CHAPTER IV

RESULTS

Both EDSBs and global hypomethylation have been proposed to cause CIN, however, how global hypomethylation associates with EDSBs is unknown. Thus, the aim of this thesis was to study if DNA methylation is associated with EDSB biological processes. Because there has been no available technique to characterize EDSB sequences, several methods were established for this thesis. These include L1-EDSB-LMPCR to quantitate EDSBs, COBRA-L1-EDSB to quantitate EDSB methylation, L1-EDSB-LM-MSP to map EDSB hot spot in LINE-1 sequences and associate with methylation. We also combined chromatin immunoprecipitation (ChIP) of γ-H2AX with COBRA-L1 to evaluate the methylation status of γ-H2AX bound DNA.

EDSB quantity

In order to determine quantity of EDSB sequences, we developed a novel technique, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (20), a commonly used PCR technique designed for the analysis of EDSBs during lymphoid development, such as V(D)J recombination and somatic hypermutation (21). Since general EDSBs are believed to occur rarely and randomly throughout the genome, repetitive sequences that widely intersperse in the human genome can be applied in a similar assay for the detection of EDSBs in their proximity, which would represent genome-wide EDSBs. Therefore, we combined LMPCR with interspersed repetitive sequence PCR (IRSPCR) (22) using LINE-1 (L1) human retrotransposons (54) (L1-EDSB-LMPCR). In this assay, linker oligonucleotides are ligated to EDSBs in high molecular weight DNA preparation and quantitatively analyzed by realtime PCR using an L1 primer and a Taqman probe complementary to the linker (Fig. 4). The figure 5 showed an example of results of L1-EDSB-LMPCR by using realtime PCR. It indicated that EDSBs could be quantitated by this technique. This technique had minimal intra-assay variations, but a larger range of inter-assay variations (Fig. 6). The quantity of EDSBs was not related to the proportion of fragmented cells (Fig. 7). While there were positive amplifications, we were unable to

detect any apoptotic fragmented DNA or cells, as determined by LMPCR ladder (66) and flow cytometry (83), respectively. Furthermore, L1-EDSB levels did not differ relative to the directions of L1 primers used (Fig. 8). Using L1-EDSB-LMPCR, significant amounts of EDSBs were determined in all samples from several cancer cell lines, including Daudi, Jurkat, Molt4, K562, SW480, and HeLa cells, as well as in normal cells, including sperm and white blood cells (WBCs), from several individuals. Additionally, different cell types bored significantly different quantities of EDSBs. We found that epithelial cells including SW480 and HeLa cells possessed less EDSBs than hematopoietic cells including Daudi, Jurkat, Molt4 and K562 (Fig. 9).

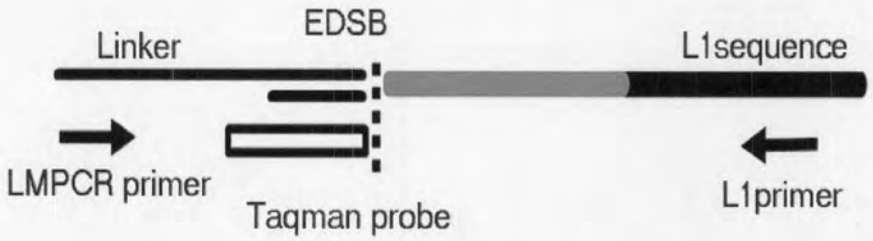

Figure 4 Schematic illustration of L1-EDSB-LMPCR. L1 sequence is ligated to linker at EDSB. The white rectangle is Taqman probe, complementary to with LMPCR linker. Arrows are PCR primers.

