

Title Page

**DNA Methyltransferase Inhibitors Coordinately Induce
Expression of the Human Reelin and GAD67 Genes¹**

Marija Kundakovic, Ying Chen, Erminio Costa, Dennis R. Grayson*

The Psychiatric Institute

Department of Psychiatry

College of Medicine

University of Illinois at Chicago

1601 W. Taylor St.

Chicago, IL 60612

Running Title Page:

DNMT inhibitors induce the human reelin and GAD₆₇ genes.

***Corresponding Author:**

Dennis R. Grayson
Department of Psychiatry
College of Medicine
University of Illinois at Chicago
1601 W. Taylor St.
Chicago IL, 60612
Phone: 312-413-4577
Fax: 312-413-4569
Email: dgrayson@psych.uic.edu

Number Text Pages: 29

Number of Tables: 0

Number of Figures: 7

Words in Abstract: 242

Words in Introduction: 742

Words in Discussion: 1,492

Abbreviations: Ac-H3, acetyl histone H3; Ac-H4, acetyl histone H4; AZA, 5-aza-2'-

deoxycytidine; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; DOXO,

Doxorubicin; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GABA, γ -aminobutyric acid;

GAD67, glutamic acid decarboxylase 67; HDAC, histone deacetylase; MeCP2, methyl-CpG

binding protein 2; NT2, N-tera 2 neuronal progenitor cells; SAM, S-adenosyl-methionine; ZEB,

Zebularine.

Abstract

Reelin and GAD67 mRNAs and protein levels are substantially reduced in post-mortem brains of schizophrenia patients. Increasing evidence suggests that the observed down-regulation of reelin and GAD67 gene expression may be caused by dysfunction of the epigenetic regulatory mechanisms operative in cortical GABAergic interneurons. To explore whether human reelin and GAD67 mRNAs are coordinately regulated through DNA methylation-dependent mechanisms, we studied the effects of DNA methyltransferase inhibitors on reelin and GAD67 expression in NT-2 neuronal precursor cells. Competitive RT-PCR with internal standards was used to quantitate mRNA levels. The data showed that reelin and GAD67 mRNAs are induced in the same dose- and time-dependent manners. We further demonstrated that the activation of these two genes correlated with a reduction in DNA methyltransferase activity and DNMT1 protein levels. Time-course Western blot analysis showed that DNMT1 protein down-regulation occurs temporally prior to the reelin and GAD67 mRNA increase. In addition, ChIP assays demonstrated that the activation of the reelin gene correlates with the dissociation of DNMT1 and MeCP2 from the promoter, and an increased acetylation of histones H3 in the region. Collectively, our data strongly imply that human reelin and GAD67 genes are coordinately regulated through epigenetic mechanisms that include the action of DNMT1. Our study also suggests that negative regulation of the reelin gene involves methylation-dependent recruitment of DNMT1, MeCP2, and certain histone deacetylases, which most likely reduce the activity of the promoter by shifting the surrounding chromatin into a more compact state.

Introduction

It is now well established that disruption of epigenetic mechanisms can give rise to a variety of disorders in humans, including some that are associated with cognitive abnormalities (Egger et al., 2004; Levenson and Sweatt, 2005). For instance, mutations in the methyl-CpG binding protein MeCP2 are responsible for 90-95% of all cases of Rett syndrome, one of the most common causes of mental retardation in females (Weaving et al, 2005). These findings, together with studies using conditional DNA methyltransferase 1 (DNMT1) mutant mice, suggest that DNA methylation is essential for proper neuronal function (Fan et al., 2001; Tucker, 2001). It appears that DNA methylation may be an important mechanism associated with the dynamic regulation of genes expressed in neurons, especially those involved in synaptic plasticity, such as reelin and brain derived neurotrophic factor (Martinowich et al., 2003; Levenson et al, 2006).

Increasing evidence indicates that the dysfunctions seen in schizophrenia may be caused by an epigenetically induced down-regulation of GABAergic neuronal markers, such as reelin and glutamic acid decarboxylase 67 (GAD67) (Costa et al, 2004; Guidotti et al., 2005). In adult brain, reelin most likely plays an important role in synaptic plasticity, learning, and memory formation (Qiu et al, 2006). GAD67 is one of the two key enzymes involved in the synthesis of GABA (Guidotti et al., 2005). The decreases in reelin and GAD67 mRNA and protein levels are among the most consistently replicated findings reported in postmortem brains of schizophrenia patients (Guidotti et al., 2000; Fatemi et al., 2000; Eastwood and Harrison, 2003; Torrey et al., 2005). A recent study demonstrated that the same GABAergic neurons which express reelin and GAD67 exhibit an up-regulation of the mRNA that encodes DNMT1 (Veldic et al, 2004). We and others have also shown that the reelin promoter is hypermethylated in the brains of schizophrenia patients compared to control subjects (Abdolmaleky et al, 2005; Grayson et al,

2005). Collectively, these findings support our hypothesis that down-regulation of reelin, GAD67, and probably other mRNAs and proteins expressed in GABAergic neurons may be caused by mechanisms mediated through DNMT1-induced hypermethylation of the corresponding CpG island-containing promoters (Grayson et al, 2006).

We have already accumulated evidence showing that the human reelin gene is epigenetically regulated through changes in the methylation status of the promoter. Using NT-2 neuronal precursor cells, we have shown that the reelin promoter is more heavily methylated when the gene is silent (Chen et al., 2002). Activation of the reelin gene by various agents, including retinoic acid, the DNA methylation inhibitor 5-aza-2'-deoxycytidine (AZA), and histone deacetylase (HDAC) inhibitors valproic acid and trichostatin A, corresponds with a decrease in promoter methylation. In addition, induction of reelin expression is accompanied by alterations that suggest a more open chromatin structure. These changes include the appearance of DNase I hypersensitive sites and increased levels of Ac-H3 and Ac-H4 histones in the vicinity of the reelin promoter (Chen et al, 2002; Mitchell et al, 2005).

Studies in mice indicate that reelin and GAD67 genes may be coordinately regulated. Treatment with L-methionine, a precursor of the methyl donor S-adenosyl-methionine (SAM), induced the down-regulation of reelin and GAD67 mRNAs and proteins *in vivo* (Tremolizzo et al., 2002) and in primary neuronal cell cultures *in vitro* (Noh et al., 2005). This effect of methionine was attenuated by co-transfection of DNMT1 antisense oligonucleotides, providing a link between the expression of DNMT1 and the regulation of reelin and GAD67 genes (Noh et al., 2005). In addition, methionine treatment also induced an increased association of the methyl CpG-binding protein MeCP2 to mouse reelin and GAD67 promoters (Dong et al., 2005).

The aim of the current study was to evaluate the hypothesis that the human reelin and GAD67 genes are coordinately regulated by DNA methylation through the action of DNMT1. To address this, we used neuronal precursor cells (NT2), and treatments with three distinct DNA methyltransferase inhibitors. Doxorubicin (DOXO) has recently been shown to act as a potent inhibitor of DNMT1 activity, most likely acting through DNA intercalation (Yokochi and Robertson, 2004). AZA and zebularine (ZEB) are nucleoside analogues that after incorporation into replicating DNA form covalent bonds with DNA methyltransferases and inhibit their function (Egger et al., 2004). Our study strongly suggests that inhibition of DNA methylation and/or DNMT1 protein down-regulation lead(s) to coordinate reactivation of human reelin and GAD67 gene expression. This study also provides evidence that transcription of the human reelin gene is repressed by the methylation-mediated recruitment of DNMT1, MeCP2, and possibly other co-repressors, including certain HDACs.

Material and Methods

Cell culture and drug treatments. NT-2 cells (Stratagene, La Jolla, CA) were maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin, 1% streptomycin, and 1% L-glutamine. DOXO, AZA and ZEB were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of the drugs were prepared by dissolving the substances in either distilled water (DOXO), 50% acetic acid (AZA) or dimethylsulfoxide (ZEB), and stored at -20 °C. For all experiments, control measurements were obtained from vehicle-treated cells. For dose-response quantitative RT-PCR experiments, cells were treated for 48 h with following concentrations of DOXO; 10, 25, 50, 100, 250 and 1000 nM. Time-course mRNA experiments and time-course Western blot analyses were carried out after cells had been treated with 100 nM DOXO for 0, 3h,

6h, 12h, 24h, 36h or 48h. For DNA methyltransferase assays and dose-response Western blot analysis, cultures were either untreated, or treated with 100 nM or 250 nM DOXO for 48h, while for cell viability assays cells were additionally treated with 2 μ M DOXO for 48 h. Chromatin immunoprecipitation assays and non-quantitative RT-PCR assay for GAD65 were performed with vehicle-treated cells and cells treated with 250 nM DOXO for 48h. For both quantitative RT-PCR experiments and Western blot analysis, cells were treated with 5 μ M AZA for 48h, and 500 μ M ZEB for either 48h or 48h followed by 48h-incubation with untreated medium.

