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**REVERSIBLE EPIGENETIC FINGERPRINT-MEDIATED GLUTATHIONE-S-
TRANSFERASE P1 GENE SILENCING IN HUMAN LEUKEMIA CELL LINES.**

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ABSTRACT

Glutathione *S*-transferase P1 (GSTP1) gene is commonly silenced by CpG island promoter hypermethylation in prostate, breast, and liver cancers. However, mechanisms leading to GSTP1 repression by promoter hypermethylation in leukemia and its relationship with pathological alterations of the chromatin structure remain poorly understood. A panel of leukemia cell lines was analyzed for their GSTP1 expression, revealing cell lines with high, moderate or no detectable GSTP1 expression. Bisulfite sequencing, methylation-specific PCR and combined bisulfite restriction analysis revealed that GSTP1 promoter was completely methylated in transcriptionally inactive RAJI and MEG-01 cell lines. In contrast, cell lines expressing GSTP1 exhibited an unmethylated and transcriptionally active promoter. Furthermore, histone marks and effector proteins associated with transcriptional activity were detected by chromatin immunoprecipitation in the GSTP1 expressing hypomethylated K-562 cell line. However, repressive chromatin marks and the recruitment of silencing protein complexes were found in the non-expressing hypermethylated RAJI and MEG-01 cell lines. Finally, we provide evidence that treatment of RAJI and MEG-01 cells with the DNA demethylating agent, 5-Aza-2'-deoxycytidine, resulted in GSTP1 promoter demethylation, drastic changes of histone modifications and promoter associated proteins and GSTP1 gene activation. In contrast, treatments with HDAC inhibitors failed to demethylate and reactivate the GSTP1 gene. Our study extends the knowledge on leukemia-specific epigenetic alterations of GSTP1 gene. Furthermore, we are showing the correlation of DNA methylation and histone modifications with the positive/negative GSTP1 transcriptional expression state. Finally, these data support the concept of the dominance of DNA methylation over HDAC inhibitor-sensitive histone deacetylation in gene silencing.

KEYWORDS

GSTP1, leukemia, DNA methylation, chromatin, epigenetic silencing

1. INTRODUCTION

It is commonly known that aberrant DNA methylation contributes to the initiation and progression of tumor development [1]. Indeed, cancer cells undergo a genome wide DNA methylation loss, which leads to the activation of oncogenes, activation of retrotransposons as well as to chromosome instability. Paradoxically, a number of tumor suppressor genes (TSGs), including genes involved in cell cycle, p53 network, DNA repair, hormonal response, carcinogen-metabolism or apoptosis, become hypermethylated in their CpG island (CGi) promoter region during carcinogenesis [2].

Mapping of aberrant promoter hypermethylation led to the identification a tumor specific “DNA methylation signature”, which can be potentially used as a translational biomarker for cancer detection and evaluation of tumor progression. In addition, the methylotype is a prospective indicator for drug susceptibility and a possible target for therapy [2, 3].

In non-cancerous cells, DNA methylation is responsible for transcriptional regulation and is implicated in gene silencing, X-inactivation in mammals, genomic imprinting and repression of harmful transposable elements or endogenous retroviruses [4]. 5-methylcytosine may directly influence transcriptional activity by inhibiting transcription factor binding or by blocking the migration of the transcriptional machinery [5, 6]. Moreover, hypermethylation on gene promoter region leads to repressor complex formation, consisted of methyl-CpG-binding proteins (MBDs), DNA methyltransferases (DNMTs), HP1/polycomb proteins and histone deacetylases (HDACs) [7, 8]. The resulting multi-DNA-protein-complex induces histone deacetylation and catalyzes the methylation of lysines 27 and 9 on histone H3, resulting in a compact nucleosomes arrangement and heterochromatin structure [9, 10].

In contrast, lack of methylated CpG sites as well as absence of DNMTs, MBDs and HDACs in gene promoters are hallmarks of transcriptionally active euchromatin. Nucleosomes in the neighbourhood of transcribed genes are more widely spread and are constituted of heavily acetylated histones H3 and H4 [11]. In addition, modifications of the lysine 4 in histone 3 such as di- (H3K4me2) or trimethylation (H3K4me3) are found to be associated with transcriptionally permissive chromatin [12, 13].

Hypermethylated glutathione-S-transferase P1 gene (GSTP1) is suggested to be a potential epigenetic biomarker for prostate cancer (Pca) [14, 15]. As a member of the GST superfamily, GSTP1 catalyzes the conjugation of glutathione to hydrophobic and electrophilic compounds, previously activated by cytochrome P450 oxygenase enzymes [16, 17]. Transcription factors AP-1, SP-1 and NF- κ B are playing an important role in the regulation of GSTP1 expression [18-20]. Lee *et al.* were the first to relate lack of GSTP1 expression with promoter hypermethylation in Pca cells [21]. *In vitro* methylation of GSTP1 promoter region and the resulting decrease in transcriptional activity confirmed the involvement of hypermethylation in GSTP1 silencing [22-24]. In addition to Pca, loss of GSTP1 expression and promoter hypermethylation was also reported for liver and breast cancer [25-28].

Here, we wanted to elucidate the epigenetic modifications and mechanisms, contributing to GSTP1 silencing in leukemia cells. In this study, we reported that GSTP1 is differentially expressed in various human leukemia cell lines and that lack of GSTP1 expression in RAJI and MEG-01 cells was associated with both promoter hypermethylation and the presence of repressive chromatin conformation. In addition, our data show that DAC-mediated demethylation induces drastic reprogramming of the chromatin structure, leading to GSTP1 re-expression in RAJI and MEG-01 cell lines.

2. MATERIALS AND METHODS

2.1. Cell culture and drug treatments

The human leukemia cell lines K-562 (chronic myeloid leukemia (CML) in blast crisis), MEG-01 (CML in megakaryocytic blast crisis), RAJI (Burkitt's lymphoma), JURKAT (acute lymphoblastic leukemia, ALL), MOLT-3 (ALL), HEL (acute myeloid leukemia, AML), TF-1 (AML), JVM-2 (chronic B-cell leukemia), HL-60 (AML) and U-937 (histiocytic lymphoma) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were cultured and maintained in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% FCS (Lonza) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. 5'-aza-2'-deoxycytidine (DAC) (Sigma–Aldrich, Bornem, Belgium), valproic acid (VPA) (Sigma–Aldrich) and suberoylanilide hydroxamic acid (SAHA) (Cayman, Bio-connect, Huissen, Netherlands) stock solutions were prepared in DMSO. Cells were cultured in presence of DAC for up to 6 days, in presence of VPA or SAHA for 16 h.

2.2. GSTP1 mRNA expression

Changes in GSTP1 mRNA level were quantified by Real-Time PCR in a two-step reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from 5.10⁶ cells with the RNA-II RNA preparation kit (Macherey-Nagel, Hoerd, France). Reverse transcription of RNA (1 µg/reaction) was performed using Superscript II reverse transcriptase (Invitrogen, Tournai, Belgium) and Oligo(dT) primers (Invitrogen) according to manufacturer's instructions. The generated first strand cDNA was diluted into a final volume of 250 µl and samples analyzed by quantitative Real-Time PCR with primers specific for GSTP1 and β-actin (Table 1).

2.3. Quantitative Real-Time PCR analysis (QRT-PCR)

Quantitative Real-Time PCR was performed using 2 μ l cDNA, 12.5 μ l Power Sybr[®] Green PCR master mix (Applied Biosystems, Halle, Belgium) and 1 μ l primers (2.5 μ M) (Table 1) in a final volume of 25 μ l. PCR conditions used for the 40-cycles reaction were as followed: 95°C for 15 sec, 60°C for 1 min. Amplification and detection were carried out using the Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was calculated using the comparative threshold cycle number (delta-Ct method).

2.4. Whole cell extracts, acid extracts, and nuclear extracts

Whole cell lysates were prepared with M-Per Mammalian Protein extraction reagent (ThermoFischer scientific, Erembodegem-Aalst, Belgium). Briefly, cell pellets from 5.10^6 cells were resuspended in M-Per Mammalian Protein extraction reagent supplemented with 25:1 complete protease inhibitor cocktail (Roche, Prohac, Luxemburg, Luxemburg). Cells were lysed under agitation for 10 min at room temperature and then subjected to centrifugation at 10 000 g for 15 min. Supernatant was stored at -80°C .

For histone isolation, cell pellets from 5.10^6 cells were resuspended in ice-cold hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM KCl, 1.5 mM MgCl_2 , 1 mM DTT, 10 mM sodium butyrate and 1 X protease inhibitor cocktail (Complete[®] minus EDTA, Roche) and incubated for 30 min at 4°C . Nuclei were collected by centrifugation at 10 000 g for 10 min at 4°C , resuspended in 0.4 N NH_2SO_4 , and incubated for 30 min at 4°C . Histones were collected by precipitation with 33% trichloroacetic acid (Sigma) and by centrifugation at 16 000 g for 10 min, washed with ice-cold acetone, dissolved in water and stored at -80°C .

Nuclear extracts of leukemia cells were prepared as described previously [29]. Protein concentrations were determined by the Bradford assay (Bio-Rad, Nazareth, Belgium) with bovine serum albumin as a standard.

2.5. Western Blot analysis

For Western Blot, protein extracts were resolved on SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare, Roosendaal, Netherlands). The membrane was probed with primary antibodies that specifically recognize the antigen of interest (Table 2). Horseradish-peroxidase-conjugated secondary antibodies in combination with the ECL Plus Western Blot Detection system (GE Healthcare) were used to visualize protein bands (Table 2). Chemiluminescence was analyzed with the Kodak image station 440 CF (Kodak, Analis, Suarlée, Belgium) and quantified with the Kodak 1D image analysis software.

2.6. GSTP1 immunostaining, flow cytometry and UV-microscopy analysis

For immunostaining, 8.10^6 cells were washed with 1X PBS, fixed in 2% paraformaldehyde (PFA) (Merck, VWR, Leuven, Belgium) /1X PBS for 15 min and permeabilized in 0.1% Triton X-100 (Merck) for 8 min at room temperature (RT). Cells were thoroughly washed and incubated with an antibody anti-GSTP1 (Table 2) and subsequently with an Alexa Fluor® 488 conjugated anti-mouse antibody (Invitrogen) for 1 h at RT, washed with 1X PBS and stored in 0.2% PFA. GSTP1 associated green fluorescence was quantitatively processed by the acquisition on a BD FACSCalibur flow cytometer with CellQuest Pro software (Becton Dickinson, Erembodegem, Belgium) and analyzed by FlowJo software (Treestar, Ashland, OR, US). GSTP1 localization was investigated by fluorescence microscopy analysis

using an IX81 (MT10) Olympus microscope (Olympus, Aartselaar, Belgium). For nuclear staining, cells were incubated 15 min at 37°C with Hoechst 33342 (Calbiochem, VWR, Leuven, Belgium).

2.7. GST activity assay

GST Fluorometric Activity Assay Kit (Biovision, Gentaur, Brussels, Belgium) using monochlorobimane (MCB) as substrate was used to measure total GST activity. Briefly, 10^6 cells were collected and subsequently homogenized by sonication with a Bioruptor (Diagenode, Liège, Belgium) in 100 µl of GST sample buffer. After centrifugation at 10 000 g for 15 min at 4°C, supernatant was collected for the subsequent assay following manufacturer's instructions. Fluorescence was read at Ex.380/Em.460nm after 1 h of incubation using a SPECTRA MAX Gemini EM (Molecular Devices, Berkshire, UK). GST activity was expressed as milliunits (mU) of GST per 10^6 cells.

2.8. CpG island analysis in the human GSTP1 promoter

A 1550-nt fragment of the human GSTP1 promoter, containing the transcription start site, was selected from the National Center for Biotechnology Information (NCBI) database using the accession number NT_167190.1. This sequence was analyzed with the Methyl Primer Express software (Applied Biosystems) using the following settings: observed/expected CpG ratio of 0.6, minimum length island of 200 nt, and minimum G+C content of 50%. A 702-nt fragment was identified that contained CpG islands and the transcription start site. Within this fragment, a 327 nt region was selected for further analysis.