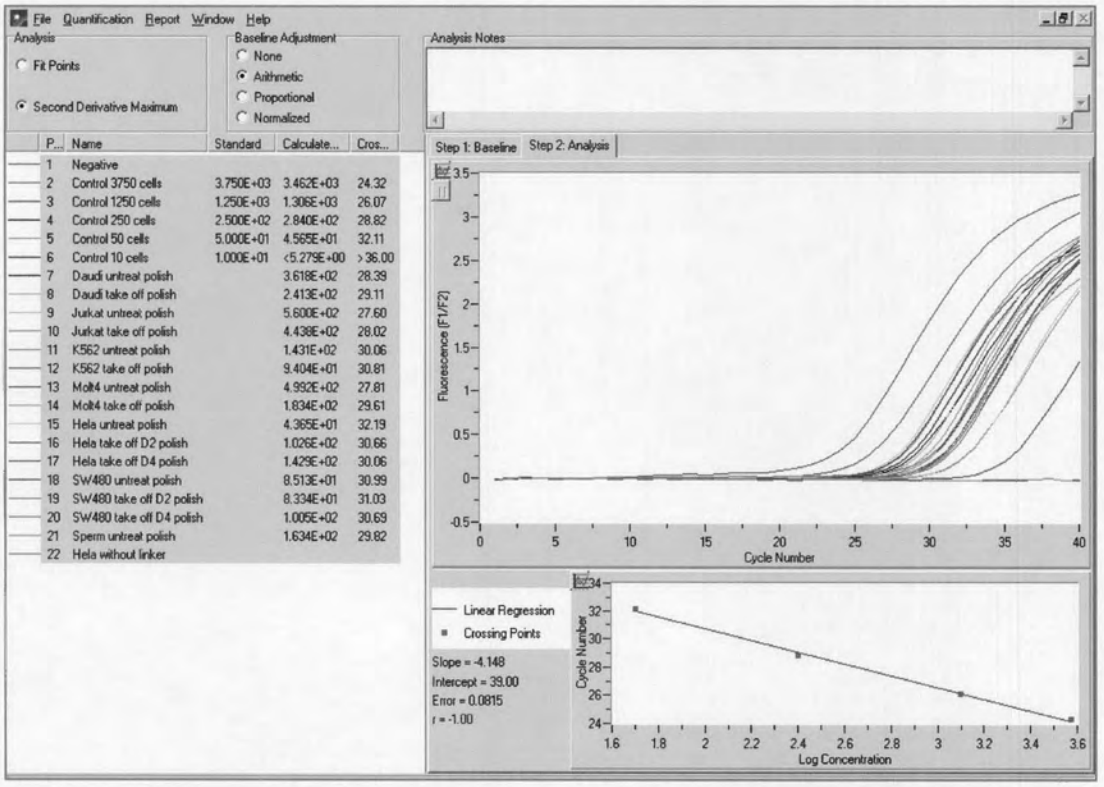

Figure 5 An example of results of L1-EDSB-LMPCR by using realtime PCR

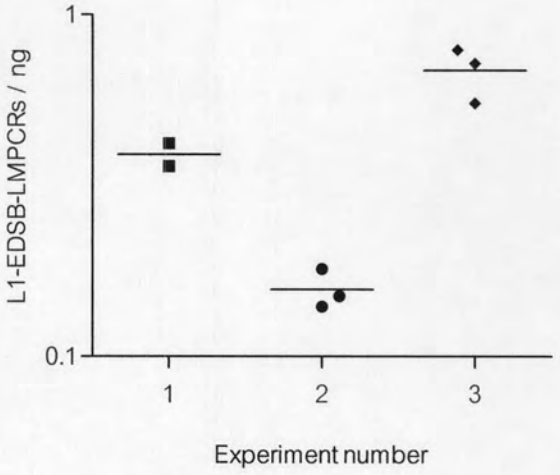

Figure 6 Intra- and inter-assay variations of L1-EDSB-LMPCR. Experiment numbers 1, 2 and 3 indicate independent assays and each spot represents a different culture flask.

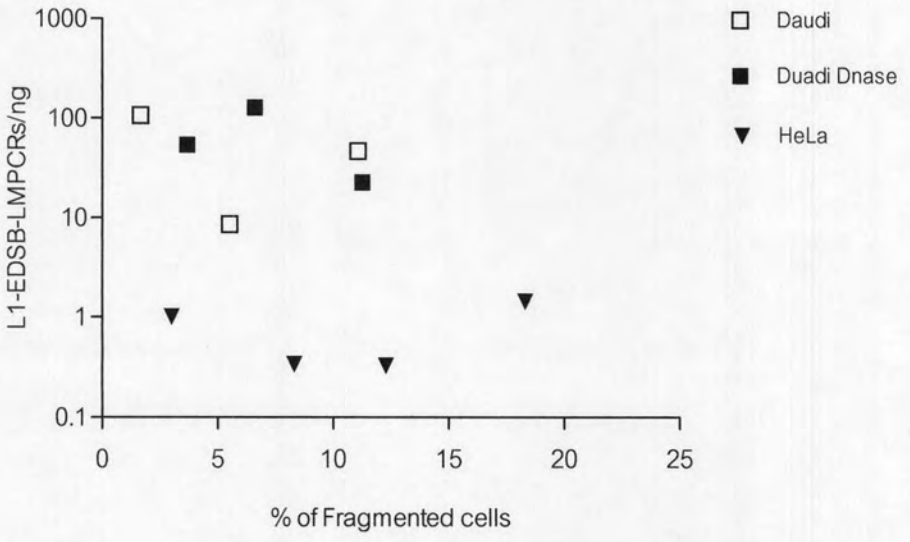

Figure 7 L1-EDSB-LMPCR quantity in relation to % fragmented cells, documented by flow cytometry. Daudi DNase represents Daudi cells treated with *DNase* I.

