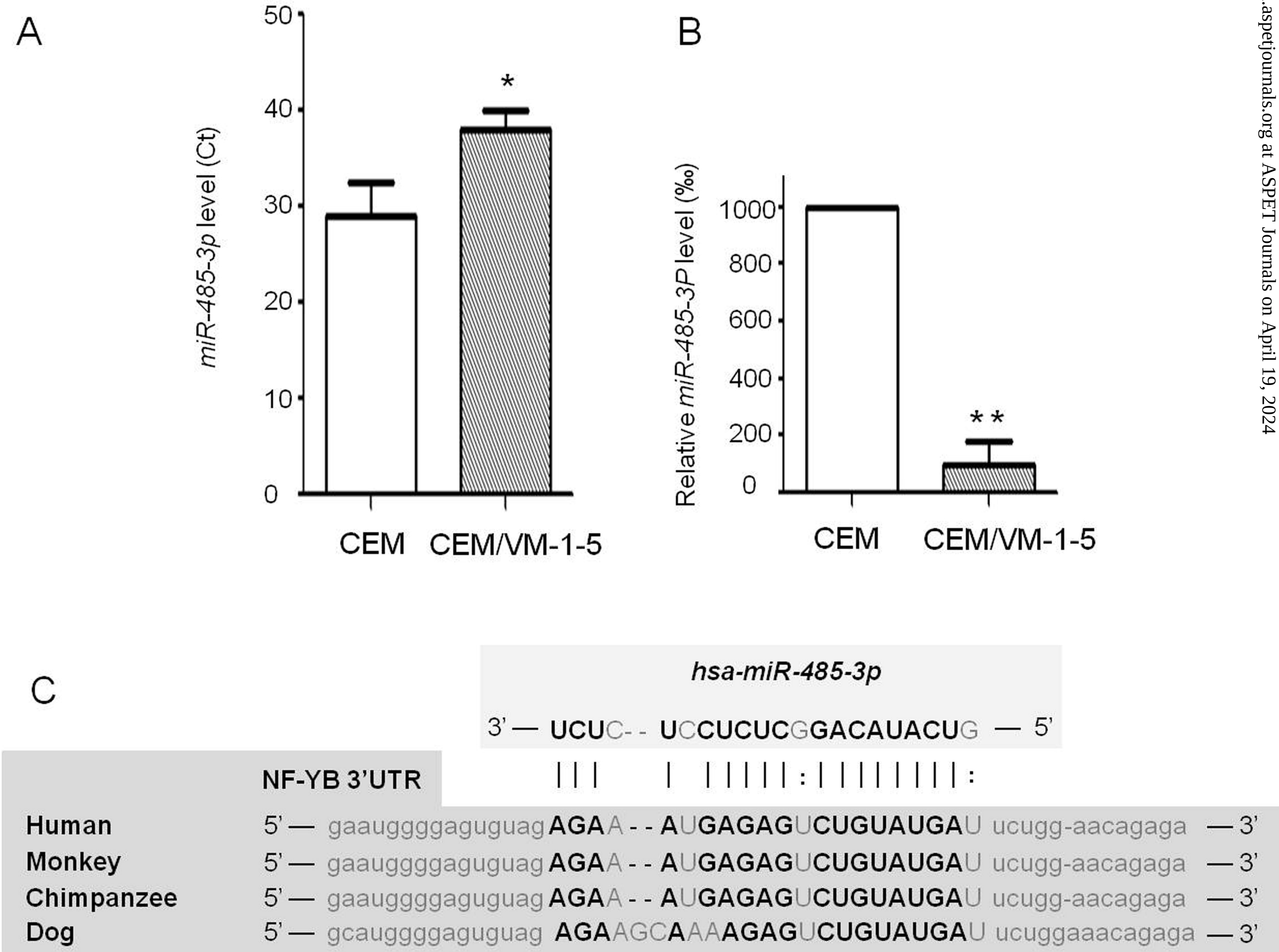

Fig.3

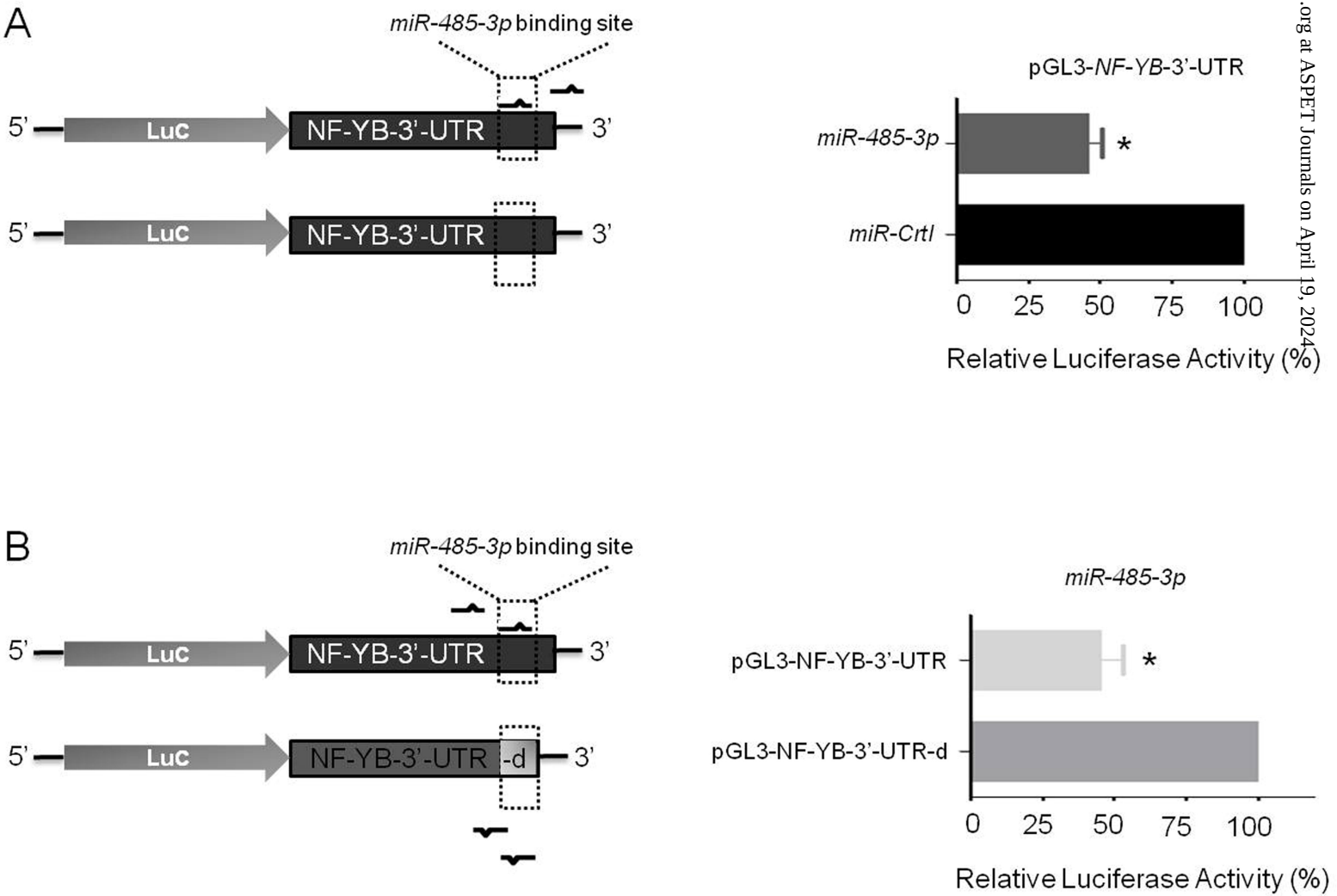

Fig.4

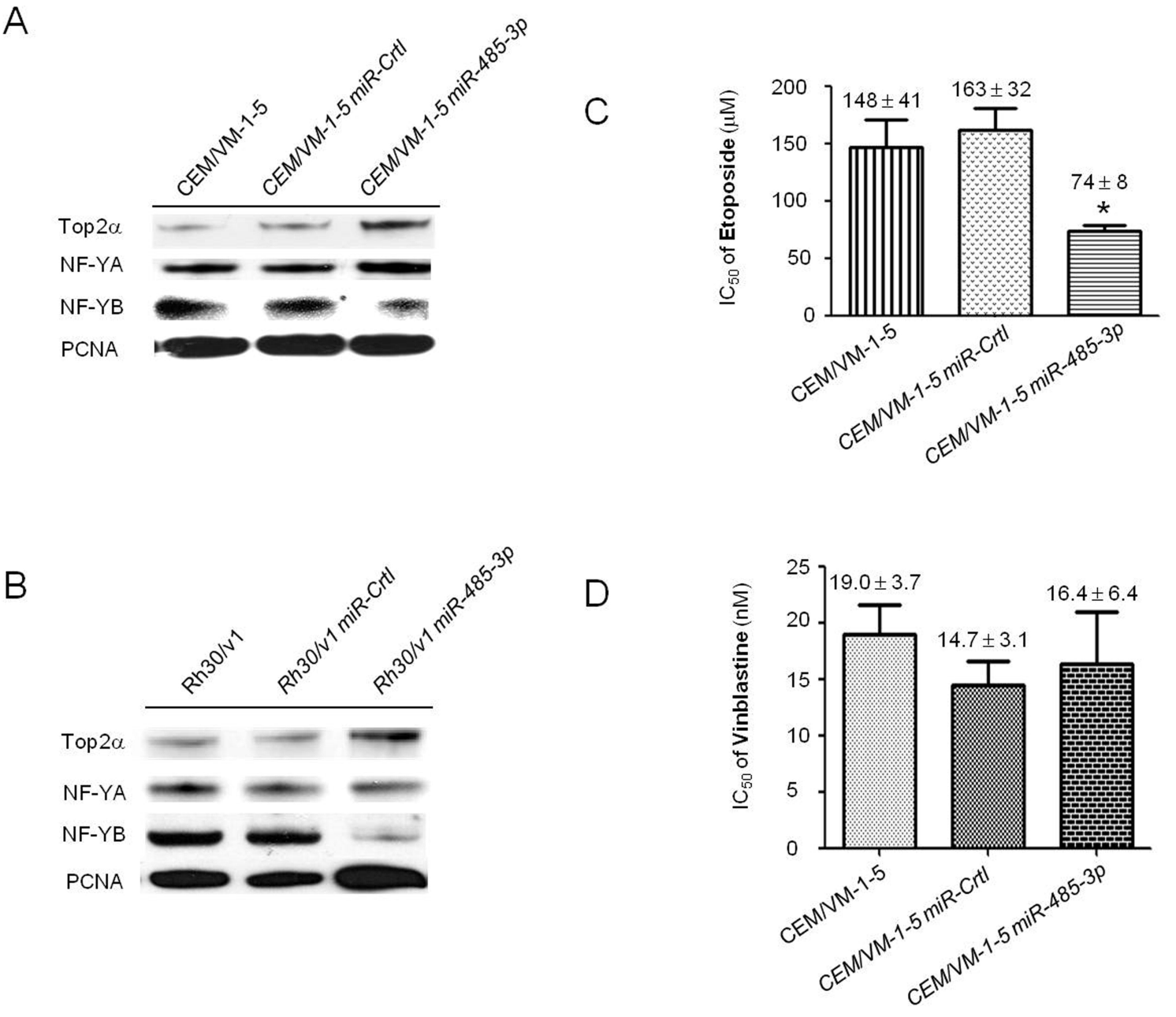
A
CEM/VM-1-5
CEM/VM-1-5 miR-Crtl
CEM/VM-1-5 miR-485-3p
Top2α
NF-YA
NF-YB
PCNA
B
Rh30/v1
Rh30/v1 miR-Crtl
Rh30/v1 miR-485-3p
Top2α
NF-YA
NF-YB
PCNA
C
IC50 of Etoposide (μM)
200
150
100
50
0
148 ± 41
163 ± 32
74 ± 8
*
CEM/VM-1-5
CEM/VM-1-5 miR-Crtl
CEM/VM-1-5 miR-485-3p
D
IC50 of Vinblastine (nM)
25
20
15
10
5
0
19.0 ± 3.7
14.7 ± 3.1
16.4 ± 6.4
CEM/VM-1-5
CEM/VM-1-5 miR-Crtl