2.9. DNA methylation analysis of the GSTP1 promoter

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. *Bgl*III restriction (Promega, Leiden, Netherlands) was performed on 1 µg of genomic DNA and then subjected to sodium bisulfite conversion using the MethylDetector™ Kit (Active motif, Rixensart, Belgium) as proposed by the manufacturer.

GSTP1 methylation pattern was analyzed by bisulfite sequencing (BSP) and combined bisulfite restriction assay (CoBRA). For BSP analysis bisulfite-modified DNA was amplified with primers specific for GSTP1 (Table 1) and the Hot start Taq™ polymerase (Qiagen). Thermal conditions were 15 min at 95°C, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension step of 10 min at 72°C. PCR products were separated on agarose gels, purified with the Qiaex Gel extraction Kit (Qiagen), cloned into pGEM-T vector (Promega) and sequenced (GATC Biotech, Konstanz, Germany). For CoBRA analysis, 50 ng of purified PCR products were restricted with *Rsa*I (NEB, Westburg, Leusden, Belgium) in a reaction volume of 20 µl. The restriction pattern was analyzed with a 2100 Bioanalyzer platform on a DNA 1000 Kit (Agilent, Diegem, Belgium) [30].

Methylation status of GSTP1 promoter region was analyzed using methylation-specific PCR (MSP). MSP was performed with primers specific for methylated (M) or unmethylated (U) sequences (Table 1). Primer specificity was determined with the Epiect DNA (Qiagen). PCR reaction was done using 2 µl of bisulfite-modified DNA and Platinum Taq DNA polymerase HIFI (Invitrogen). Amplification was carried out with an initial denaturation 2 min at 94°C and 35 three-step cycles (15 sec at 94°C, 30 sec at the annealing temperature listed in table 1 and 30

sec at 68°C). Amplicons were separated, stained with ethidium bromide (Promega) and visualized under UV illumination.

2.10. Chromatin Immunoprecipitation (ChIP)

10⁷ cells were cross-linked with 1% formaldehyde (MP Biomedicals, Illkirch, France) for 8 min at room temperature. After cross-linking, the reaction was quenched with 0.125 M of glycine for 10 min at room temperature. Cells were washed twice with ice-cold PBS, pelleted by centrifugation, resuspended in 1 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% IGEPAL) and 1X protease inhibitor cocktail (Complete[®] minus EDTA), and incubated 30 min at 4°C under agitation. After centrifugation, nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS and 1X protease inhibitor cocktail) and incubated for 10 min on ice. The soluble chromatin with a size range of 0.5 kb to 0.9 kb was prepared by sonication using a Bioruptor (Diagenode). After centrifugation, to remove cell debris, chromatin was pre-cleared 1 h at 4°C with a 50% gel slurry of protein A/G-agarose beads saturated with salmon sperm DNA and bovine serum albumin (Upstate, Millipore, Brussels, Belgium), diluted in IP dilution buffer (0.01% SDS, 0.5% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 100 mM NaCl and 1 X protease inhibitor cocktail), and 10% of the supernatant was used as input. The diluted chromatin was incubated overnight at 4°C with the antibodies of interest (Table 2) and the immune complexes were recovered by 1 h incubation at 4°C with a 50% gel slurry of protein A/G-agarose beads (Upstate). The precipitated complexes were washed sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl and 1X protease inhibitor cocktail), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl and 1X protease inhibitor

cocktail), LiCl buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1 % Igepal, 1% deoxycholic acid and 1X protease inhibitor cocktail) and twice with Tris-EDTA buffer (1 mM EDTA and 20 mM Tris-HCl pH 8.1), and extracted twice with freshly prepared elution buffer (100 mM NaHCO₃ and 1% SDS) with mild vortexing. The cross-linking between DNA and proteins was reversed by an overnight incubation at 67°C with 0.3 M NaCl in the presence of RNase A (Roche). Samples were then digested with proteinase K (Roche) at 45°C for 1 h. DNA was purified using QIAquick PCR purification kit (Qiagen) and analyzed by QRT-PCR. PCRs were performed using primers that covered different regions of the GSTP1 promoter (Table 2).

2.11. Evaluation of the doubling time of stable transfectants of GSTP1 in RAJI and MEG-01 cells

Stably transfected cells were obtained by electroporation using a BioRad Gene Pulser (Bio-Rad). Briefly, 4.10⁶ cells with 5 µg of pcDNA3.1 plasmid with or without the GSTP1 gene (Kindly provided by Dr. Yongkui Jing, Mount Sinai School of Medicine, New York, USA) were electroporated at the following settings: 250 V for RAJI cells and 275 V for MEG-01 cells at a capacity of 950 µF. Clone selection was done by limiting dilution into regular medium supplemented with G418 (Sigma). Cell clones resistant to G418 were screened for high GSTP1 expression by western blot. Cells were counted and doubling time calculated using a web-based software (<http://www.doubling-time.com/compute.php>).

2.12. Statistical Analysis

Data are presented as means ± SD, and analyzed by the Student's *t*-test. *p*-values below 0.05 were considered as statistically significant.

3. RESULTS

3.1. Heterogeneous basal GSTP1 expression levels in leukemia cell lines.

GSTP1 gene expression was assessed at the level of mRNA (Figure 1-A) and protein (Figure 1-B) by QRT-PCR and Western Blot, respectively. A panel of 10 human cell lines, representing various types of hematological malignancies were analyzed. The highest amount of GSTP1 was detected in the CML cell line K-562, whereas GSTP1 was undetectable in RAJI and MEG-01 cells (Figure 1). Furthermore, high GSTP1 expression was detected in the cell lines JURKAT, TF-1, HL-60 and U-937, in contrast to the moderate GSTP1 expression level detected in JVM-2, HEL and MOLT-3 cells (Figure 1).

3.2. DNA demethylating agent but not HDAC inhibitors restores GSTP1 expression in non-expressing cells.

To evaluate a possible link between epigenetic silencing mechanisms and the lack of GSTP1 expression in RAJI and MEG-01 cell lines, we assessed the effects of the DNA demethylating agent, 5-aza-2'-deoxycytidine (DAC), and histone deacetylases inhibitors (HDACi) on GSTP1 expression. DAC had only a moderate but significant inducing effect on the basal GSTP1 expression of K-562 cells whereas DAC drastically increased GSTP1 expression in RAJI and MEG-01 cell lines (Figure 2-A, -B). The effect of DAC on GSTP1 expression was time-dependent but concentration-independent for the tested range of concentrations (Figure 2-A, -B, Supplemental Figure 1). Moreover, DAC had a significant positive effect on GSTP1 expression in HEL, MOLT-3 and JVM-2 after 3 days of treatment (Figure 2-C).

Furthermore, HDACi alone did not have a significant effect on constitutive GSTP1 expression in K-562 and failed to restore GSTP1 expression in RAJI and MEG-01 cells (Figure

2-D, Supplemental Figure 2). Combinatorial treatments of RAJI and MEG-01 cells with DAC and HDACi caused a moderate but significant increase in GSTP1 expression, compared to DAC treatment alone (Figure 2-D).

To evaluate the localization of GSTP1 protein on cellular level before and after DAC treatment, GSTP1-immunostained K-562, RAJI and MEG-01 cells were analyzed by microscopy. GSTP1 expression in K-562 cells remained mainly unaffected after DAC treatment whereas DAC treatment induced GSTP1 expression in a restricted subpopulation of RAJI and MEG-01 cell lines (Figure 3-A). Quantification of the DAC-induced GSTP1 immunostaining by flow cytometry determined a drastic and significant 4.4-fold increase of GSTP1 expression in RAJI cells after 3 days of treatment (Figure 3-B). In correlation with microscopy results, only a subpopulation of about 65% of the DAC-treated RAJI population was GSTP1 positive compared to untreated cells (Figure 3-C). DAC treatment of MEG-01 cells for 3 days resulted in a significant relative induction of GSTP1 protein expression of about 1.6-fold (Figure 3-B). In contrast, 6 days were required to induce GSTP1 expression in 33.8% of the MEG-01 cell population (Figure 3-C).

We next evaluated whether DAC-induced GSTP1 expression was enzymatically active. The highest basal GST activity was measured in K-562 and MEG-01 cell lines in contrast to the minor GST activity in RAJI cells (Figure 3-D). Furthermore, DAC treatment increased GST activity in a time-dependent manner and reached 3- and 12-fold induction of GST activity in 3- and 6-days treated RAJI cells, respectively (Figure 3-E). A slight but significant GST activity induction was observed in DAC treated K-562 and MEG-01 cells (Figure 3-E).

3.3. Methylation status of the GSTP1 regulatory region in leukemia cell lines.

The effect of the demethylating agent DAC on GSTP1 expression prompted us to hypothesize that methylation of the GSTP1 promoter region could correlate with the lack of GSTP1 expression in RAJI and MEG-01. *In silico* CpG density analysis identified a CGi associated with the 5' upstream GSTP1 promoter region, potentially responsible for GSTP1 gene silencing by hypermethylation (Figure 4-A, -B). BSP technique was applied to determine the methylation status of the GSTP1 CGi in K-562 and RAJI cell lines. GSTP1 promoter region of K-562 was hypomethylated (Figure 4-C) in contrast to the hypermethylated status in RAJI cells (Figure 4-D). Sequence analysis of GSTP1 amplicon clones revealed a decrease from 96.3% methylation in untreated cells to 64.5% in RAJI cells after 3 days of 2 μ M DAC treatment. Interestingly, the resulting demethylation was restricted to specific sequences without any CpG site preference (Figure 4-E). BSP results were confirmed by CoBRA analysis (Supplemental Figure 3).

3.4. Profiling DNA methylation pattern of the GSTP1 promoter before and after DAC treatment in various leukemia cell lines.

MSP was used to test the GSTP1 methylation status of further leukemia cell lines as well as to analyze the influence of the demethylating agent, DAC, on methylation pattern. MSP primer sets were located at the transcription start region (MGP) and the NF-KB binding site (MGD) (Figure 5-A). Methylation specificity was demonstrated with fully unmethylated, methylated converted as well as with unmethylated unconverted DNA (Figure 5-B). MSP results of K-562 and RAJI cells correlated with the previous BSP and COBRA results. Furthermore, MSP analysis of the GSTP1 promoter in JVM-2, MOLT-3 and HEL cell lines demonstrate an

hypomethylated GSTP1 promoter (Figure 5-C). Interestingly, MSP technique revealed dense methylation on GSTP1 promoter in the CML-derived cell line MEG-01 (Figure 5-C). Treatments with the demethylating agent DAC did not have any effect on the GSTP1 hypomethylation status in K-562, JVM-2, MOLT-3 and HEL cell lines (Figure 5-D). In addition, DAC treatment reduced dramatically GSTP1 methylation pattern of RAJI and MEG-01 cells in a time-dependent manner. MSP results are similar for both analyzed GSTP1 promoter sites (Figure 5-D).

3.5. GSTP1 transcriptional activity correlates with the DNA methylation status as well as with the chromatin structure conformation.

We performed ChIP analysis to determine repressor/activator protein complexes and histone marks associated with GSTP1 promoter region. Enrichment of promoter fragments in ChIP assays using specific antibodies was detected by PCR using primer sets targeting GSTP1 basal promoter region (CG3, Figure 6-A). As shown in Figure 6-B, transcription factors (Sp1 and Sp3) and RNA polymerase II were associated to this region in K-562 cells. Moreover, GSTP1 promoter in K-562 cells was highly enriched for di- and trimethylation of lysine K4 on histone H3, for acetylation of histones H3 and H4 as well as for their associated HAT proteins p300 and CBP (Figure 6-B). Concerning RAJI and MEG-01 cells, GSTP1 basal promoter was enriched for repressive histone modifications such as trimethylation of H3K9 and H3K27. In contrast, the recovery of acetylated histones H3 and H4 at this position was reduced in RAJI and MEG-01 cell lines. In addition, GSTP1 promoter of RAJI and MEG-01 cells was enriched for DNA methylation- and heterochromatin- associated proteins such as DNMTs, MBDs and HDACs, respectively (Figure 6-B). Finally, we used primers targeting a region 10kb upstream of the basal

promoter as a negative control (CG5, Figure 6-A). As expected, this region was not enriched for proteins related to active transcription (Figure 6-B).