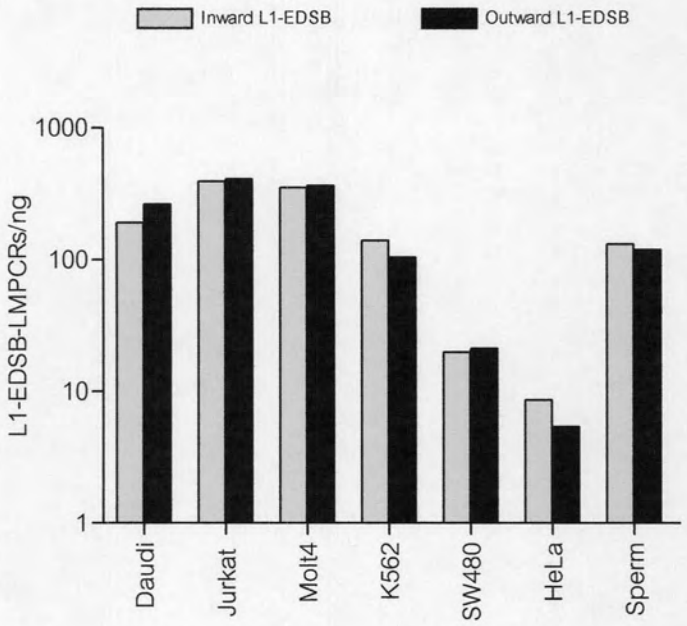

Figure 8 L1-EDSB-LMPCR quantities in several cell types by using different direction of L1 primers. L1 inward primer amplifies inside L1 sequence while L1 outward primer amplifies outside. Inward L1-EDSB-LMPCR was normalized by the proportion of number of L1 oligonucleotide sequence copies in the human genome (www.ncbi.nlm.nih.gov).

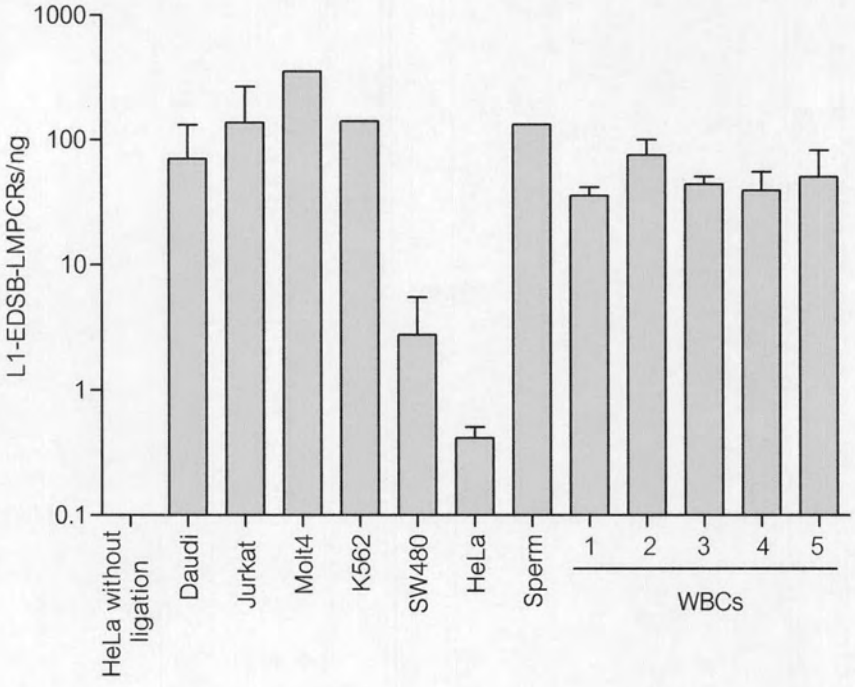

Figure 9 L1-EDSB-LMPCR quantities in several cancer cells and normal cells including sperm cell and white blood cells (WBCs) from several individuals. HeLa without ligation was a negative control.