Nuclear extracts. Nuclear extracts of untreated and treated NT-2 cells were obtained using NEPER Nuclear and Cytoplasmic Extraction kit as recommended by the manufacturer (PIERCE Biotechnology, Rockford, IL). The protein concentrations in the extracts were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Quantitative RT-PCR analysis. RNA was isolated following ultracentrifugation through CsCl (Chen et al, 2002). Reelin, GAD67, G3PDH, and DNMT1 mRNA contents were measured by competitive RT-PCR with internal standards as previously described (Grayson and Ikonovic, 1999). Primers were designed to minimally cross at least one exon/intron boundary. For example, the 5' Dnmt1 primer resides in exon 23 while the 3' primer was taken from exon 27 (Ramchandani et al., 1998). For measuring reelin mRNA (Genbank #NM_005045), the internal standard was generated by deleting 160 bp in the middle of the 674 amplicon using overlap-extension PCR (Auta et al., 2006). PCR was conducted using the forward primer (+2344) 5'-ATCCGTGGTGCTGAAGTCAGCTTT-3' and the reverse primer (+3018) 5'-TGAGTACTCCAGCTTCACCTGGTT-3' (annealing T=68°C, 30 cycles). For GAD67 mRNA (Genbank #M81883), the internal standard was generated by deleting 74 bp of the 414 bp amplicon, and the following primers were used for PCR: the forward primer (+1855) 5'-

CTTCCAGCCAGACAAGCAGTATGA-3' and the reverse primer (+2269) 5'-TGGGTTGGAGATGACCATCCGGAA-3' (annealing T=60°C, 30 cycles). For G3PDH (Genbank #: BC083511), the internal standard was generated by deleting 216 bp of the 683 bp amplicon, and PCR was carried out using the forward primer (+237) 5'-CTGAGAACGGGAAGCTTGTCATCA-3' and reverse primer (+920) 5'-TGTCGCTGTTGAAGTCAGAGGAGA-3' (annealing T=60°C, 30 cycles). For measuring DNMT1 mRNA (Genbank #BC092517), the internal standard was generated by deleting 196 bp of the 509 bp amplicon, and PCR was carried out using the forward primer (+2228) 5'-AATCGCATCTCTTGGGTCGGAGAA-3' and the reverse primer (+2737) 5'-ACGGGCACAGCTCACACAGAATTT-3' (annealing T=65°C, 30 cycles). The following primers were used for the PCR amplification of GAD65 cDNA (Genbank #NM_000818): the forward primer (+989) 5'-TTTCTCTCAAGAAGGGAGCTGCAG-3' and the reverse primer (+1788) 5'-GGGTTGGTAGCTGACCATTGTGG-3' (annealing T=60°C, 34 cycles).

DNMT assay

To measure DNA methyl transferase activity we used a modification of a previously published method (Szyf et al., 1991). A typical methylation reaction (30 μ l) contained 1 μ g oligonucleotides [Poly(dI-dC)-poly(dI-dC)] (GE Healthcare Bio-Sciences, Piscataway, NJ), an appropriate volume of nuclear extract containing 13 μ g of protein and 12.2 nM S-adenosyl-L-[methyl-3H] methionine (specific activity 82 Ci/mmol, GE Healthcare Bio-Sciences, Piscataway, NJ) in reaction buffer (20 mM Tris, pH 7.6, 25% glycerol, 10 mM EDTA, 28 mM 2-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride). The reaction mixtures were incubated at 37°C for 3 h, followed by incubation at 65°C for 10 min. Afterwards, 1 ml of 10% trichloroacetic acid was added and samples were incubated overnight at 4°C. Mixtures were then

filtered through Whatman GF/C glass microfiber filters and washed twice with 2 ml trichloroacetic acid. Filters were immersed in 3 ml of scintillation cocktail (ScintiVerse, Fisher Scientific, Pittsburgh, PA) for radioactivity counting.

Western blots. Nuclear extract proteins were separated on 4-20% (DNMT1) or 10-20% (MeCP2) Tris-Glycine gels and transferred overnight (DNMT1) or for 2 h (MeCP2) to nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membranes were blocked with PBS/Tween-20 (0.1 %) containing 5% nonfat dry milk for 1 h, followed by an overnight incubation at 4°C with DNMT1 polyclonal antibody (1:1000 dilution; New England Biolabs, Ipswich, MA) or MeCP2 polyclonal antibody (1:500 dilution, Abcam, Cambridge, MA). Membranes were then rinsed three times in PBS and incubated with peroxidase-labeled secondary antibody (1:3000, GE Healthcare Biosciences, Piscataway, NJ). Immunoreactive bands were visualized using the ECL plus Western blotting detection system (GE Healthcare Biosciences, Piscataway, NJ). The intensity of β -actin immunofluorescence was determined on the same blots using β -actin monoclonal antibodies (1:5000 dilution; Sigma-Aldrich, St. Louis, MO) and the corresponding signals were used for a comparative estimation of the amounts of protein applied to the gels. Blots were scanned and bands were visualized using a Storm 860 Phosphor Imager (Molecular Dynamics, Sunnyvale CA). Band intensities were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale CA).

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using the ChIP assay kit protocol (Upstate, Lake Placid, NY) as previously described (Mitchell et al., 2005). Briefly, 10^7 non-treated or DOXO-treated cells were fixed using 1% formaldehyde at room temperature for 10 min. Cells were washed twice in ice-cold PBS, resuspended in SDS-lysis buffer and sonicated until crosslinked chromatin was sheared to an average DNA fragment

length of 200-800 bp. 5% of the sonicated lysate was used to quantitate the total amount of DNA present in different samples before immunoprecipitation (inputs). Chromatin preparations were immunoprecipitated using anti-DNMT1 monoclonal antibody (Imgenex, San Diego, CA), anti-MeCP2 and anti-acetyl-histone polyclonal antibodies (Upstate, Lake Placid, NY). Non-immunoprecipitated samples were used as negative controls. Precipitated complexes were bound to protein G Agarose, washed and then eluted in 1% SDS/0.1M NaHCO₃. Cross-linking between DNA and proteins was reversed by heating the samples at 65°C overnight, followed by Proteinase K digestion at 65°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and 4 µl of 20 µl sample was analyzed by PCR. The primers for the reelin promoter region were 5'-CCGGGACACGTGTGGCGGCG-3' (forward, -220 bp) and 5'-AAAGCGGGGGTAATAGCCAGCCGC-3' (reverse, + 262 bp). The protocol included an initial denaturation cycle (5 min, 94°C), 40 cycles of denaturation (1 min, 94°C), annealing (1 min, 62°C) and extension (1 min, 72°C), followed by the final extension cycle (7 min, 72°C). For the β-globin locus control region, the forward primer (+3961 bp) was 5'-AGACACTTGCTCTTTCCAGGACTT-3', while the reverse primer (+4250 bp) was 5'-TGCCAGTATATGTGCTTCGATAGG-3'. The amplification included an initial denaturation cycle (5 min, 94°C), 40 cycles of denaturation (1 min, 94°C), annealing (1 min, 55°C) and extension (1 min, 72°C), followed by the final extension cycle (7 min, 72°C). PCR amplification products were separated on 1.6% agarose gels and optical density readings were determined using a computer-assisted densitometry program (Kodak EDAS 290, Eastman Kodak Co., Rochester, NY). For all experiments, input and immuno-precipitated DNA samples were below saturation levels following PCR.

Cell viability assays. Cell cultures were treated with vehicle containing medium, or medium supplemented with 100 nM, 250 nM or 2 μ M DOXO. After 48 h, medium was removed and replaced with control medium containing 50 μ M propidium iodide (a marker of cell damage) and 1 μ M calcein-AM (a marker of cell viability). After 10 min. of incubation, the fluorochrome-containing medium was removed and replaced with control medium and cell density and viability were examined by fluorescence microscopy.

Statistical Analyses. All experimental results are expressed as mean \pm SEM of three independent experiments (a minimum of 3 separate measurements were obtained per experiment). Student's t test (for ChiP results) and one-way ANOVA followed by the Bonferroni multiple comparison test (for all other results) were used to assess significance of the differences between groups. Analyses were conducted using SigmaStat software (SysStat, Richmond, CA). In addition, dose-response curves for reelin and GAD67 gene induction were obtained using GraphPad Prism Version 4 (GraphPad Software, San Diego, CA).

Results

DOXO increases reelin and GAD67 gene expression in a similar dose- and time-dependent manner. The initial step in our study was to explore whether treatment of human neural progenitor (NT2) cells with DOXO, a drug that acts as a DNMT1 inhibitor, would lead to changes in reelin and GAD67 mRNA expression. In NT-2 cells, background levels of reelin mRNA are barely detectable (0.010 ± 0.0015 pg reelin mRNA/ μ g total RNA), while these cells show significant expression of GAD67 mRNA (0.12 ± 0.013 pg GAD67 mRNA/ μ g total RNA). We observed a dose-dependent increase in both reelin and GAD67 mRNA levels following 48 h of DOXO treatment (Figs. 1A, 1B). The data showed that expression of the reelin mRNA increased up to 92 fold, where a maximal response was achieved using 250 nM DOXO ($0.92 \pm$

0.15 pg reelin mRNA/ μ g total RNA). Similarly, 250 nM DOXO led to a 20-fold (maximal) induction of GAD67 mRNA (2.5 ± 0.23 pg GAD67 mRNA/ μ g total RNA). To demonstrate the specificity of the reelin and GAD67 mRNA induction by DOXO, we examined the expression of two additional genes, G3PDH and GAD65, following the same treatment. In contrast to the changes in reelin and GAD67 mRNAs, G3PDH mRNA levels were not significantly changed independent of the concentration of drug used (Figs. 1A, 1B). Similarly, GAD65 mRNA, which is not expressed in NT-2 cells at readily detectable levels, was not induced even with the DOXO treatment that maximally activated reelin and GAD67 mRNAs (Fig. 1D). We next constructed dose-response curves to compare EC_{50} values for reelin and GAD67 mRNA induction in response to DOXO. These analyses revealed the EC_{50} values to be 102 nM and 103 nM for reelin and GAD67 mRNA increases, respectively (Fig. 1C). Both the maximal induction at 250 nM concentration of the drug together with the nearly equal EC_{50} values for these mRNAs, suggest that reelin and GAD67 are activated in similar dose-dependent manner.