Finally, we mapped the effect of DAC on histone modifications and promoter associated proteins by ChIP assay (Figure 6-A). Results showed that DAC treatment induced important changes in histone modification marks and protein recruitment to the GSTP1 promoter in RAJI and MEG-01 cells, which correspond to a shift from a repressive to a more permissive chromatin state, close to the one observed in K-562. Accordingly, this chromatin remodelling is in agreement with DAC-induced changes in transcriptional activity and DNA methylation pattern (Figure 6-C). The lack of protein recruitment was not due to a lack of expression (Supplemental Figure 4).

3.6. Restoration of GSTP1 expression in GSTP1 non-expressing cells lines fail to affect cell proliferation.

GSTP1 expression has been implicated in the regulation of cell proliferation through direct interaction with the c-Jun N-terminal kinase (JNK) [31]. Therefore, loss of GSTP1 expression may provide a selective growth advantage in RAJI and MEG-01 cells. To determine whether GSTP1 affect cell growth, its expression was restored by generating stable transfectants of RAJI and MEG-01 cells. We compared the doubling time of RAJI or MEG-01 cell lines between wild type and various clones presenting high GSTP1 expression levels (Table 3). Restoration of GSTP1 expression in RAJI and MEG-01 cells had no consistent effect on their cell growth (Table 3).

4. DISCUSSION

In 1989, Greger *et al.* described for the first time the association between DNA hypermethylation and TSG silencing in human cancer based on a study on the Retinoblastoma gene (Rb) [32]. Nowadays, long lists of TSGs (*e.g.* DNA repair or metastasis genes) are showing aberrant methylation and thus gene silencing in cancerous cells [33]. This inventory includes the detoxification gene GSTP1, which is hypomethylated and widely expressed in benign tissue, but hypermethylated and transcriptionally silenced in prostate, breast and liver cancer cells [21, 25, 26, 28, 34]. Since leukemia-specific epimutations in the GSTP1 loci are mostly unknown, the data obtained in this study could open up new strategies to detect and treat haematological malignancies.

In the current study, we investigated GSTP1 promoter methylation and the contribution of transcriptional regulators such as histone modifications or promoter-associated proteins in relation to GSTP1 expression levels. Screening of various human leukemia cell lines confirmed the constitutively high level of GSTP1 expression in K-562 cell line and the lack of GSTP1 expression in RAJI cells [23, 35]. Our results identified several leukemia cell lines (JVM-2, HEL, MOLT-3) with moderate GSTP1 expression, and an additional GSTP1-negative CML cell line (MEG-01). MSP, BSP and CoBRA assays and DAC-induced GSTP1 expression pointed out accordantly the hypermethylation of the GSTP1 regulatory region in RAJI and MEG-01 cells. In contrast, GSTP1 promoter was hypomethylated in K-562 cells. Therefore, promoter methylation status and GSTP1 expression pattern are correlating in K-562, RAJI and MEG-01 cell lines. Taking together, these results prompt us to assume that, accordingly to what is observed in prostate cancer, aberrant GSTP1 promoter hypermethylation could be linked to early leukemogenesis .

Previous studies discussed the possibility to detect aberrant methylation on cell-free DNA, isolated from serum, plasma or urine of patients with prostate, lung and colon cancer [15, 36-38]. This idea was implemented to detect urological malignancies during a minimal invasive DNA-based routine screening by analyzing GSTP1 methylation pattern on circulating DNA [15, 39]. According to our results, aberrant GSTP1 promoter methylation could also be a characteristic of certain leukemia cells, thus implying that the detection of GSTP1 hypermethylation in serological biopsies could not only stem from prostate cancer but could also originate from hematological malignancies.

GSTP1 immunostaining followed by UV-microscopy and flow cytometry analyses showed that DAC treatment of RAJI and MEG-01 cells led to GSTP1 re-expression in only a limited subpopulation of cells, resulting in an heterogeneous mixed population of GSTP1 expressing and non-expressing cells. Furthermore, only a limited number of entirely demethylated GSTP1 promoter sequences were observed after 3 days in DAC-treated RAJI cells. This limited demethylation could be explained by a partial genomic demethylation related to low efficiency of DAC in those cells. However, methylation specific restriction assay showed a strong genomic demethylation (data not shown). Interestingly, DAC-mediated heterogeneous cell population was already reported in a publication from dos Santos *et al.* about synovial sarcoma X gene expression in human cell lines [40]. Currently, it remains unclear why only a part of the DAC-treated cells loses GSTP1 promoter methylation and thereupon re-expresses GSTP1.

Although DNA methylation and histone deacetylation act as synergistic layers in tumor-associated gene silencing, we demonstrate that HDACi alone were unable to restore GSTP1 expression in non-expressing cell lines. Our findings are in agreement with a former study on

1
2
3
4 cyclin-dependent kinase inhibitor 2A (p15) and 2B (p16), tissue inhibitor of metalloproteinase 3,
5
6 and DNA mismatch repair protein (MLH1) [41]. However, the failure of HDACi to restore
7
8 GSTP1 expression was not due to lack of inhibitory activity (Supplemental Figure 2). Therefore,
9
10 our results are showing that dense CGi methylation of the GSTP1 promoter region is dominant
11
12 for the permanent transcriptional silencing of the GSTP1 loci and has to be removed before
13
14 HDACi can affect GSTP1 expression.
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19 The two Sp1 binding sites on the GSTP1 proximal promoter region are essential *cis*-
20
21 elements required for basal gene activity [18, 28]. Furthermore, the binding of Sp1 transcription
22
23 factor to its recognition site has been shown to be insensitive to cytosine methylation [42, 43].
24
25 However, our study showed that neither Sp1 nor Sp3 is associated to hypermethylated GSTP1
26
27 promoter region in RAJI and MEG-01 cell lines. This finding is in accordance to the current
28
29 hypothesis that DNA methylation can induce conformational changes in chromatin structure,
30
31 which block the accessibility of Sp1 to its binding site and contribute to GSTP1 transcriptional
32
33 silencing.
34
35

36
37
38 GSTP1 expression and gene methylation patterns are correlated to the chromatin structure
39
40 of its promoter. Indeed, ChIP analyses identified typical proteins and histone marks associated to
41
42 a transcriptionally active GSTP1 promoter in K-562 cells. However, the hypermethylated GSTP1
43
44 promoter region in RAJI and MEG-01 cells was enriched for repressive protein and histone
45
46 marks, correlating with the lack of GSTP1 expression. GSTP1 expression is therefore not
47
48 exclusively regulated by DNA methylation, but also synergistically on level of histone
49
50 modifications and transcription factor recruitment. In addition, the effect of DAC was not limited
51
52 to the methylation pattern of GSTP1. Indeed, GSTP1 demethylation is linked to drastic changes
53
54 of GSTP1 promoter-associated proteins including the release of MBDs, HDACs, and DNMTs in
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both non-expressing RAJI and MEG-01 cell lines, which is correlated to GSTP1 re-expression [44-46]. Thus, we clearly confirmed that DAC treatment triggered DNA demethylation and induced a substitution of repressive marks by active ones on the GSTP1 promoter in RAJI and MEG-01 cells.

Data regarding the influence of GSTP1 expression on cell proliferation (Table 3) are suggesting that GSTP1 does not likely function as a classical TSG in leukemogenesis. Nonetheless, loss of expression by CpG hypermethylation of GSTP1 promoter region could be an early event in leukemogenesis. Indeed, in its function as a 'caretaker' gene, GSTP1 inactivation might promote susceptibility to other somatic genomic alterations, caused by electrophilic or oxidative carcinogens, and therefore providing a selective growth advantage for cancer cells [47, 48]. Consequently, avoidance of GSTP1 promoter hypermethylation is not only beneficial for cell detoxification, integrity and survival but also for cancer prevention. This is confirmed by enhanced cancer susceptibility, detected in GSTP1 deficient mice [49].

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TABLES

Table 1: Oligonucleotide primers.

Oligonucleotide primers for gene expression and ChIP analysis were generated using Primer3 software (<http://frodo.wi.mit.edu/primer3/>). Primer pairs for methylation analysis were designed with Methyl Primer Express software from Applied Biosystems (Halle, Belgium). All primers were obtained from Eurogentec (Liège, Belgium). BSP: Bisulfite sequencing, CoBRA: Combined bisulfite restriction assay, MSP: Methylation specific PCR, ChIP: Chromatin immunoprecipitation, UMGD/P: unmethylated GSTP1 distal/proximal, MMGD/P: methylated GSTP1 distal/proximal.

Application	Primer	Sequence (5'→3')	Annealing temp (°C)	Amplicon length (bp)
mRNA expression	β-actin	F: CTCTTCCAGCCTTCCTTCCT R: AGCACTGTGTTGGCGTACAG	60	116
	GSTP1	F: GGCAACTGAAGCCTTTTGAG R: GGCTAGGACCTCATGGATAC	60	129
BSP/ CoBRA MSP	GSTP1	F: GGAAAGAGGGAAAGGTTTTTT R: ACTCTAAACCCCATCCCC	55	292
	UMGD	F: GTGAAGTGGGTGTGTAAGTTTT R: ACAAAAAAAAAAACCAACAAA	60	106
	MMGD	F: AAGCGGGTGTGTAAGTTTC R: CAAAAAAAAAACCGCAACG	62	106
	UMGP	F: GTGGGATTTTTTAGAAGAGT R: CACATACTCACTAATACCAAA	58.9	140
	MMGP	F: CGGGATTTTTTAGAAGAGC R: CGCGTACTCACTAATAACGA	63	140
ChIP	CG1	F: CTCTATGGGAAGGACCAGCA R: GATGTATTTGCAGCGGAGGT	60	81
	CG2	F: CCAGTTCGAGGTAGGAGCAT R: GATAAGGGGGTTCGGATCTC	60	103
	CG3	F: GCAGCGGTCTTAGGGAATTT R: CTTTCCCTCTTTCCCAGGTC	60	131
	CG4	F: AAGTAGGCAGCAAAGCCAAA R: GTCCCTGCAAAGGACATGAT	60	77
	CG5	F: AAGCCCAGGAACCTCAAGAT R: TGATCAGCCTGTGCCTGTAG	60	86

Table 2: Antibodies.

Target	Company	Reference No.	Application
AcH3	Upstate,	06-599	ChIP
AcH4	Upstate	06-866	WB, ChIP
β -actin	Sigma-Aldrich	A5441	WB
CBP	Abcam	ab2832	ChIP
CBP	Santa Cruz	sc-369	WB
DNMT1	Active motif	39204	WB, ChIP
DNMT3A	Active motif	39206	ChIP
DNMT3A	Santa Cruz	sc-20703	WB
DNMT3B	Active motif	39207	ChIP
DNMT3B	Abcam	ab2851	WB
GSTP1	BD Biosciences	610718	WB, IP, IF
H4	Upstate	05-858	WB
H3K4Me2	Abcam	ab7766	ChIP
H3K4Me3	Abcam	ab8580	ChIP
H3K9Me3	Upstate	07-442	ChIP
H3K27Me3	Upstate	07-449	ChIP
HDAC1	Upstate	05-100	WB, ChIP
HDAC2	Abcam	ab7029	WB, ChIP
MBD1	Abcam	ab2846	WB, ChIP
MBD2	Upstate	07-198	ChIP
MBD2	Santa Cruz	sc-9397	WB
MBD3	Abcam	ab3755	ChIP
MBD3	Santa Cruz	sc-9402	WB
MeCP2	Upstate	07-013	WB, ChIP
p300	Upstate	05-257	WB, ChIP
RNA pol II	Upstate	05-623	ChIP
Sp1	Active motif	39058	WB, ChIP
Sp3	Santa Cruz	sc-644	WB, ChIP
donkey anti-goat IgG-HRP	Santa Cruz	sc-2020	WB
donkey anti-rabbit IgG-HRP	Santa Cruz	sc-2313	WB
donkey anti-sheep IgG-HRP	Santa Cruz	sc-2473	WB
goat anti-mouse IgG-HRP	Santa Cruz	sc-2005	WB

WB: Western Blot, ChIP: Chromatin immunoprecipitation, IF: Immunofluorescence

Table 3: Restoration of GSTP1 expression in non-expressing leukemia cells fails to reduce cell proliferation.