CEM/VM-1-5 miR-485-3p

Fig.5

	cell lines	NF-YB	Top2α	
	CO:HCT_15	0.73	1.29	
	LE:CCRF_CEM	1.26	1.27	<<<<
	LC:HOP_62	0.68	1.17	
	CNS:SF_295	0.04	0.96	
	LC:NCI_H460	0.24	0.8	
	PR:DU_145	0.8	0.79	
	OV:SK_OV_3	0.81	0.69	
POSITIVE	LC:EKVX	0.03	0.59	
CORRELATED	LE:MOLT_4	1.68	0.56	
# 14	BR:MCF7	1.1	0.42	
	LC:NCI_H23	1.45	0.33	
	CO:SW_620	0.25	0.26	
	RE:SN12C	1.77	0.24	
	OV:OVCAR_8	1.24	0.13	
	LC:NCI_H226	2.27	-0.02	
	LE:HL_60	0.13	-0.06	
	RE:CAKI_1	1.04	-0.07	
	RE:ACHN	1.03	-0.13	
	CO:KM12	0.44	-0.14	
	LC:NCI_H322M	0.02	-0.14	
	BR:T47D	1.48	-0.18	
	CO:HCC_2998	0.06	-0.5	
	OV:OVCAR_3	0.09	-0.62	
	PR:PC_3	0.04	-0.71	
	RE:UO_31	0.52	-1	
	OV:NCI_ADR_RES	0.49	-2.92	<<<<
INVERSELY	CNS:SNB_19	-0.43	1.74	
CORRELATED	LC:A549	-0.13	1.62	
# 25	BR:MDA_MB_231	-0.11	1.53	
	CNS:SF_268	-0.27	1.43	
	OV:OVCAR_5	-0.83	1.32	
	CNS:SNB_75	-0.82	0.86	
	LC:HOP_92	-0.65	0.59	
	RE:786_0	-0.45	0.58	
	LC:NCI_H522	-0.4	0.49	
	CO:COLO205	-0.68	0.28	
	LE:SR	-0.13	0.27	
	BR:BT_549	-0.33	0.16	
	ME:M14	-0.8	0.07	
	ME:UACC_62	-1.04	-0.03	
	CNS:U251	-0.28	-0.11	
	CO:HT29	-0.22	-0.12	
	ME:LOXIMVI	-0.1	-0.13	
	ME:UACC_257	-0.51	-0.14	
	BR:HS578T	-0.78	-0.17	
	ME:MALME_3M	-0.01	-0.33	
	LE:K_562	-0.12	-0.35	
NEGATIVE	OV:IGROV1	-0.29	-0.36	
CORRELATED	CNS:SF_539	-0.8	-0.43	
# 21	RE:RXF_393	-0.6	-0.57	
	RE:TK_10	-0.44	-0.59	
	ME:MDA_N	-1.21	-0.71	
	ME:SK_MEL_2	-0.43	-0.95	
	ME:MDA_MB_435	-1.58	-1.03	
	RE:A498	-0.61	-1.04	
	ME:SK_MEL_28	-1.81	-1.15	
	LE:RPMI_8226	-1.38	-1.31	
	CO:HCT_116	-0.26	-1.37	
	ME:SK_MEL_5	-1.02	-1.69	
	OV:OVCAR_4	-0.61	-1.72