EDSB methylation status

Methylation status of L1s have been extensively studied in several cancers and normal tissues by PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (3). To compare percentage of methylation level between genomic DNA and EDSBs, matched pair degree of methylation between L1 and L1-EDSB sequences was examined by COBRA-L1 and COBRA-L1-EDSB, respectively. COBRA-L1 was performed as previously described to quantify genome-wide methylation status. For COBRA-L1-EDSB, all ligated HMW DNA samples were chemically modified by bisulfite. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. Subsequently, the PCR products of bisulfite 5'L1 sequences were digested with *Taq*I and *Tas*I as restriction enzymes. While *Taq*I detected methylated L1 sequences, *Tas*I detected nonmethylated L1 sequences. The percentage of *Taq*I digestible amplicon was measured as COBRA-L1 methylation level by α-^{32}P-labeled-bisulfite-L1-outward, B-L1-outward, radiation intensity. For COBRA-L1-EDSB, the same protocol was adopted but the B-L1-inward primer was replaced by a linker primer, B-LMPCR (Fig. 10 and 11). Similar to L1-EDSB-LMPCR, this technique had minimal intra-assay variations, but a larger range of inter-assay variations (Fig. 12). Although fluctuation of EDSB methylation status was observed among different experiments, EDSBs were hypermethylated across all tested cells, including several cancer cell lines, sperm and WBCs (Fig. 13). This result indicated that EDSBs were associated with DNA methylation.

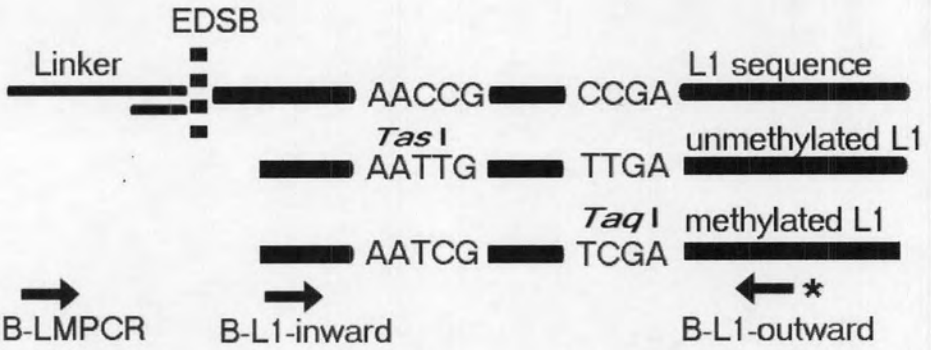

Figure 10 Schematic illustration of COBRA-L1 and COBRA-L1-EDSB. L1 sequence is ligated to linker at EDSB. Arrows are PCR primers. Asterisk indicates α-^{32}P-labeled primer for COBRA. AACCG and CCGA are L1 sequences. After these sequences are treated with bisulfite and PCR, unmethylated AACCG and methylated CCGA will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site), respectively. The both sequences can be digested with *Tas*I and *Taq*I, respectively.