Cell line	GSTP1 expression [‡]	Doubling time (hours)
RAJI	-	27.3±2.1
RAJI-vector	-	28.8±5.4
RAJI-GSTP1-1	+	29.2±3.9
RAJI-GSTP1-3	+	28.4±3.1
RAJI-GSTP1-6	+	30.1±1.9
MEG-01	-	49.4±3.6
MEG-01-vector	-	50.7±4.2
MEG-01-GSTP1-4	+	52.1±4.9
MEG-01-GSTP1-9	+	51.2±1.2
MEG-01-GSTP1-10	+	51.1±2.7

[‡]GSTP1 expression was assessed by western blot. Data represent the mean ± SD of 5 independent experiments. vector: empty pcDNA3.1 plasmid, GSTP1: pcDNA3.1 plasmid with the sequence of the human GSTP1 gene.

LEGENDS TO FIGURES

Figure 1: Analysis of basal GSTP1 expression in various human leukemia cell lines.

(A) Total RNA was extracted from various human leukemia cell lines and analyzed by QRT-PCR with primers specific for GSTP1 and β -actin. Results represent the relative ratio of GSTP1/ β -actin. (B) Total protein extractions from the indicated leukemia cell lines were analyzed by Western Blot with an antibody specific for GSTP1. Chemiluminescence was quantified and normalized with respect to the housekeeping gene β -actin. The relative level of GSTP1 protein in various cell lines is expressed compare to the level in K-562 cells set to 100%. Data represent the mean \pm SD of 3 independent experiments.

Figure 2: Effect of DAC and HDAC inhibitors on GSTP1 expression in various leukemia cell lines.

K-562, RAJI and MEG-01 cells were treated with 2 μ M DAC. (A) Total RNA was isolated after various time points and GSTP1 mRNA expression assessed by QRT-PCR. Results represent the ratio GSTP1/ β -actin mRNA. Data show the means \pm SD of more than 3 independent experiments (* p <0.05, ** p <0.01 vs. control). (B) Cells were harvested after various time points of DAC treatment for Western Blot analysis with a GSTP1 specific antibody. Pictures are representative of three independent experiments. Relative level of GSTP1 protein expression in various cell lines is normalized against β -actin and expressed compare to the GSTP1 expression level after 6 days of DAC treatment. (C) Total RNA from various untreated (-) and DAC-treated (+) (3 days, 2 μ M) leukemia cell lines was isolated and analyzed by QRT-PCR with primers specific for GSTP1 and β -actin. Results represent the ratio GSTP1/ β -actin mRNA expression

level. (D) Total RNA was extracted from leukemia cell lines K-562, RAJI and MEG-01 treated either with 2 μ M DAC for 3 days, 1 mM VPA, 2 μ M SAHA for 16 h or co-treated with 2 μ M DAC for 3 days followed by 16 hours in presence of 1 mM VPA or 2 μ M SAHA. QRT-PCR analysis was performed with GSTP1 and β -actin primers. The results represent the ratio GSTP1/ β -actin mRNA expression level. Data are the mean \pm SD of 3 independent experiments (* p <0.05, ** p <0.01 vs. control; # p <0.05, ## p <0.01 vs. DAC).

Figure 3: DAC treatment induces heterogeneous GSTP1 expression and promotes GST activity in human leukemia cell lines.

K-562, RAJI and MEG-01 cells were treated with 2 μ M DAC for 3 days and GSTP1 expression was assessed by immunostaining using anti-GSTP1 and secondary Alexa Fluor 488-conjugated antibodies, and nuclear staining with Hoechst 33342. (A) Pictures were taken by fluorescence microscopy and are representative for 3 independent experiments. (B) GSTP1 fluorescence intensity of untreated K-562, RAJI and MEG-01 cells was quantified by flow cytometry and expressed as the ratio of GSTP1 immunostained cells to the isotype control. DAC-mediated GSTP1 induction is presented as the fold induction vs. control of GSTP1 protein expression after 3 days of treatment with 2 μ M DAC. Data are the mean \pm SD of 3 independent experiments (* p <0.05, ** p <0.01 vs. control). (C) One dimensional GSTP1 frequency histogram of 10^5 control and 3 or 6 days DAC-treated RAJI and MEG-01 cell lines (red peak: mock; blue peak: DAC-treated). Data are means \pm SD of 3 independent experiments. Untreated and DAC-treated K-562, RAJI and MEG-01 cell lines were harvested to determine basal intracellular GST activity by measuring the fluorescence level of monochlorobimane, a dye that reacts with glutathione. (D) Fluorescence results of untreated K-562, RAJI and MEG-01 cells were interpolated by a

calibration curve to get GST activity (mU/10⁶ cells). Data are the mean \pm SD of 10 independent experiments. (E) GST activity induction in K-562, RAJI and MEG-01 cells after 3 and 6 days of treatment with 2 μ M DAC was expressed as fold induction vs. untreated control cells. Data are the mean \pm SD of at least 3 independent experiments (* $p < 0.05$, ** $p < 0.01$ vs. control).

Figure 4: Mapping of the GSTP1 promoter CpG island and analysis of the methylation pattern in the leukemia cell lines K-562 and RAJI.

(A) The Methylprimer express software was used to analyze *in silico* the CpG dinucleotide density of the GSTP1 promoter region. The physical map is showing the distribution of CpG dinucleotides (vertical black bars) and their relative positions to the transcription start site (+1) in the 5' regulatory area and the first exon region of GSTP1 gene. Arrows highlight the positions of bisulfite sequencing primers on GSTP1 promoter region. (B) Database sequence containing 38 CpG dinucleotides (gray boxes) selected for methylation analysis of the GSTP1 5'upstream regulatory. Genomic DNA, extracted from untreated K-562 (C), untreated (D) and DAC-treated (3 days, 2 μ M) RAJI cells (E) were bisulfite converted. The specific methylation pattern was revealed by PCR amplification, subcloning, and sequencing of the region of interest (BSP). Graphical illustration of the BSP results for the analysis of the GSTP1 promoter CGi methylation status in control K-562 cells (C), untreated (D) and DAC-treated (E) RAJI cells. Open and closed circles indicate unmethylated and methylated sites, respectively.

Figure 5: Effect of DAC on GSTP1 promoter methylation in various leukemia cell lines.

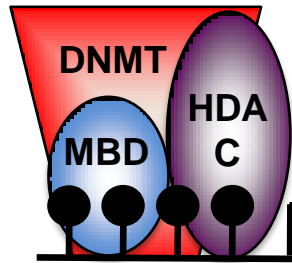
(A) Physical map generated with the Methylprimer software is showing the distribution of CpG dinucleotides (vertical black bars) and the positions of the MSP primer pairs MGD and MGP on the GSTP1 promoter region. (B) MSP control reactions were necessary to determine the primer specificity for the unmethylated (U) or methylated (M) proximal (MGP) and distal (MGD) primer sets. MSP assays with the following control samples as templates has been carried out: complete bisulfite conversion procedure without genomic DNA (Bi), unmethylated converted (UMC), methylated converted (MC) and unconverted unmethylated DNA (UC). (C) Genomic DNA was extracted from the indicated leukemia cell lines, bisulfite converted and used as DNA templates for MSP with primers specific for the unmethylated (U) and methylated (M) state of MGD and MGP in order to define the methylation status of the GSTP1 promoter. The MSP amplicons were separated by gel electrophoresis and stained with ethidium bromide ($n>3$). Images are representative of three independent experiments. (D) Genomic DNA from various leukemia cell lines, treated up to 6 days with DAC were bisulfite converted and analyzed by MSP.

Figure 6: Characterization of GSTP1 chromatin structure in K-562, RAJI and MEG-01 leukemia cell lines and its evolution after DAC treatment by ChIP analysis.

(A) Schematic representation of the GSTP1 5' upstream region with PCR primers (CG1 to 5) positions used to map the promoter in ChIP analysis. (B) Chromatin from K-562, RAJI and MEG-01 cell lines was immunoprecipitated with specific antibodies. Associated DNA was amplified using primers specific for the GSTP1 promoter region (CG3 and CG5) and PCR products were separated on a gel stained with ethidium bromide. (C) Chromatin immunoprecipitations were performed as previously mentioned on untreated K-562, RAJI and

MEG-01 cells as well as on RAJI and MEG-01 cells treated with 2 μ M DAC for 3 days. The enrichment was measured by QRT-PCR and results expressed as recovery (% of total input) = $((Ct_{IP} - Ct_{IgG})/Ct_{input})$. Data are the mean \pm SD of 3 independent experiments.