To explore the time frame in which changes in reelin and GAD67 mRNA expression occur, NT-2 cells were treated with 100 nM DOXO (EC_{50} value) for various lengths of time. Data from these experiments showed that both reelin and GAD67 mRNA levels were increased in a similar temporal manner as well (Fig. 2A, 2B). Most importantly, the initial induction of both mRNAs occurred 12 h after initiating drug treatment (Fig. 2B). As measured by competitive RT-PCR, incubation of NT-2 cells with 100 nM DOXO for 12 h increased reelin mRNA levels 5.3 fold (from 0.019 ± 0.003 to 0.1 ± 0.002 pg reelin mRNA/ μ g total RNA) and GAD67 mRNA levels 4 fold (from 0.15 ± 0.015 to 0.63 ± 0.057 pg GAD67 mRNA/ μ g total RNA). Together with the dose-response study, these results suggest that DOXO treatment leads to a coordinated up-regulation of reelin and GAD67 mRNA expression.

Induction of reelin and GAD67 genes is associated with reduced DNMT enzymatic activity and decreased DNMT1 protein levels. The next goal was to examine whether DOXO inhibits DNA methyltransferase enzyme activity in the same concentration range in which it induces changes in reelin and GAD67 mRNA levels. For this purpose, we employed an *in vitro* enzymatic assay using nuclear extracts prepared from non-treated and DOXO-treated NT-2 cells. Extracts were used to measure methyltransferase activity with [³H]-S-adenosylmethionine and an artificial DNA substrate. The data showed that 100 nM and 250 nM DOXO treatment of NT2 cells (48 h) resulted in a significant 70% and 83% inhibition of nuclear DNMT activity, respectively (Fig. 3).

To determine if the reduction of DNMT enzymatic activity was, at least partly, due to decreased DNMT1 protein levels, we performed Western blot analyses. We observed a significant down-regulation of nuclear DNMT1 protein following 100 nM and 250 nM DOXO treatments (73% and 83%, respectively, Figs. 4A, 4B). Although DNMT1 protein is predominantly localized in the nucleus of NT-2 cells, a small cytoplasmic fraction exists. The cytoplasmic DNMT1 protein showed a similar trend toward decrease following DOXO treatment (data not shown). To assess whether DNMT1 mRNA also showed a similar decrease, we examined the expression of DNMT1 mRNA under the same conditions. The corresponding mRNA was not reduced by 100 nM and 250 nM DOXO (Figs. 4C-D), implying that DOXO-induced DNMT1 protein down-regulation is a post-transcriptional event. Since it was of interest to determine if the decrease in DNMT1 protein levels paralleled temporally the induction of reelin and GAD67 mRNAs, we performed time-course Western blot analysis. As shown in Figs. 4E-F, 100 nM DOXO treatment led to a time-dependent decrease in DNMT1 protein levels. The

reduction of DNMT1 protein was apparent as early as 6 h following drug treatment (78% of the control levels), while the amount of the protein dropped by half after 24h.

AZA and ZEB treatments induce reelin and GAD67 gene expression associated with DNMT1 protein depletion. To confirm that reelin and GAD67 mRNAs are coordinately regulated through methylation-dependent mechanisms, we employed two additional methylation inhibitors, AZA and ZEB (Fig. 5A-D). NT-2 cells were treated with 5 μ M AZA for 48h, since it was previously shown that in the same cell system this treatment leads to a significant increase in reelin mRNA levels (Chen et al, 2002), along with a decrease in reelin promoter methylation (Mitchell et al, 2005). As expected, AZA treatment increased reelin mRNA levels around 11 fold (from 0.0095 ± 0.0018 pg to 0.10 ± 0.015 reelin mRNA/ μ g total RNA). However, in the current study, we showed that the same AZA treatment also led to a 10-fold induction of GAD67 mRNA levels (from 0.12 ± 0.011 to 1.25 ± 0.13 pg GAD67 mRNA/ μ g total RNA). We also performed a dose-response study with ZEB, treating NT-2 cells with the following concentrations for 48 h; 0.5, 5, 50 and 500 μ M. As reported by another group using different cell lines, low concentrations of ZEB failed to induce their gene of interest (Cheng et al, 2004). Similarly, we found no changes in either reelin or GAD67 mRNAs at low concentrations of ZEB (data not shown). However, at 500 μ M of ZEB treatment (48 h), the expression of reelin mRNA was increased around 9 fold (0.09 ± 0.004 reelin mRNA/ μ g total RNA), while GAD67 mRNA levels were elevated 2.5 fold (0.32 ± 0.03 pg GAD67 mRNA/ μ g total RNA). Since the previous study (Cheng et al., 2004) suggested that ZEB induced the demethylation of the p16 gene with a 2 day delay, we also treated NT-2 cells with 500 μ M ZEB for 48 h and then allowed cells to grow in a fresh medium containing no-drugs for an additional 48 h before RNA isolation (2+2 D). This treatment led to a more significant induction of both genes, with an almost 20-fold increase in

reelin (0.19 ± 0.02 pg reelin mRNA/ μ g total RNA) and 12.5-fold increase in GAD67 mRNA levels (1.6 ± 0.05 pg GAD67 mRNA/ μ g total RNA). As in the case of DOXO treatment, neither AZA nor ZEB treatments were associated with significant changes in G3PDH mRNA levels (Fig 5B). However, all three treatments led to an almost complete depletion of DNMT1 protein, as shown by Western blot analysis (Figs. 5C-5D).

Activation of reelin mRNA by DOXO is accompanied by the dissociation of DNMT1 and MeCP2 and increased histone acetylation from the promoter region. We next sought to understand the mechanisms by which DNMT inhibitors induce the reelin and GAD67 genes. We examined changes at the level of the reelin promoter, since we had previously established cis-regulatory elements that are operative in its regulation (Chen et al., 2002). We also have strong evidence showing that the reelin promoter in NT-2 cells is silenced by methylation, while the activation of the reelin gene corresponds with a decreased methylation of the promoter. Using ChIP assays, we explored the possibility that inhibition of methylation results in a release of repressor proteins from the reelin promoter. Proteins were first cross-linked and chromatin was sonicated to an average DNA fragment size from 200 to 800 bp (Fig. 6A). Using specific antibodies for immunoprecipitation, we examined the association of MeCP2 and DNMT1 proteins with the promoter both before and after 250 nM 48 h DOXO treatment. ChIP data showed that DNMT1 and MeCP2 are bound to the reelin promoter in untreated NT-2 cells (Fig. 6B-C). In contrast, the induction of reelin mRNA by DOXO corresponded with a dissociation of these proteins from the promoter region (Fig. 6B-C). By using acetyl H3 pull down assays, we further explored whether changes in DNMT1 and MeCP2 binding were accompanied by changes in the acetylation status of histone H3 in the vicinity of the promoter. As shown (Fig. 6B-C), DOXO significantly increased the amount of acetyl H3 histone associated with the same region,

implying that this treatment also alters chromatin structure in the vicinity of the reelin gene. The specificity of these changes with the reelin promoter was demonstrated by amplifying the β -globin promoter region in parallel after pull-down assays using the same antibodies. As expected for a gene that is not epigenetically regulated, none of the examined proteins was bound to the β -globin promoter either before or following DOXO treatment (Fig. 6B). Lack of DNMT1 association with the reelin promoter correlated with almost complete depletion of DNMT1 protein from NT-2 cell nuclear extracts following 250 nM 48 h-DOXO treatment. However, neither 100 nM nor 250 nM 48 h-DOXO treatment led to significant changes in MeCP2 protein levels (Figs. 6D and E).

100 nM and 250 nM 48h-DOXO treatments are not associated with significant NT-2 cell loss. Besides acting as a DNMT1 inhibitor, DOXO can act as a DNA damaging agent that activates p53 and induces apoptosis (Esteve et al, 2005). Previous studies using HCT116 cells showed that only at a 1×10^{-6} M concentration (and not lower), DOXO induced significant cell death which was related to apoptosis (Yokochi and Robertson, 2004). Here we report that maximal induction of reelin and GAD67 genes was associated with 250 nM DOXO. As compared to EC₁₀₀ treatment, the 1 μ M treatment was associated with slightly reduced reelin and GAD67 mRNA levels (Fig. 1B), which could be explained by the effect of DNA damage at that concentration of DOXO. To confirm that reelin and GAD67 gene induction is not related to apoptosis, we performed cell viability assays following 100 nM, 250 nM and 2 μ M (48h) DOXO treatments. As shown in Fig. 7, 100 nM and 250 nM DOXO treatment was not associated with significant NT-2 cell death. However as anticipated, 2 μ M DOXO induced considerable cell loss due to apoptosis and possibly to necrosis.