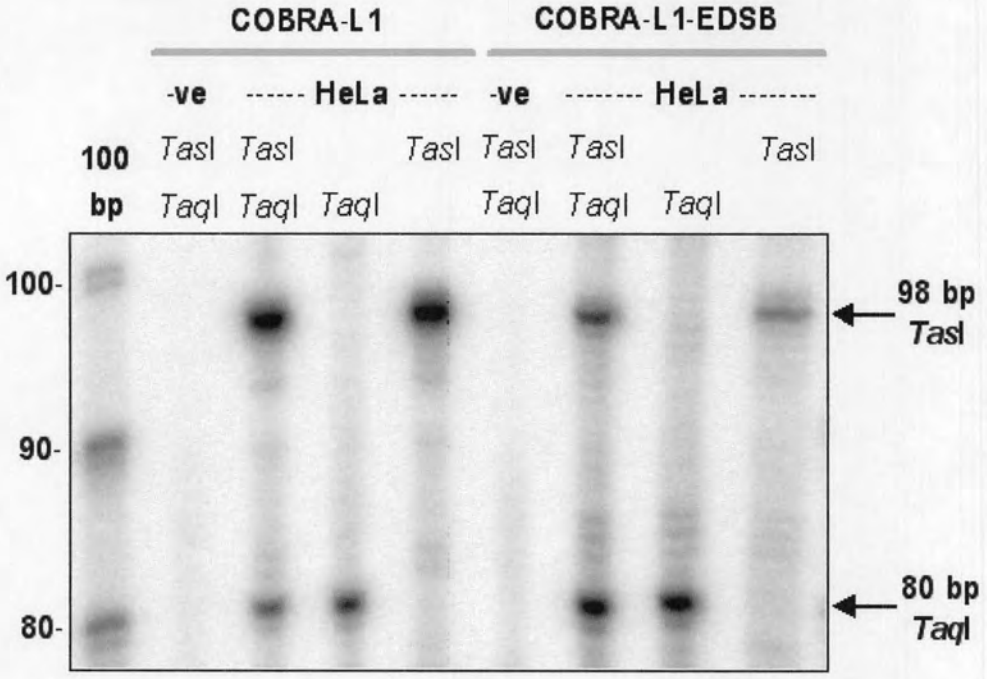

Figure 11 A typical example of result from COBRA-L1 and COBRA-L1-EDSB experiments. The arrow at 98 bp indicates *Tas*I digested unmethylated L1 sequences and the arrow at 80 bp indicated *Taq*I digested methylated L1 sequences. Negative control (–ve) was dH_2O for COBRA-L1 and nonligated HMW DNA for COBRA-L1-EDSB. *Tas*I and *Taq*I are enzymes, added in each experiment.

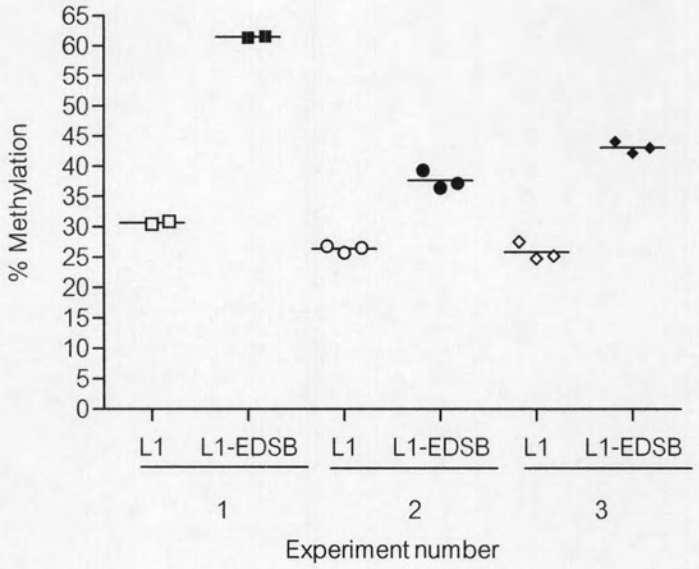

Figure 12 Intra- and inter-assay variations of COBRA-L1 (L1) and COBRA-L1-EDSB (L1-EDSB). Experiment numbers 1, 2 and 3 indicated independent assays and each spot represents a different culture flask.