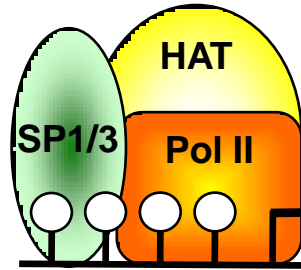
*Graphical Abstract
RAI1, MEG-01



5-Aza-2'-Deoxycytidine



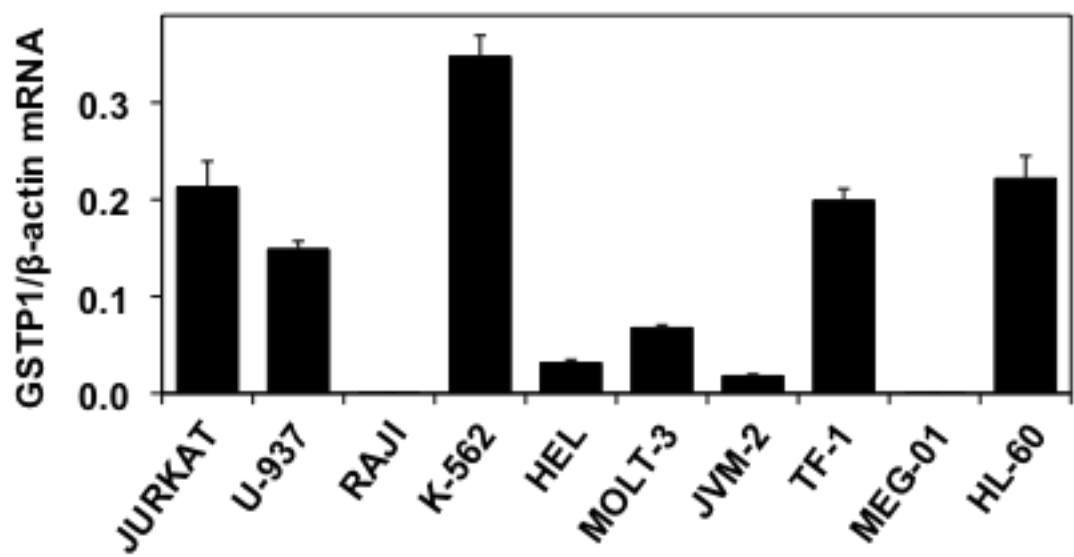
K-562



GSTP1

Epigenetic regulation of GSTP1 expression in human leukemia cell lines

A



B

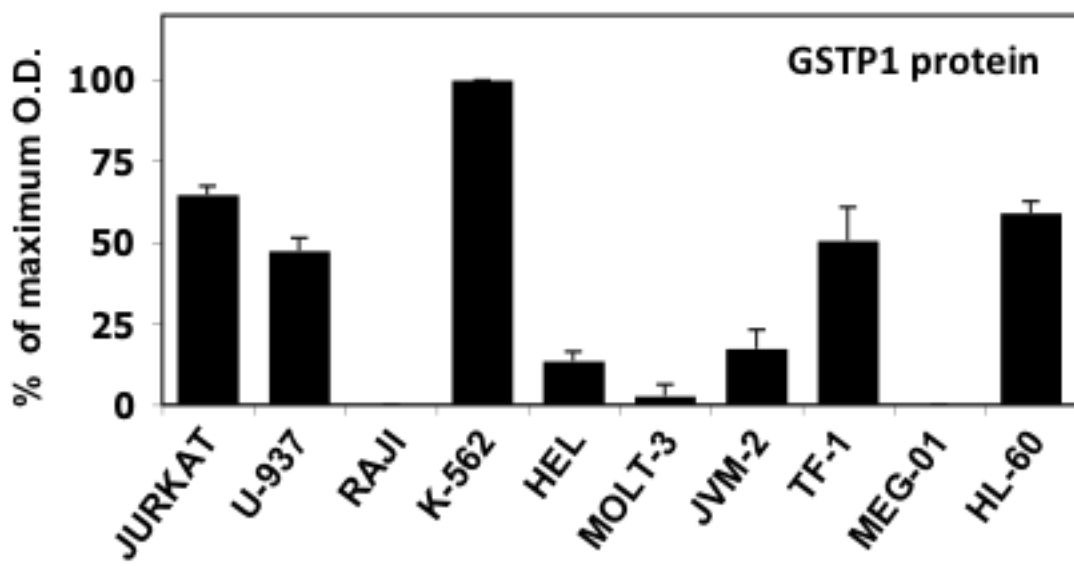
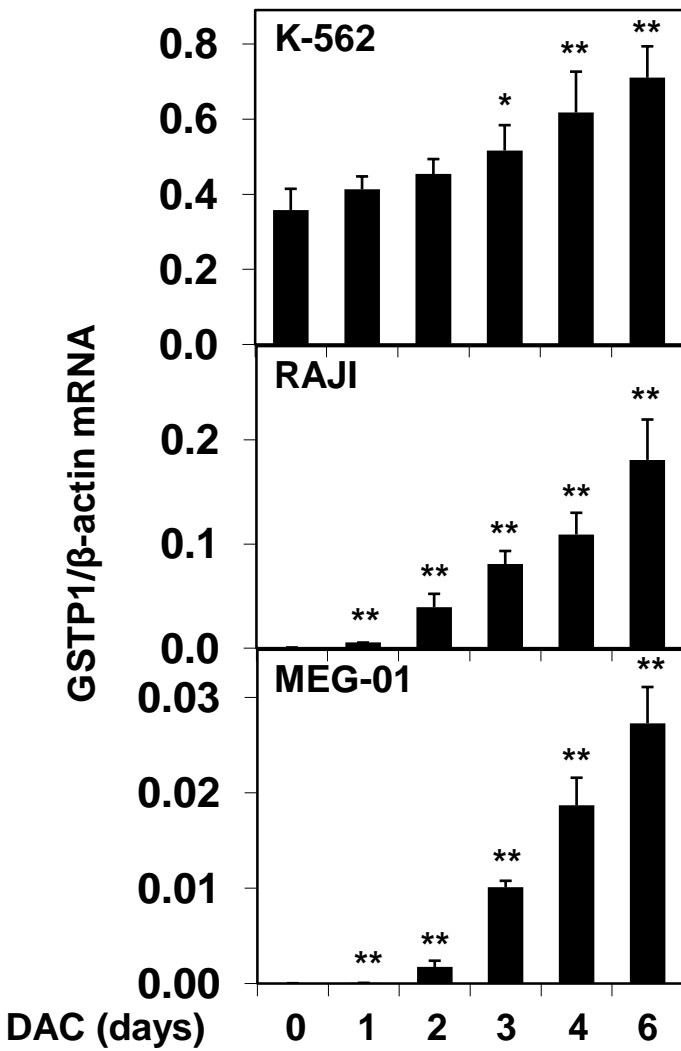
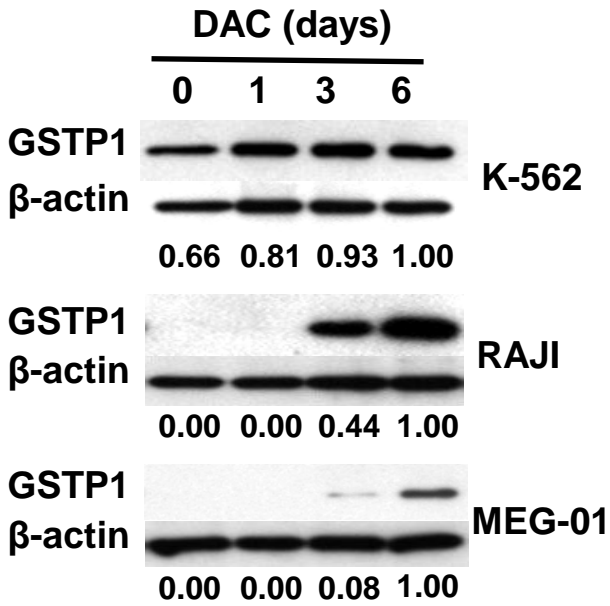


Figure 2

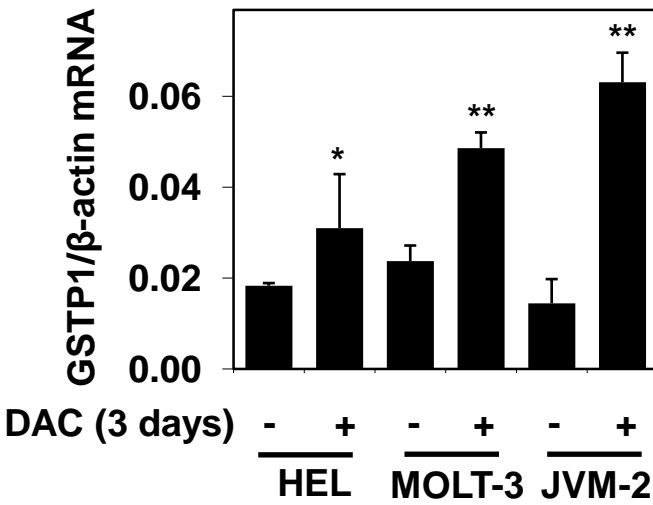
A



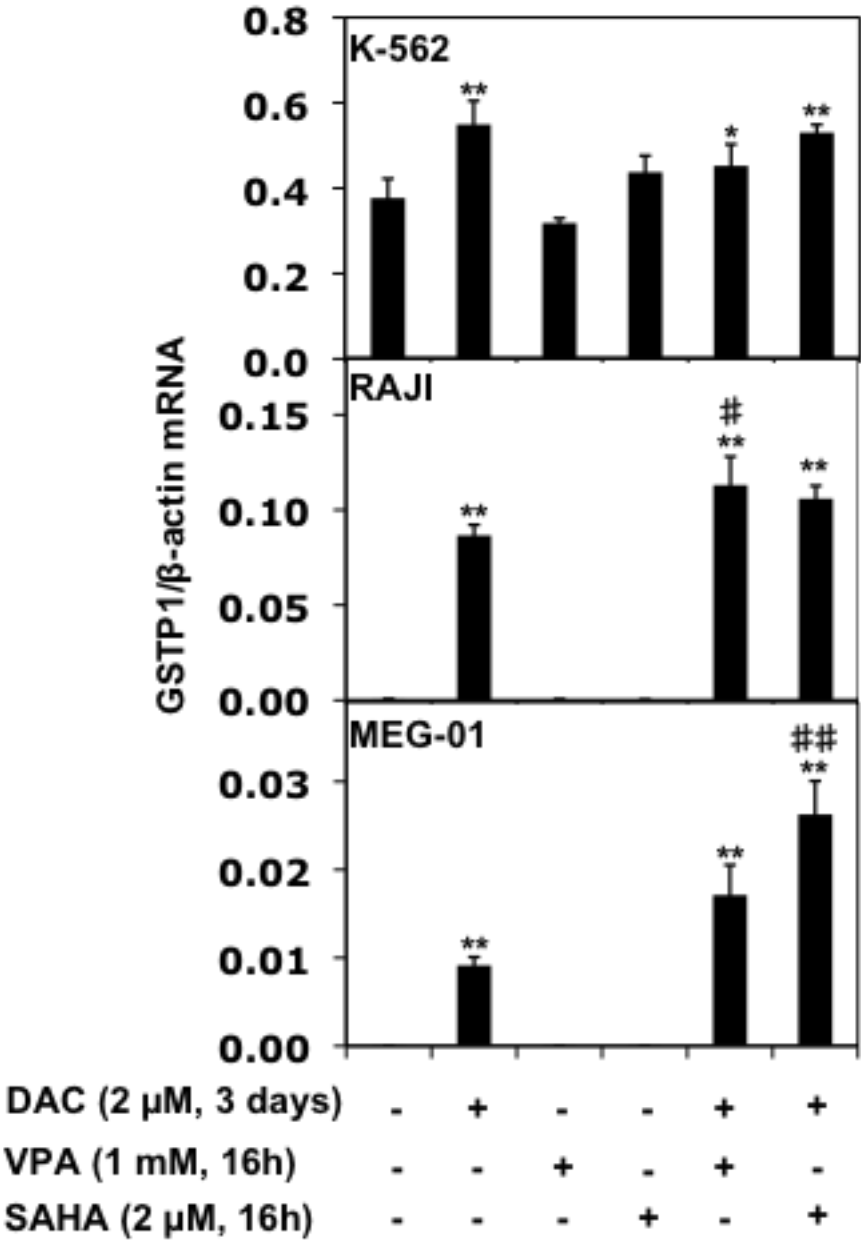
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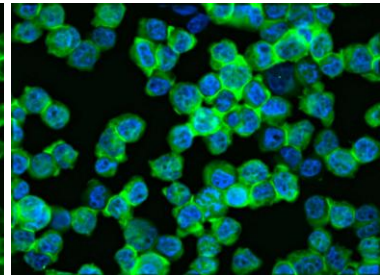
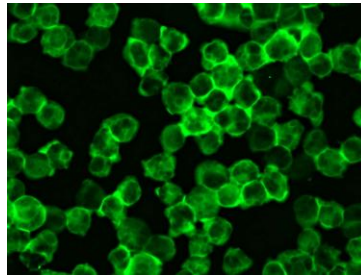
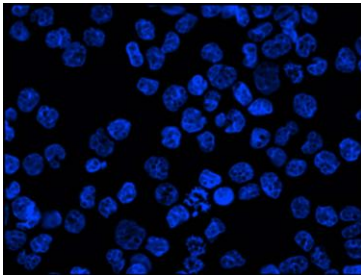
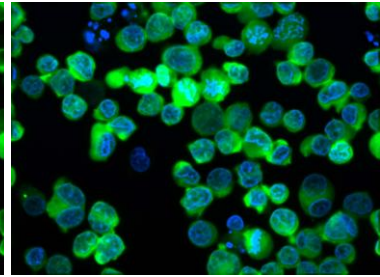
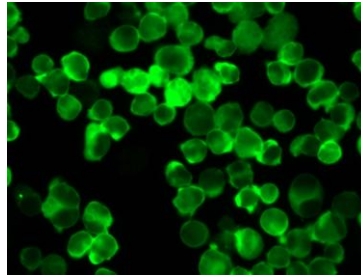
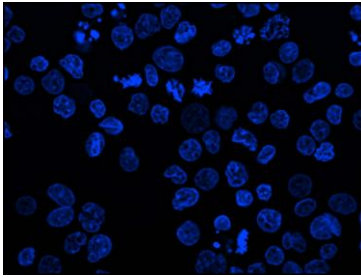
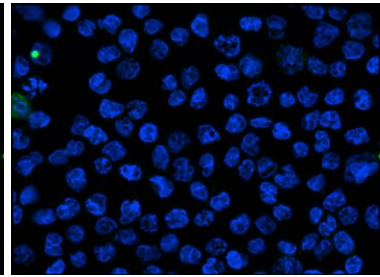
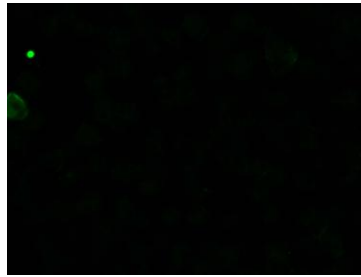
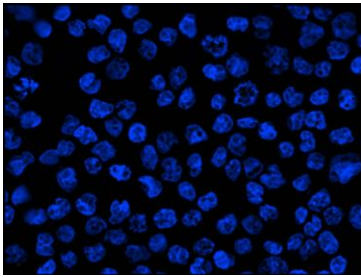
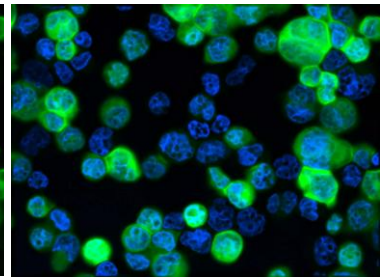
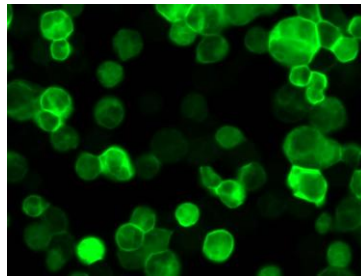
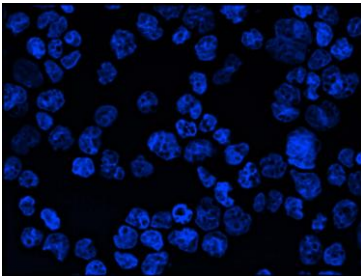
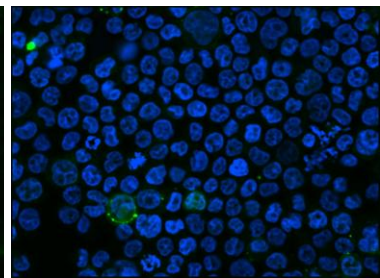
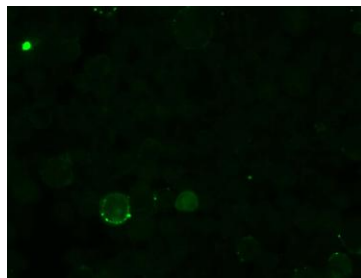
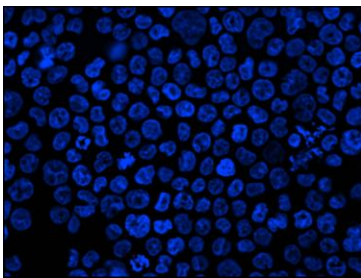
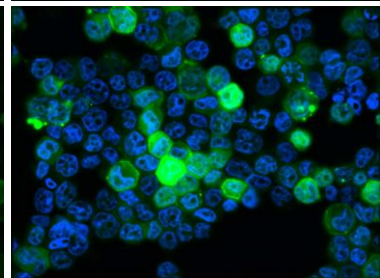
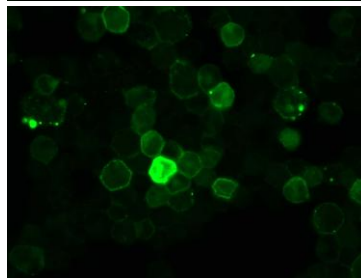
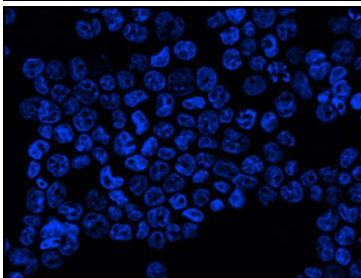