Discussion

The data presented here clearly demonstrated that both reelin and GAD67 mRNA expression was significantly induced by three different DNMT inhibitors, namely DOXO, AZA, and ZEB. Most importantly, the detailed study with DOXO showed that this induction occurs: a) in a similar dose-dependent manner (as shown by the same EC₅₀ and EC₁₀₀ values for the induction of both mRNAs); and b) within the same time frame (both mRNAs begin to be induced after ~12h). The similar concentration-dependent and temporal activation patterns of the reelin and GAD67 mRNAs strongly support our hypothesis that these two genes are coordinately regulated. Moreover, the finding that DOXO inhibits DNA methyltransferase activity in the same concentration range that induces reelin and GAD67 mRNA expression provides additional evidence that reelin and GAD67 genes are activated epigenetically. However, the assay we used measures total DNA methyltransferase activity, and likely reflects the activities of so-called maintenance methyltransferase (DNMT1), as well as the activity of de novo methyltransferases (DNMT 3A and DNMT 3B). Using recombinant DNMT1 protein, Yokochi and Robertson, 2004 previously showed that DOXO inhibits the enzymatic activity of DNMT1. As indirect evidence that this drug inhibits DNMT1 enzymatic activity under the conditions that we applied, we showed that the reduction of total DNMT activity is highly correlated with the reduction in DNMT1 protein levels following the same DOXO treatment. Furthermore, AZA and ZEB treatments, which induced reelin and GAD67 mRNAs, led to a complete depletion of nuclear DNMT1 protein. Consistent with this, a previous study of ours demonstrated that the knock-down of DNMT1 protein is associated with an up-regulation of mouse reelin and GAD67 mRNA levels in cortical neurons in vitro (Noh et al, 2005). Taken together, these data imply a possible role for DNMT1 in the coordinated regulation of the reelin and GAD67 genes. Having said this,

we cannot exclude the possibility that DNMT 3A and/or 3B might also play a role in these events.

In dividing cells such as NT-2 cells, DNMT1 is thought to be mainly involved in the methylation of hemimethylated DNA. This process predominates during DNA replication. Accordingly, we anticipate that drugs which inhibit DNMT1 enzymatic activity, such as DOXO, AZA and ZEB, may require several cell divisions to induce significant changes in promoter methylation status along with changes in mRNA expression (Egger et al, 2004). Interestingly, we observed an induction of both reelin and GAD67 mRNAs as early as 12 h after initiating DOXO treatment. In contrast, 20-24 h are needed to complete one cell cycle. This observation led us to consider a possible co-repressor role of DNMT1 protein in regulating reelin and GAD67 mRNA expression. It has been shown that DNMT1 represses gene transcription through its non-catalytic domain independent of its methyltransferase function. This action occurs through the recruitment of MeCP2 and HDACs (Fuks et al., 2000; Kimura and Shiota, 2003; Burgers et al, 2002). As an example, it has recently shown that DNMT1 can suppress the activity of the metallothionein-I gene promoter regardless of its methylation status (Majumder et al., 2006). To explore if this is also the case with the reelin and GAD67 genes, we first checked the time frame of DNMT1 protein down-regulation. Strikingly, DNMT1 protein levels start to decrease 6 h before the induction of reelin and GAD67 mRNAs occurs. Our data demonstrated that the down-regulation of DNMT1 occurs post-transcriptionally, since we show that DOXO does not induce changes in DNMT1 mRNA levels. It seems likely that DNMT1 may get trapped in a DOXO-DNA complex, which subsequently targets DNMT1 for degradation (Yokochi and Robertson, 2004). This event can be replication-independent, since it has been shown that in addition to S phase, DNMT1 is continuously loaded onto chromatin during the G2 and M phases of the cell cycle (Easwaran et

al, 2004). In support of our finding, another group reported that 2 h of AZA treatment is sufficient to induce significant replication-independent reduction in DNMT1 protein levels (Ghoshal et al., 2005). Furthermore, we demonstrated that DNMT1 protein decreases in a time-dependent fashion, very similar to that seen for the increases in reelin and GAD67 mRNA levels.

As additional evidence for the co-repressor role of DNMT1, we showed (using ChIP assays) that this protein is bound to the reelin promoter when the gene is silent or transcriptionally inactive. In contrast, the maximal induction of the reelin mRNA is accompanied by a complete dissociation of DNMT1 from the promoter regulatory region. These data suggest that DNMT1 is involved in keeping the reelin promoter in a repressed state in NT-2 cells. It seems likely that the slight activation of the reelin (and probably GAD67) gene(s), seen 12 h after beginning DOXO treatment, is triggered by decreased amounts of DNMT1 in these cells and the subsequent decreased binding of DNMT1 to the reelin promoter. This, in turn, might lead to the release of the repressor complex from the reelin promoter. Evidence suggests that this repressor complex likely includes MeCP2 and certain HDACs. Recently it has been shown that the down-regulation of reelin and GAD67 mRNAs corresponds with increased recruitment of MeCP2 to the mouse reelin and GAD67 promoters (Dong et al., 2005). Here we demonstrate that the maximal activation of the reelin gene by DOXO is associated with the dissociation of MeCP2 from the promoter region and an increase in H3 histone acetylation in the vicinity of the promoter. Since MeCP2 binds specifically to methylated cytosines, our data also suggest that 48 h of DOXO treatment induces changes in the methylation status of the reelin promoter. This conclusion is strengthened by the finding that unlike DNMT1, MeCP2 protein levels did not change after the same treatment. We have previously shown that all treatments that induce reelin expression, including AZA, also decrease reelin promoter methylation (Chen et al, 2002; Mitchell et al,

2005). However, as previously noted, it seems likely that the 12 h treatment which produces a slight induction of reelin and GAD67 mRNAs, may not be sufficient to induce changes in promoter methylation status. Accordingly, we suggest that promoter methylation per se may not be sufficient to keep the reelin promoter in a fully repressed state. Complete silencing of the reelin promoter probably requires the fully assembled repressor complex and highly condensed chromatin maintained by the recruitment of DNMT1. Therefore, we believe that the reelin promoter must be demethylated for the maximal activation of the gene to occur. Additional studies are needed to confirm this speculation.

In conclusion, we would like to highlight several implications of these data. First, the study suggests a mechanism by which reelin and GAD67 mRNAs might be coordinately regulated in GABAergic neurons of the adult brain. It seems likely that both genes may be regulated by methylation of the corresponding promoters. DNMT1 probably has a dual role in this process. One could be its well-established enzymatic (DNA methyltransferase) role, by which it controls the methylation status and the activity level of the reelin, GAD67, and possibly other epigenetically-regulated promoters. Another role of DNMT1 could be to participate in the formation of the transcriptional repressor complex, by recruiting MeCP2, HDACs, and other co-repressors. This may lead to the generation of a more condensed chromatin structure that subsequently limits promoter accessibility. As mentioned above, while we have focussed on the role of DNMT1 in this process, we cannot exclude a contributing role for either DNMT 3A and or DNMT 3B.

Second, this study gives new insight into the molecular mechanisms that underly the down-regulation of reelin and GAD67 mRNAs in the brains of schizophrenia patients. We propose that the reported up-regulation of DNMT1 (Veldic et al., 2004) leads to the hypermethylation and

increased binding of DNMT1 to the reelin and GAD67 promoters. Furthermore, we suggest that there is a subsequent increased recruitment of MeCP2, HDACs, and possibly additional co-repressor proteins. However, studies with postmortem human brains will be necessary to confirm this hypothesis. Third, we would like to suggest a new approach in the treatment of schizophrenia that focuses on the reactivation of expression of genes that are down-regulated due to modifications in the epigenome. Thus far, epigenetic drugs (DNA methylation inhibitors and HDAC inhibitors) have been used in cancer treatment, since they often times selectively reactivate tumor suppressor genes that are silenced by CpG island promoter methylation (Egger et al, 2004). It seems likely that this may be one of the mechanisms that contributes to the therapeutic benefits of DOXO in some types of cancer. However, of specific interest in the context of schizophrenia research, we report that DOXO concentrations that do not induce significant cell death lead to a robust induction of the reelin and GAD67 mRNAs. Furthermore, our data suggest that DOXO induces changes in the methylation status of the reelin promoter, which has been shown to be hypermethylated in the brains of schizophrenia patients (Abdulmaleky et al., 2005; Grayson et al., 2005). While this remains to be addressed experimentally, the changes in methylation most likely occur only at specific promoters, since another group reported no changes in global methylation of genomic DNA after DOXO treatment (Yokochi and Robertson, 2004). We propose that drugs that induce promoter hypomethylation and/or DNMT1 down-regulation might be useful in correcting the reelin and GAD67 mRNA insufficiencies associated with schizophrenia. This means that DNMT1 and HDACs may represent possible new molecular targets to treat schizophrenia patients. At the same time, since many of these drugs are toxic to cells and may have global effects in the

nervous system that have yet to be determined, the safety of these compounds needs to be fully tested in animal models prior to adopting their use in humans.