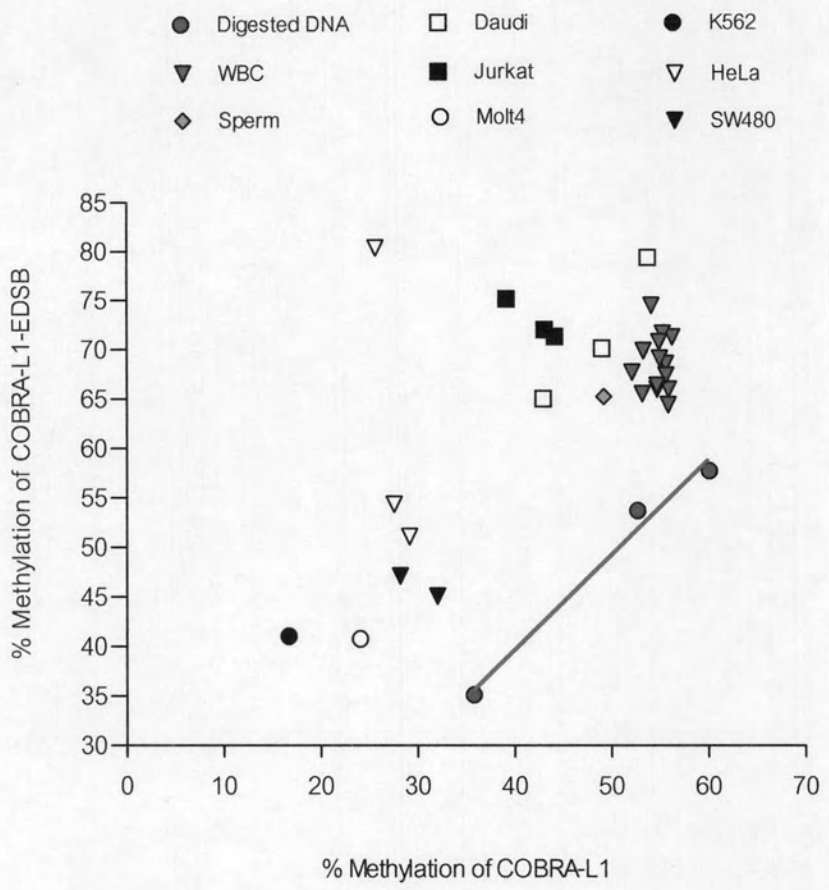

Figure 13 COBRA-L1 and COBRA-L1-EDSB comparison among cell types. Digested DNA was HeLa, Jurkat and Daudi DNA, digested with *Alu*I and *EcoR*V, as controls.

EDSB hot spots and the relation to methylation

There are two types of chromatin structure in higher eukaryotic cells, heterochromatin and euchromatin. Heterochromatin is highly condensed form and comprises of methylated DNA. In contrast, euchromatin is loosely condensed form and contains unmethylated DNA. Thus, we hypothesized that the loosely packaged area may be susceptible to DNA damage. If EDSBs occur at both methylated and unmethylated DNA, they should be induced by different mechanisms. We therefore can detect different pattern of EDSBs (EDSB hot spots). To prove this hypothesis, we screened for EDSB hot spots between methylated and unmethylated DNA and identified if they possessed methylation bias by L1-EDSB-LM methylation specific PCR (L1-EDSB-LM-MSP). This technique applied nested PCR to increase sensitivity and specificity. In the first PCR, all ligated HMW DNA samples were treated with bisulfite and amplified by using B-LMPCR 1^{st} primer as forward primer and either Met-L1-inward or Unmet-L1-inward primers. Met-L1-inward primer was complementary to methylated L1 sequence while unmet-L1-inward was complementary to unmethylated L1 sequence, directed into L1 sequence (Fig. 14). In the second PCR, amplicons of both primer sets were amplified by using α-^{32}P-labeled B-LMPCR and L1-Bihot primers (Fig. 14). L1-EDSB-MSP was interpreted by analyzing the band patterns of methylated and unmethylated EDSBs as DNA fingerprinting for mapping the locations of EDSBs. Additionally, we further investigated whether EDSBs hot spots associated with methylation status. We hypothesized that hypomethylated DNA induced by 5-aza-2-deoxycytidine (5-azadC) treatment have more EDSBs than hypermethylated DNA (5-azad-untreated cells). Therefore, EDSB hot spots of 5-azadC-treated cells should be detected in higher quantity than 5-azadC-untreated cells at unmethylated DNA. To prove this hypothesis, we increased hypomethylated DNA in cancer cell lines by using 5-azadC as DNA methylation inhibitor. It can convert methylated sequences to unmethylated sequences. To evaluate the fingerprint patterns, we compared the methylation level of bands between untreated and treated cells. We observed common and random band patterns in both 5-azadC-treated and 5-azadC-untreated Daudi, Jurkat, Molt4, SW480 and HeLa cells (Fig 15). Moreover, in both band patterns, the methylation of L1-EDSBs was higher

than methylation of genomic DNA (Fig 16). From these results, it implied that although the mechanisms inducing common and random band patterns of EDSB hot spots were different, the methylation status of EDSBs in both categories was similar. We therefore concluded that the mechanism inducing EDSBs hot spots were DNA methylation independent.

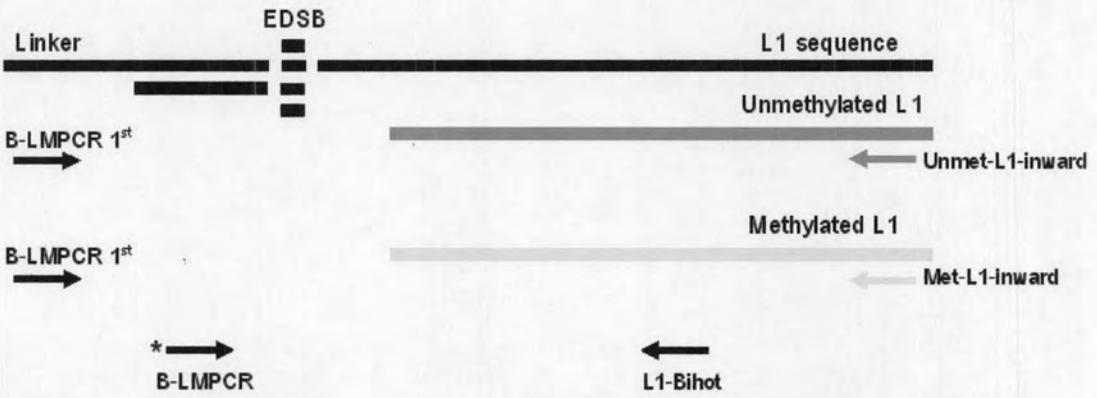

Figure 14 Schematic illustration of L1-EDSB-LM-MSP. L1 sequence was ligated to linker at EDSB. Arrows are PCR primers. Asterisk indicates α-^{32}P-labeled primers.