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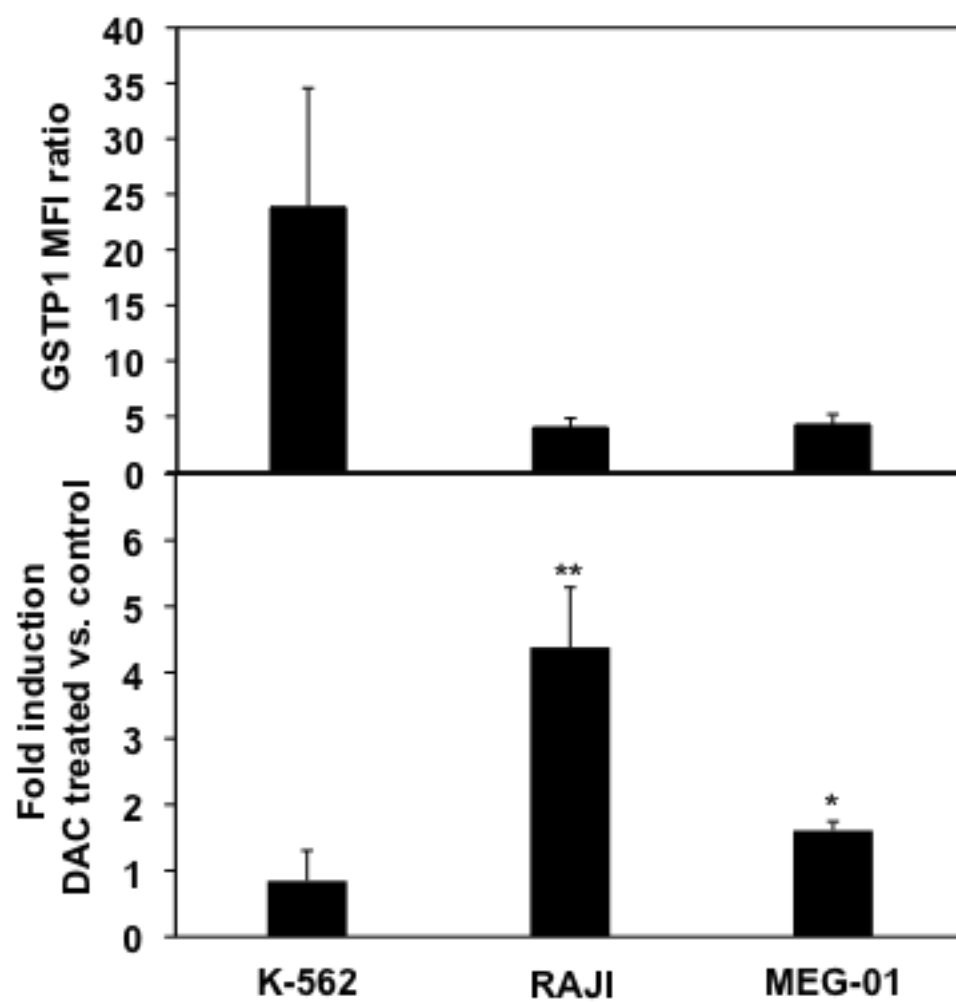


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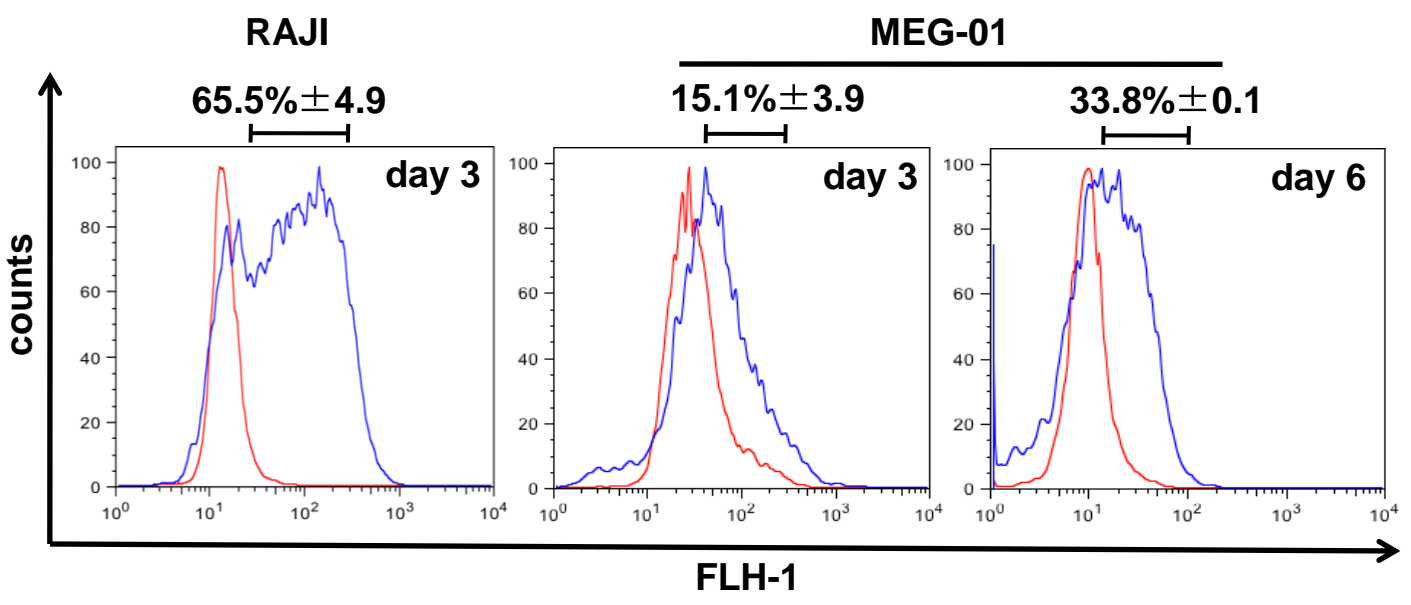


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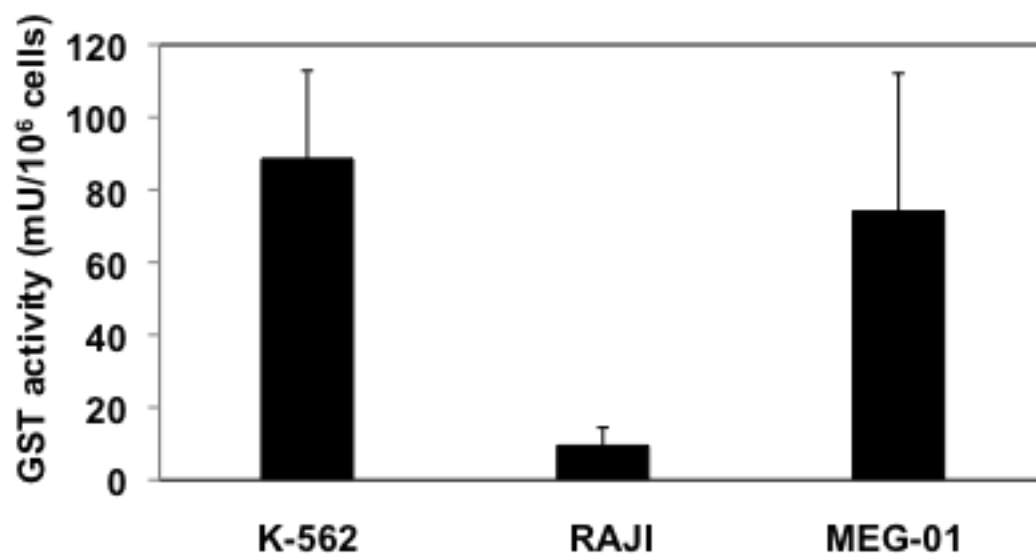
B