References

- Abdolmaleky HM, Cheng K, Russo A, Smith CL, Faraone SV, Wilcox M, Shafa R, Glatt SJ, Nguyen G, Ponte JF, Thiagalingam S and Tsuang MT (2005) Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: A preliminary report. *Am J Med Genet B Neuropsychiatr Genet* **134**:60-66.
- Auta J, Chen Y, Ruzicka, WB, and Grayson DR (2006) Nucleic acid quantitation using the competitive polymerase chain reaction. *Handbook of Neurochemistry and Molecular Neurobiology: Practical Neurochemistry Methods, 3rd Edition*. (Lajtha, Abel, Glen B. Baker, Susan M. J. Dunn and Andrew Holt eds), Kluwer Academic Publishers, New York, N.Y., pp. 341-361.
- Burgers WA, Fuks F and Kouzarides T (2002) DNA methyltransferases get connected to chromatin. *Trends Genet* **18**:275-277.
- Chen Y, Sharma RP, Costa RH, Costa E and Grayson DR (2002) On the epigenetic regulation of the human reelin promoter. *Nuc Acids Res* **30**:2930-2939.
- Cheng JC, Weisenberger DJ, Gonzales FA, Liang G, Xu G, Hu Y, Marquez VE and Jones PA (2004) Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. *Mol Cell Biol* **24**: 1270-1278.
- Costa E, Davis JM, Dong E, Grayson DR, Guidotti A, Tremolizzo L and Veldic M (2004). A GABAergic cortical deficit dominates schizophrenia pathophysiology. *Crit Rev Neurobiol* **16**:1-23.
- Dong E, Agis-Balboa RC, Simonini MV, Grayson DR, Costa E and Guidotti A (2005) Reelin and glutamic acid decarboxylase₆₇ promoter remodeling in an epigenetic methionine-induced mouse model of schizophrenia. *Proc Natl Acad Sci USA* **102**:12578-12583.

- Eastwood S. L. and Harrison P. J. (2003) Interstitial white matter neurons express less reelin and are abnormally distributed in schizophrenia: towards an integration of molecular and morphologic aspects of the neurodevelopmental hypothesis. *Mol Psychiatry* **769**:821-831.
- Easwaran HP, Schermelleh L, Leonhardt H and Cardoso MC (2004) Replication-independent chromatin loading of Dnmt1 during G2 and M phases. *EMBO Rep* **5**:1181-1186.
- Egger G, Liang G, Aparacio A and Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429**: 457-463.
- Esteve P, Chin HG and Pradhan S (2005) Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. *Proc Natl Acad Sci USA* **102**:1000-1005.
- Fan G, Beard C, Chen RZ, Csankovszki G, Sun Y, Siniaia M, Biniszkiwicz D, Bates B, Lee PP, Kuhn R, Trumpp A, Poon C, Wilson CB and Jaenisch R (2001) DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. *J Neurosci* **21**:788-797.
- Fatemi SH, Earle JA and McMenomy T (2000) Reduction in reelin immunoreactivity in hippocampus of subjects with schizophrenia, bipolar disorder and major depression. *Mol Psychiatry* **5**:654-663.
- Fuks F, Burgers WA, Brehm A, Hughes-Davies L and Kouzarides T (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet* **24**:88-91.
- Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T and Jacob ST (2005) 5-azadeoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol* **25**:4727-4741.

- Grayson DR, Ikonovic S (1999) Competitive RT-PCR to quantitate steady-state mRNA levels, in *Neuromethods, vol 34: In Vitro Neurochemical Methods* (Boulton, Baker, Bateson, eds) pp 127-151, Humana Press, New Jersey.
- Grayson DR, Jia X, Chen Y, Sharma RP, Mitchell CP, Guidotti A and Costa E (2005) Reelin promoter hypermethylation in schizophrenia. *Proc Natl Acad Sci USA* **102**:9341-9346.
- Grayson DR, Chen Y, Costa E, Dong E, Guidotti A, Kundakovic M and Sharma RP (2006) The human reelin gene: transcription factors (+), repressors (-) and the methylation switch (+/-) in schizophrenia. *Pharmacol Ther* **111**:272-286.
- Guidotti A, Auta J, Davis JM, DiGiorgi Gerevini V, Dwivedi Y, Grayson DR, Impagnatiello F, Pandey G, Pesold C, Sharma R, Uzunov D and Costa E (2000) Decrease in reelin and glutamic acid₆₇ (GAD₆₇) expression in schizophrenia and bipolar disorder. *Arch Gen Psychiatry* **57**:1061-1069.
- Guidotti A, Auta J, Davis JM, Dong E, Grayson DR, Veldic M, Zhang X, Costa E (2005) GABAergic dysfunction in schizophrenia: new treatment strategies on the horizon. *Psychopharmacol (Berl)* **180**:191-205.
- Jiang Y, Bressler J and Beaudet (2004) Epigenetics and human disease. *Ann Rev Genomics Hum Genet* **5**:479-510.
- Kimura H and Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. *J Biol Chem* **278**:4806-4812.
- Levenson JM and Sweatt JD (2005) Epigenetic mechanisms in memory formation. *Nat Rev Neurosci* **6**:108-118.

- Levenson JM, Roth TL, Lubin FD, Miller CA, Huang I, Desai P, Malone LM and Sweatt JD (2006) Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *J Biol Chem* **281**:15763-15773.
- Majumder S, Kutay H, Datta J, Summers D, Jacob ST and Ghoshal K (2006) Epigenetic regulation of metallothionein-I gene expression: differential regulation of methylated and unmethylated promoters by DNA methyltransferases and methyl CpG binding proteins. *J Cell Biochem* **97**:1300-1316.
- Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G and Sun YE (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* **302**:890-893.
- Mitchell CP, Chen Y, Kundakovic M, Costa E and Grayson DR. (2005) Histone deacetylase inhibitors decrease reelin promoter methylation in vitro. *J Neurochem* **93**:483-492.
- Noh JS, Sharma RP, Veldic M, Salvacion AA, Jia X, Chen Y, Costa E, Guidotti A and Grayson DR (2005) DNA methyltransferase 1 regulates reelin mRNA expression in mouse primary cortical cultures. *Proc Natl Acad Sci USA* **102**:1749-1754.
- Qiu S, Korwek KM, Weeber EJ (2006) A fresh look at an ancient receptor family: emerging roles for low density lipoprotein receptors in synaptic plasticity and memory formation. *Neurobiol Learn Mem* **85**:16-29.
- Ramchandani S, Bigey P, Szyf M (1998) Genomic structure of the human DNA methyltransferase gene. *Biol Chem* **379**:535-540.
- Szyf M, Bozovic V, Tanigawa G (1991) Growth regulation of mouse DNA methyltransferase gene expression. *J Biol Chem* **266**:10027-10030.

- Torrey EF, Barci BM, Webster MJ, Bartko JJ, Meador-Woodruff JH and Knable MB (2005) Neurochemical markers for schizophrenia, bipolar disorder, and major depression in postmortem brains. *Biol Psychiatry* **57**:252-60.
- Tremolizzo L, Carboni G, Ruzicka WB, Mitchell CP, Sugaya I, Tueting P, Sharma R, Grayson DR, Costa E and Guidotti A (2002) An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability. *Proc Natl Acad Sci USA* **99**:17095-17100.
- Tucker K L (2001) Methylated cytosine and the brain: a new base for neuroscience. *Neuron* **30**:649-652.
- Veldic M, Caruncho HJ, Liu S, Davis J, Satta R, Grayson DR, Guidotti A and Costa E (2004) DNA-methyltransferase 1 mRNA is selectively overexpressed in telencephalic GABAergic interneurons of schizophrenia brains. *Proc Natl Acad Sci USA* **101**:348-353.
- Weaving LS, Ellaway CJ, Gecz J and Christodoulou J (2005). Rett syndrome: clinical review and genetic update. *J Med Genet* **42**:1-7.
- Yokochi T and Robertson KD (2004) Doxorubicin inhibits DNMT1, resulting in conditional apoptosis. *Mol Pharm* **66**:1415-1420.

Footnotes.

Support: This work was supported by a grant from the National Institutes of Mental Health, MH 62682-05 to D.R.G.

¹The authors would like to dedicate this manuscript to the memory of their close friend and scientific colleague Robert H. Costa who died September 1st, 2006 from complications associated with pancreatic cancer.

Figure Legends.

Fig. 1. DOXO treatment leads to dose-dependent increase in reelin and GAD67 mRNA levels.

(A) Representative gels showing typical non-quantitative RT-PCR analysis of reelin, GAD67, and G3PDH mRNAs. (B) Bars showing results of quantitative analysis of reelin, GAD67, and G3PDH mRNA levels in NT-2 cells treated with different concentrations of DOXO for 48 h. Data are presented as amount (pg) of reelin, GAD67, or G3PDH mRNA per 1 μ g total RNA at the indicated concentrations of DOXO (x-axis). (C) Dose-response curves for reelin and GAD67 mRNA induction following 48 h DOXO treatment plotted as the log of drug concentration (X-axis). To compare reelin and GAD67 dose-response curves, the response is expressed as a percent of the maximal reelin or GAD67 mRNA increase (Y-axis). A baseline correction was performed: Y axis values are normalized so that the smallest (baseline) mRNA values are defined as 0% response, whereas the highest values (mRNA levels that correspond to maximal gene induction) are defined as 100% response. EC₅₀ – effective concentration of drug that leads to 50% of maximal reelin or GAD67 gene induction. (D) Representative gel showing RT-PCR analysis of GAD65 mRNA expression in vehicle- or DOXO-treated (250 nM, 48 h) NT-2 cells. RNA isolated from untreated mouse primary neuronal cultures and distilled water were used as positive (PC) and negative controls (NC), respectively. Data represent mean \pm SEM. ***, $p < 0.001$; **, $p < 0.01$ vs control group. (One-way ANOVA followed by Bonferroni test).