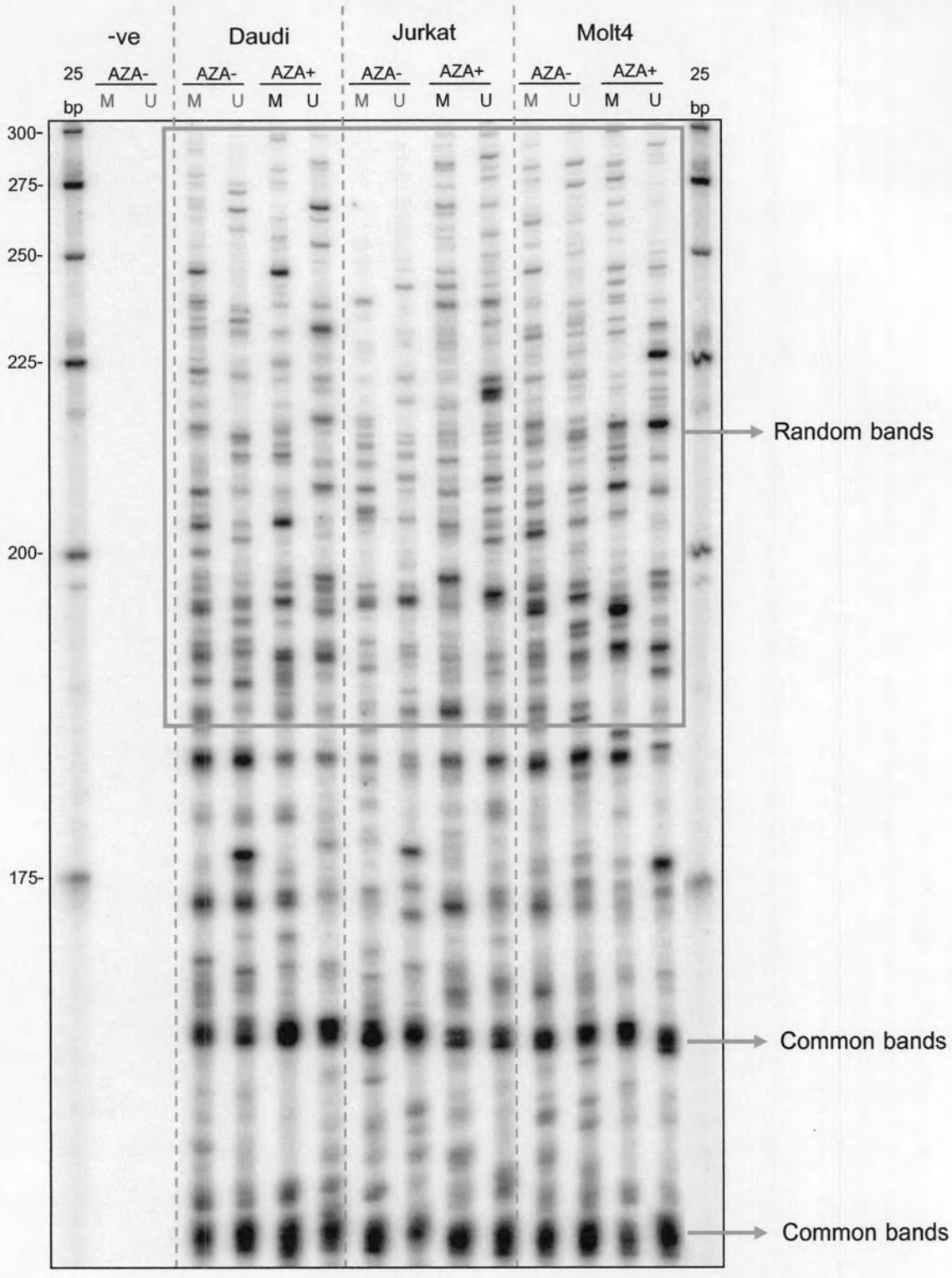

Figure 15 An example of results of EDSB hot spots from L1-EDSB-LM-MSP. Several cell lines were untreated with 5-aza-2-deoxycytidine (AZA-) and treated with 5-aza-2-deoxycytidine (AZA+) and then analyzed band patterns by L1-EDSB-LM-MSP. M indicated band patterns of methylated EDSBs and U pointed to band patterns of unmethylated EDSBs. Negative control (–ve) was dH_20.