C



D



E

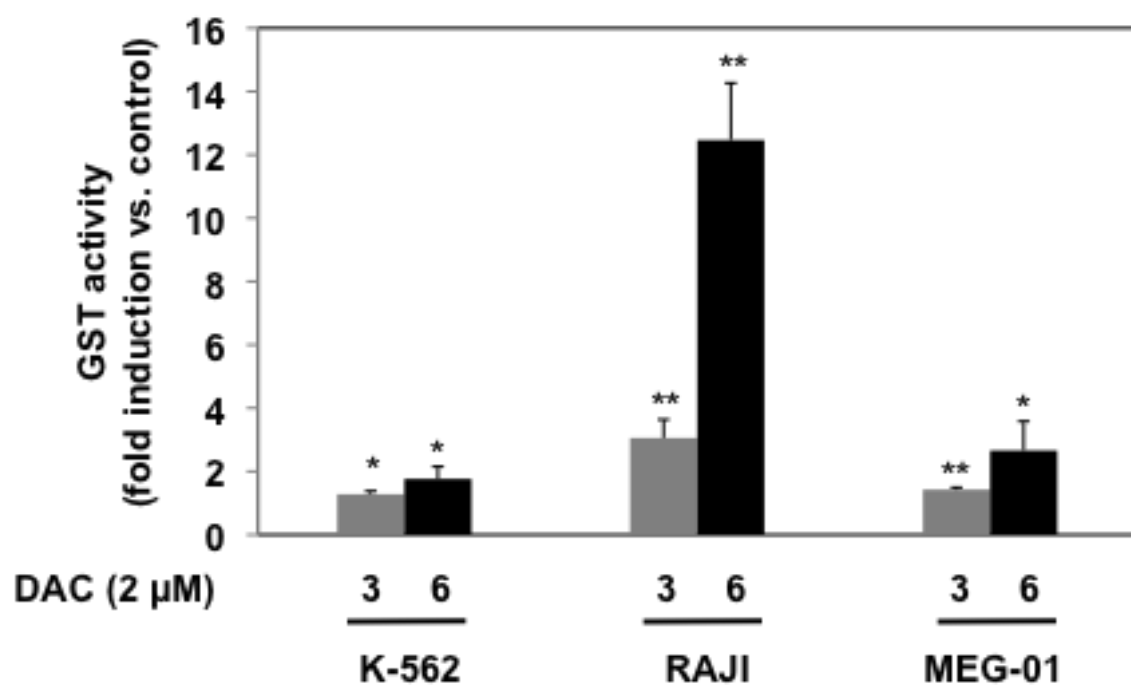
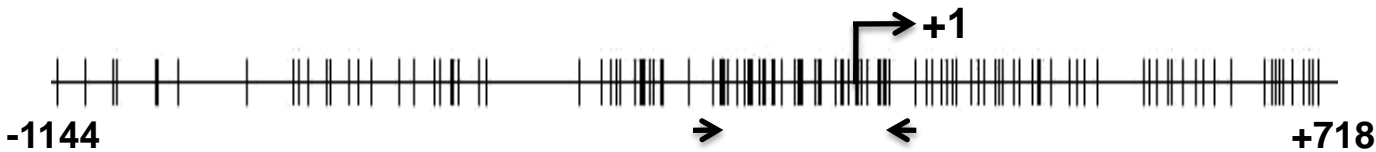


Figure 4

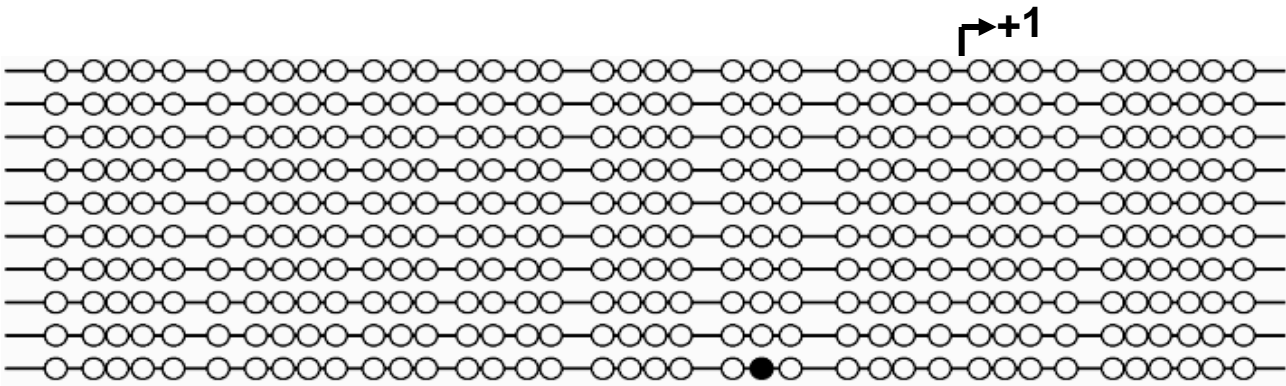
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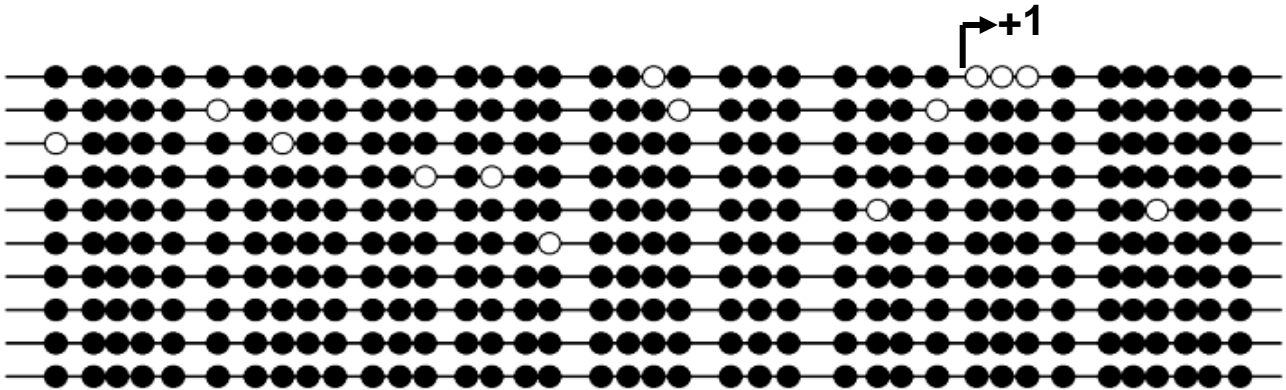
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C



D



E

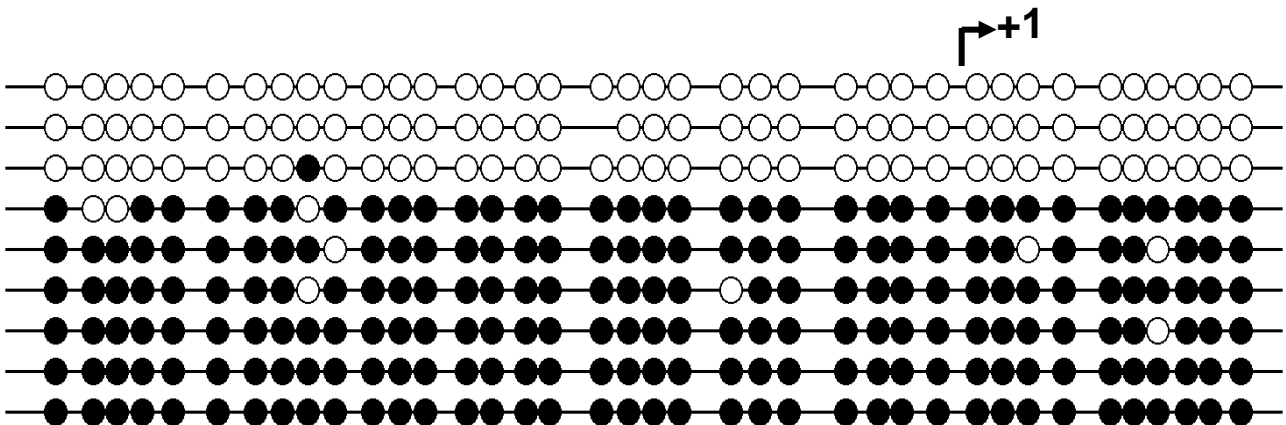
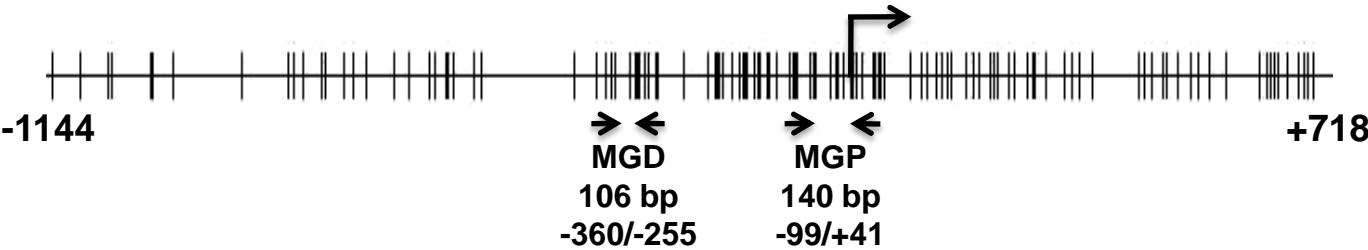
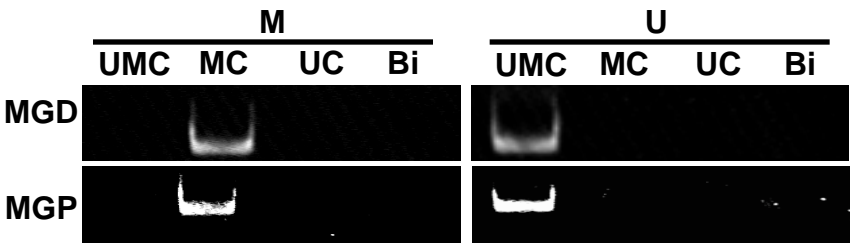