Fig. 2. DOXO treatment induces reelin and GAD67 genes in a time-dependent manner. (A) Representative gels showing non-quantitative RT-PCR analysis of reelin, GAD67, and G3PDH mRNAs following DOXO treatment for the indicated times. (B) Bars showing the results of quantitative analysis using competitive RT-PCR and internal standards for the reelin, GAD67 and G3PDH mRNA levels in NT-2 cells treated with 100 nM DOXO for various times. Results

are presented as amount (in pg) of reelin, GAD67 or G3PDH mRNA per 1 μ g total RNA. Data represent mean \pm SEM. ***, $p < 0.001$; * $p < 0.05$ vs control group. (One-way ANOVA followed by Bonferroni test).

Fig. 3. DOXO reduces the DNA methyltransferase activity of NT-2 cells. Total DNA methyltransferase activity of nuclear extracts from untreated or DOXO-treated cells was assayed *in vitro*, by measuring the incorporation of [3 H]-labeled methyl group donor, S-adenosyl-L-methyl-methionine, into the DNA substrate (poly(dI-dC)·poly(dI-dC) oligonucleotide). Data are expressed as specific radioactivity (total radioactivity – non-specific radioactivity) normalized to the amount of the protein present in the corresponding nuclear extracts. Data represent mean \pm SEM. ***, $p < 0.001$ vs control group. (One-way ANOVA followed by Bonferroni test).

Fig. 4. DOXO down-regulates DNMT1 protein levels post-transcriptionally. The representative Western immunoblots and the ratio of the DNMT1 band over the area of the β -actin band in nuclear extract protein samples from untreated cells (Control) and cells treated with either 100 and 250 nM DOXO for 48h (A and B) or 100 nM DOXO for various times (E and F). (1x corresponds to 2.5 μ g of protein; time-course analysis was done using 5 μ g of protein). (C) Representative RT-PCR gels with internal standards and (D) bars showing DNMT1 mRNA levels in vehicle-treated and DOXO-treated cells, obtained using competitive RT-PCR assay. In each tube, competition was carried out among various concentrations of the DNMT1 internal standard (IS) and 100 ng total RNA extracted from untreated cells (Control) or cells treated with 100 nM and 250 nM DOXO for 48 h. Data are presented as amount of DNMT1 mRNA (in pg) per 1 μ g total RNA. Data represent mean \pm SEM. ***, $p < 0.001$; * $p < 0.05$ vs control group. (One-way ANOVA followed by Bonferroni test).

Fig. 5. The effects of AZA and ZEB treatments on reelin and GAD67 mRNA expression and DNMT1 protein levels. (A) Representative gels of non-quantitative RT-PCR analysis and (B) bars showing the results of quantitative analysis of the reelin, GAD67, and G3PDH mRNA levels in NT-2 cells treated with 5 μ M AZA for 48h, and 500 μ M ZEB for either 48h (ZEB 48h) or 48h followed by 48h-incubation with untreated medium (ZEB 2+2D). Results are presented as the amount (pg) of reelin, GAD67 or G3PDH mRNA per 1 μ g total RNA. (C) Representative DNMT1 and β -actin Western immunoblots and (D) the ratio of the DNMT1 band over the area of the β -actin band in nuclear extract protein samples of control and AZA- or ZEB-treated NT-2 cells (1x corresponds to 5 μ g of protein). Data represent mean \pm SEM. ***, $p < 0.001$; ** $p < 0.01$ vs control group. (One-way ANOVA followed by Bonferroni test).

Fig. 6. DOXO induces dissociation of DNMT1 and MeCP2 and increased acetylation of H3 histones in the reelin promoter region. (A) Chromatin samples containing 200-800 bp DNA fragments were generated from cells treated with either control medium (C, Control) or 250 nM DOXO for 48 h (D, DOXO). (B) For both treatments, the reelin promoter region (482 bp band) and β -globin gene fragments (289 bp band) were PCR-amplified from non-immunoprecipitated input (1:10 and 1:4 dilutions), samples immunoprecipitated with DNMT1 antibody (DNMT1 IP), MeCP2 antibody (MeCP2 IP) or anti-acetyl histone H3 antibody (Ac-H3 IP), and negative control (no antibody) (C) Results of semi-quantitative analysis of the occupancy of DNMT1 and MeCP2 to the reelin promoter in vehicle- and DOXO-treated cells normalized to input DNA (1:4 dilution). For comparison, the amounts of acetylated histone H3 is shown following treatment. Data are presented as a ratio of relative optical densities (ROD) of the bands within the immunoprecipitated sample (IP) and input lanes derived from ethidium bromide stained gels. Data represent mean \pm SEM. ***, $p < 0.001$. Control vs DOXO (Student's t-test). (D)

Representative MeCP2 and β -actin Western immunoblots and (E) the ratio of the MeCP2 band over the area of the β -actin band from nuclear extract protein samples of vehicle-treated (Control) and DOXO-treated NT-2 cells (1x corresponds to 5 μ g of protein). Data represent mean \pm SEM. (One-way ANOVA followed by Bonferroni test).

Fig. 7. DOXO toxicity in NT-2 cell culture. Cell cultures were either vehicle-treated (Control) or treated with 100 nM (EC_{50} value), 250 nM (EC_{100} value) and 2 μ M DOXO for 48 h. Cell density and viability were examined by fluorescence microscopy, following the incubation with medium containing calcein-AM (green, live cells) and propidium iodide (red, dead cells) (magnification: x 5).

Figure 1

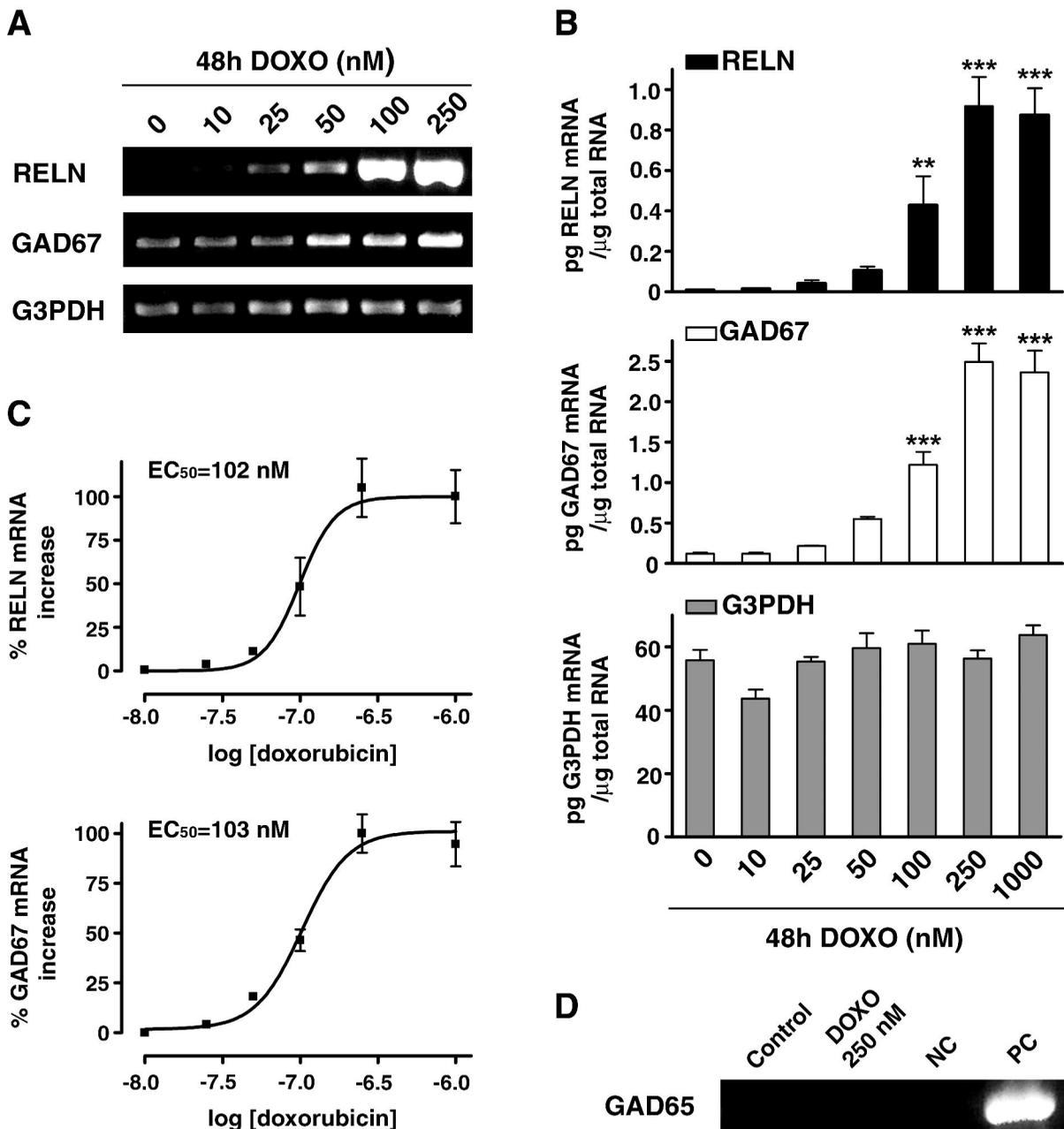


Figure 2

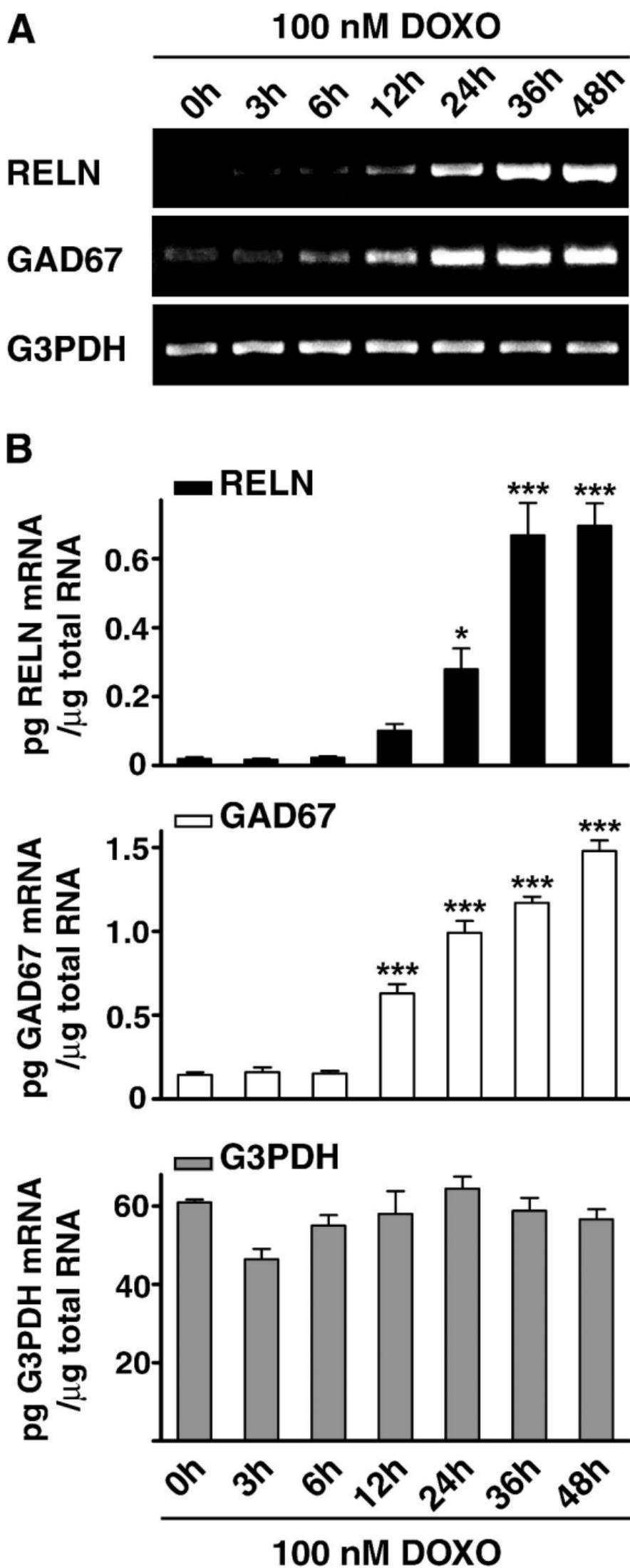


Figure 3

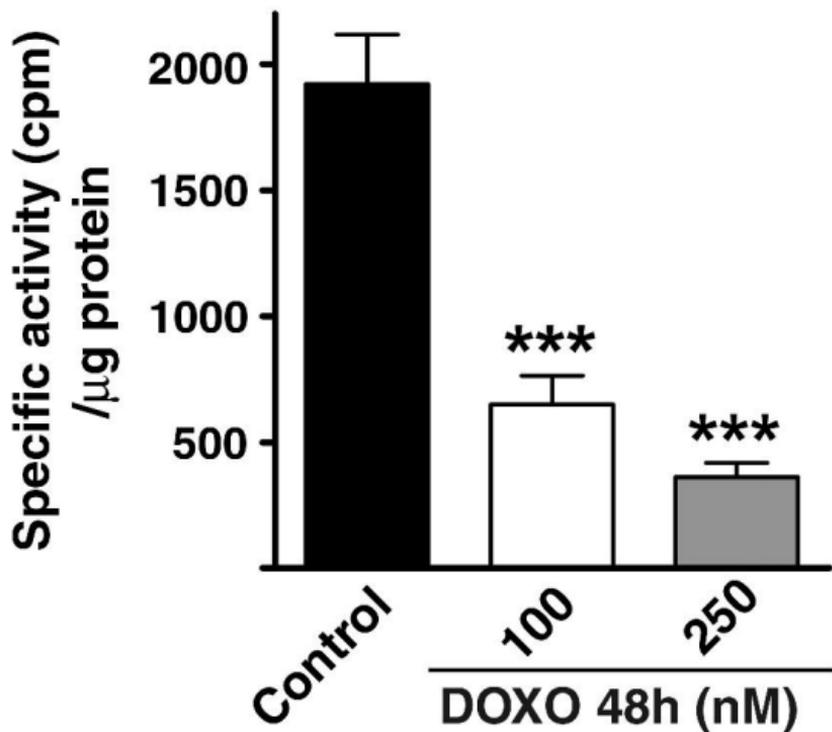
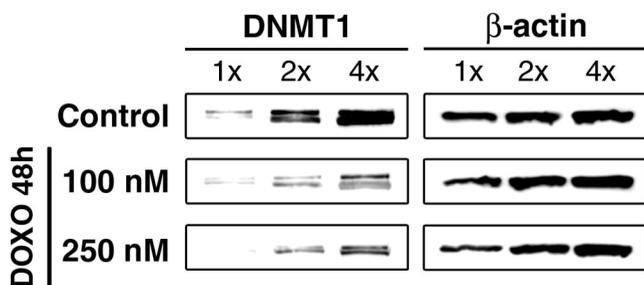
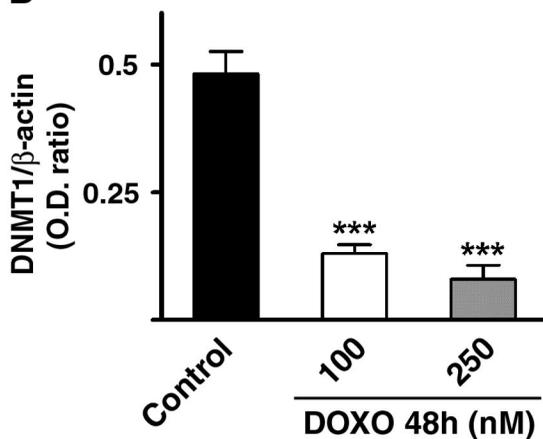