Figure 5

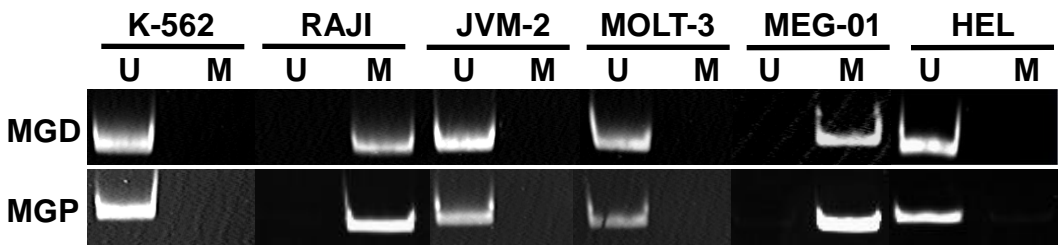
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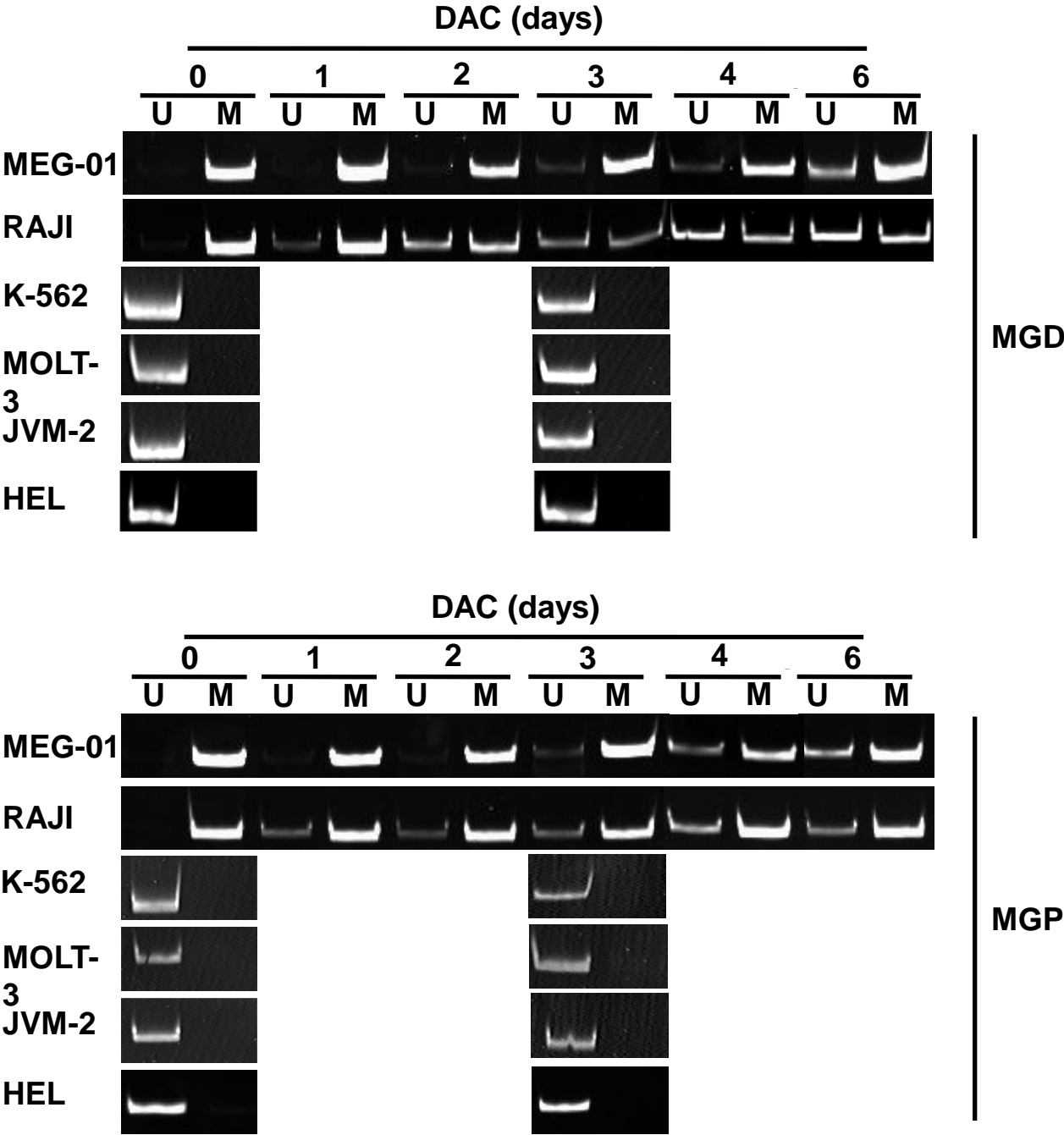
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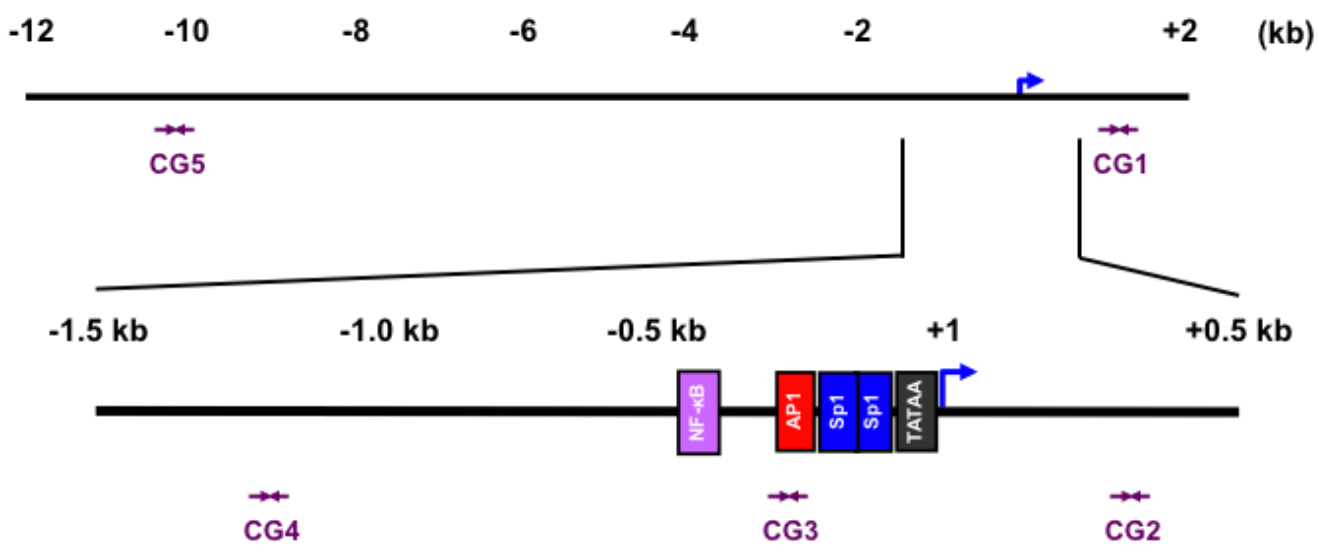
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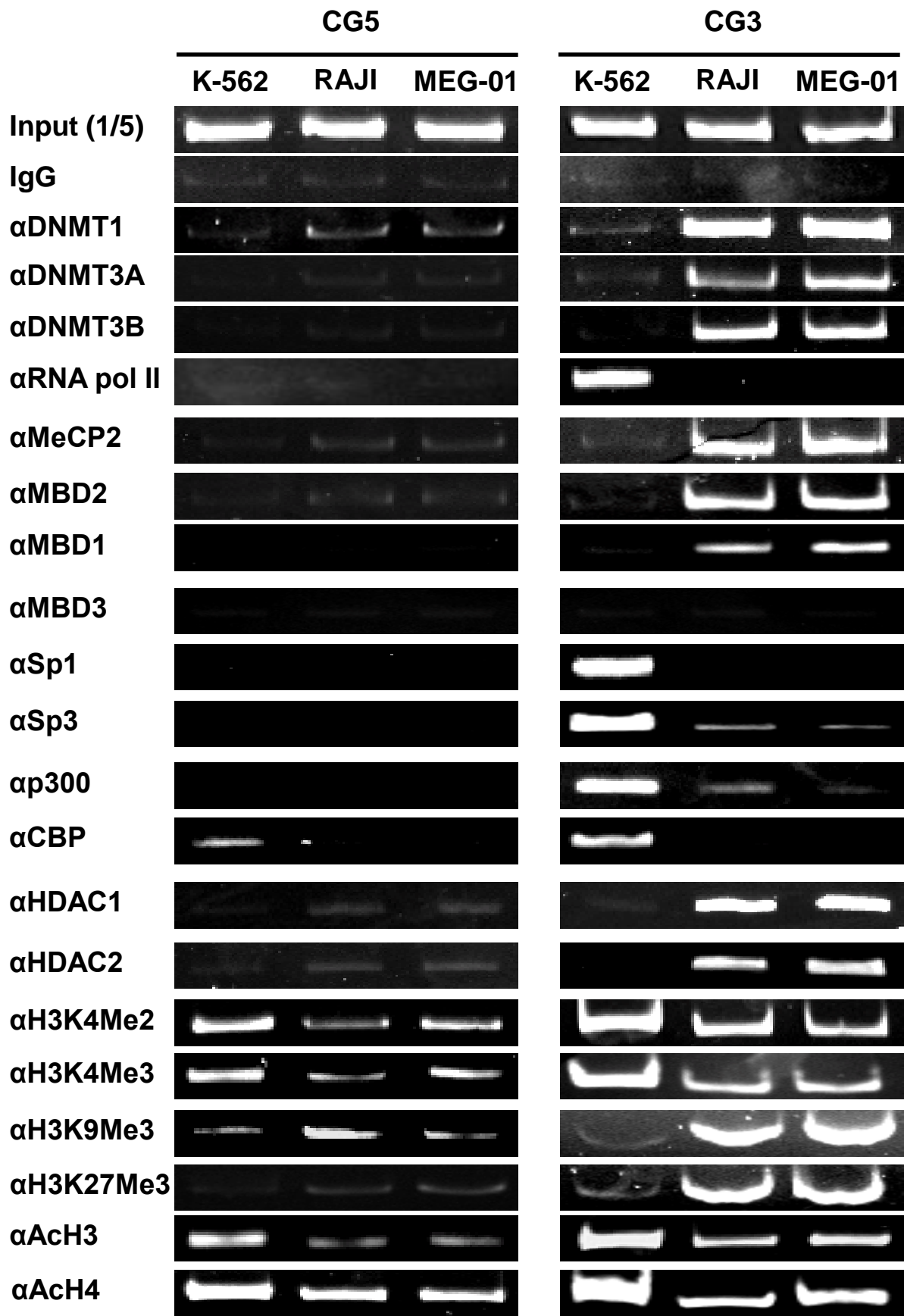
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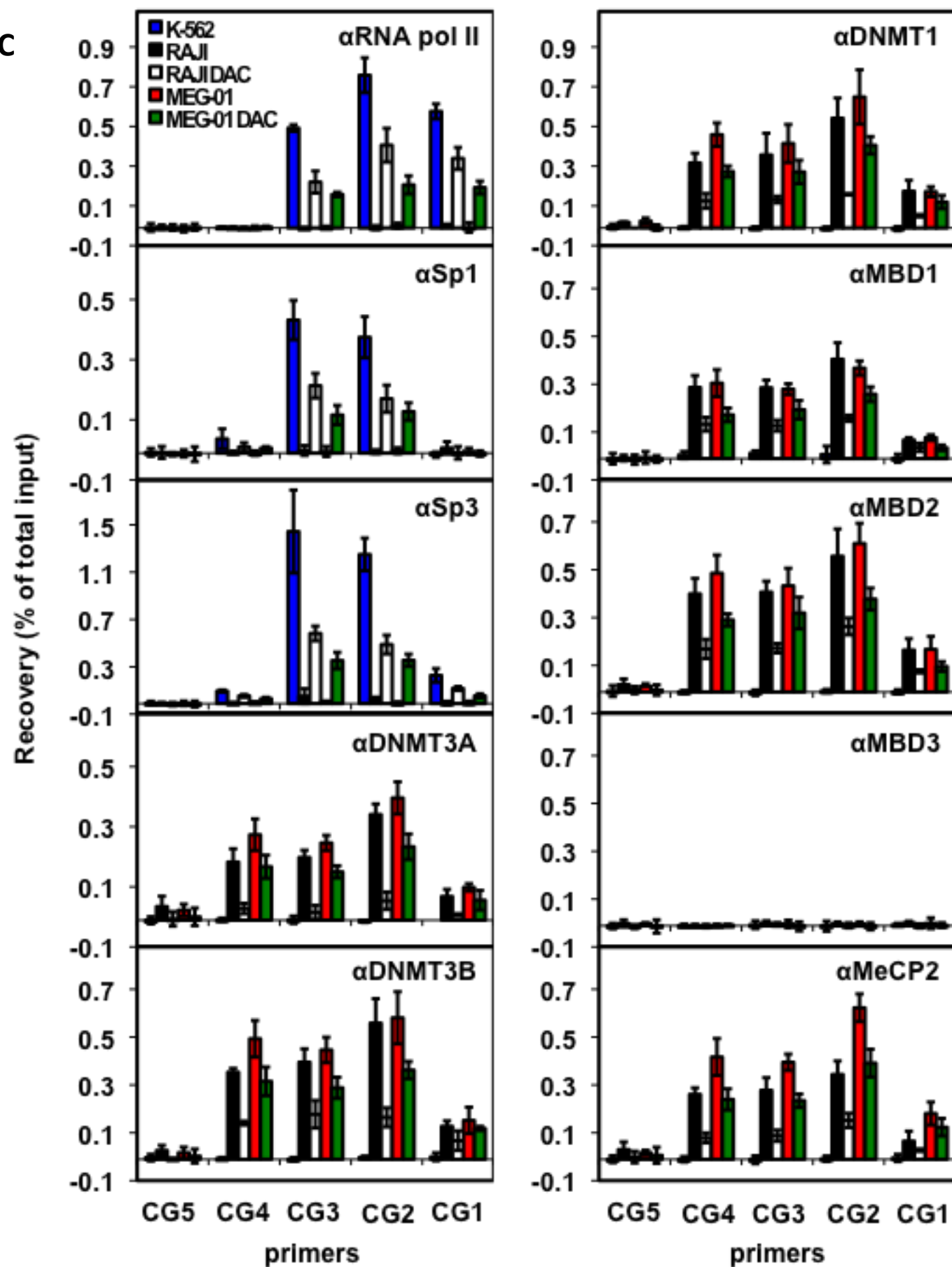
A



B



C



C