Figure 4

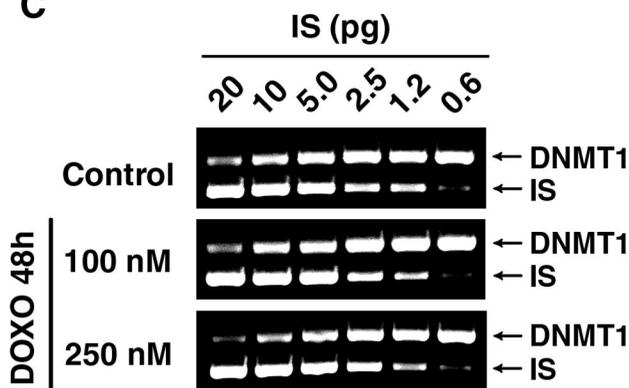
A



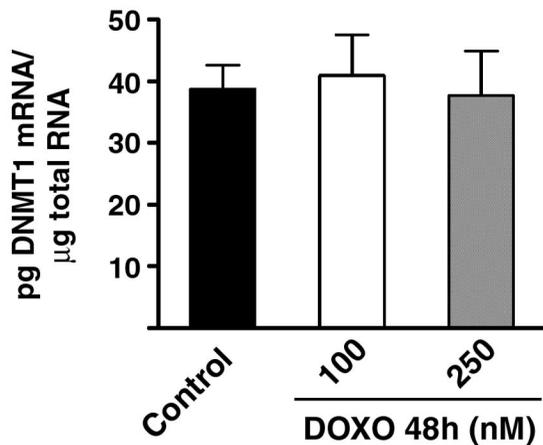
B



C



D



E



F

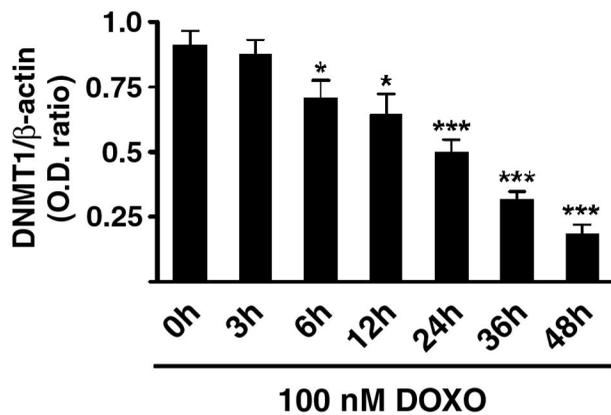


Figure 5

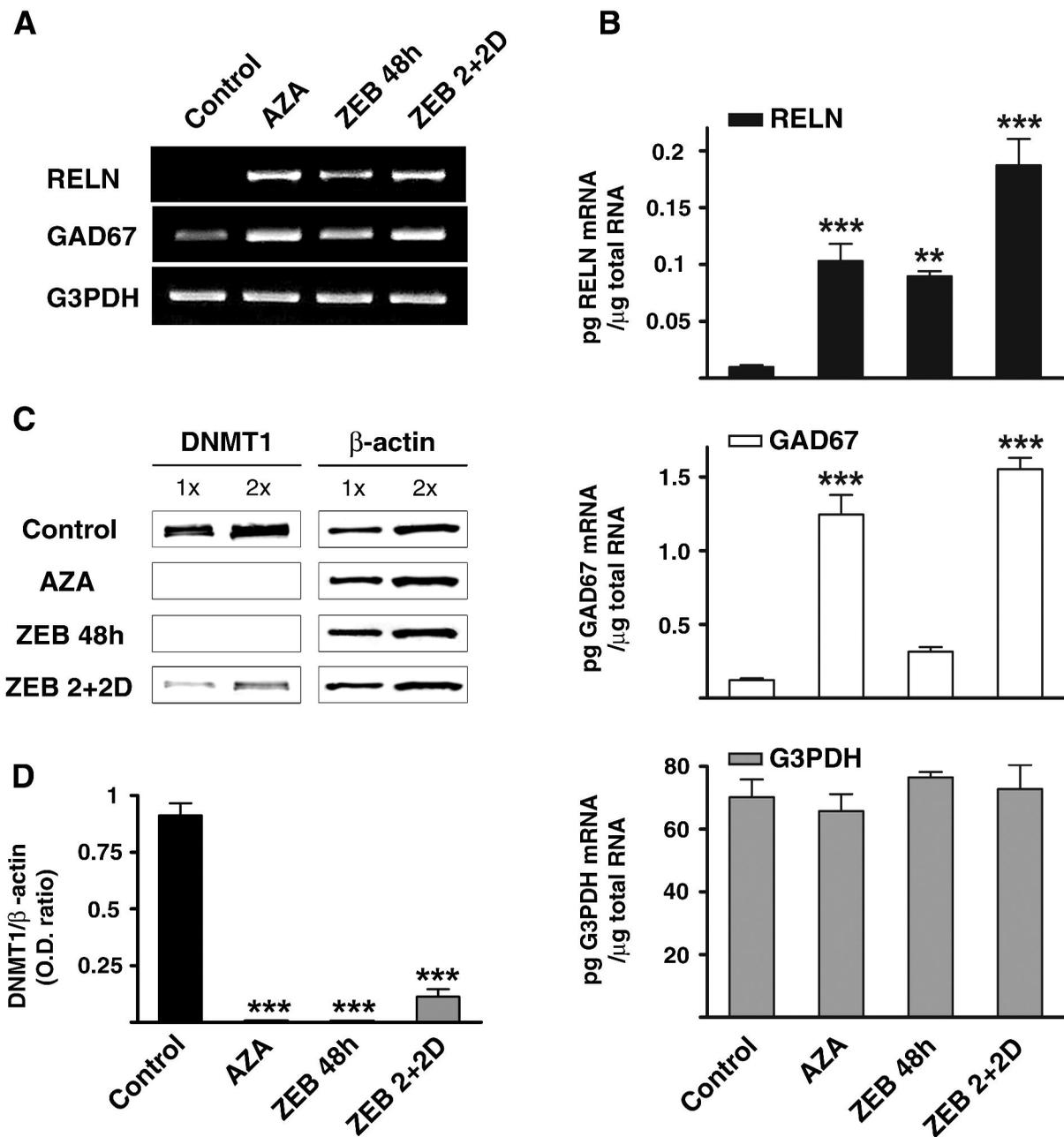


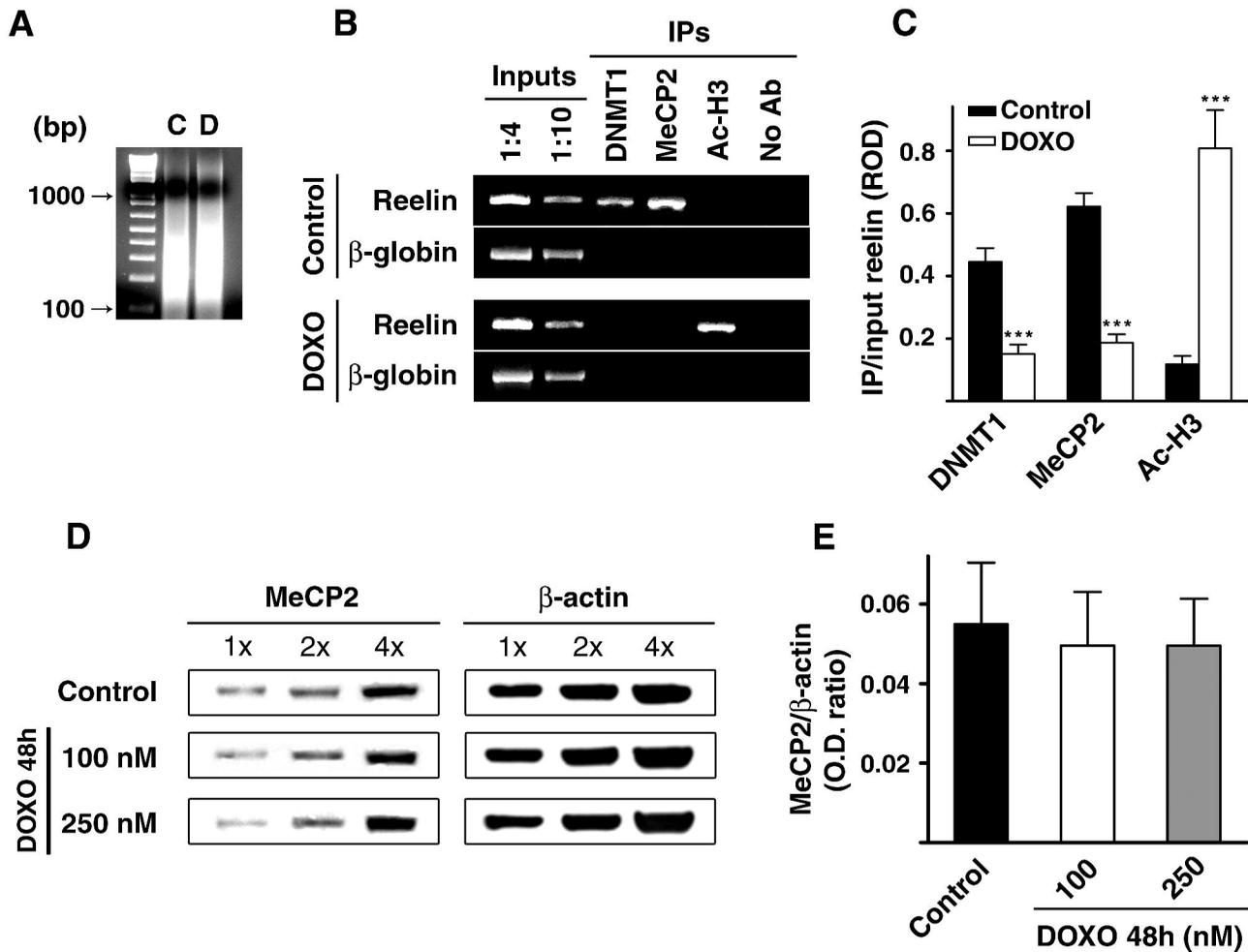
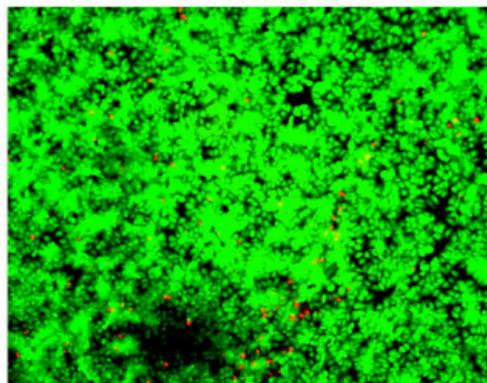
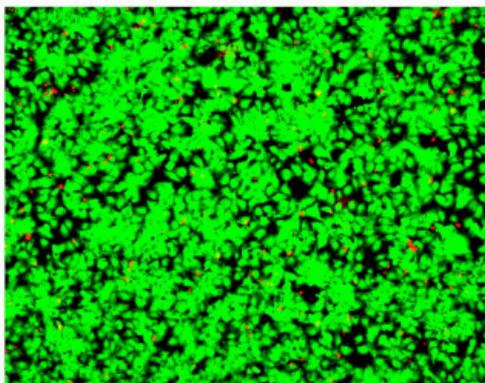
Figure 6

Figure 7

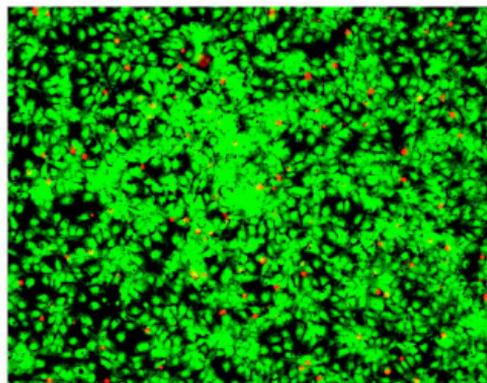
Control



100 nM DOXO



250 nM DOXO



2 μ M DOXO