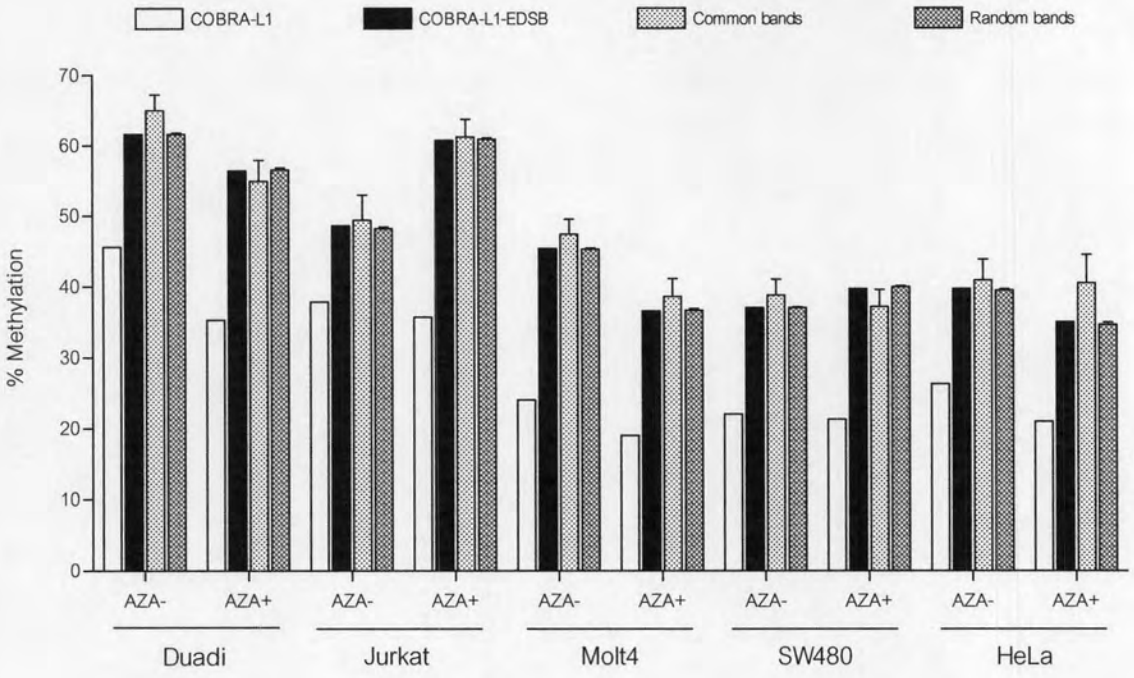

Figure 16 Percentage of methylation level in several cell lines from COBRA-L1, COBRA-L1-EDSB and L1-EDSB-LM-MSP. Common and random bands were obtained by L1-EDSB-LM-MSP. All cell lines were untreated with 5-aza-2-deoxycytidine (AZA-) and treated with 5-aza-2-deoxycytidine (AZA+)

DNA replication increases EDSBs but not EDSB methylation

From our study by COBRA-L1-EDSB, we found that the majority of EDSBs were methylated. Therefore, we hypothesized that hypermethylated EDSBs may arise from one of two causes: either methylated DNA carries a higher rate of EDSB production or a lower rate of EDSB repair. To distinguish between these two hypotheses, we first examined EDSB production. Since EDSBs were proposed to be preferentially produced in S phase from the conversion of single strand lesions (7), we assessed the amount of EDSBs and their methylation status in several cell cycle phases, G0, G1/S and S, in HeLa cells. Cell lines were synchronized in each cell cycle before theses cells were measured EDSB level by L1-EDSB-LMPCR and methylation status by COBRA-L1 and COBRA-L1-EDSB. As predicted, the result demonstrated that G0 phase bore the least EDSBs, compared with active replications and showed statistical significance (Fig. 17). In addition, we observed that EDSBs were hypermethylated in most examined cell phases. Especially, G0 phase illustrated the most statistical significance (Fig. 18). This data suggested that hypermethylation of EDSBs was DNA replication-independent.

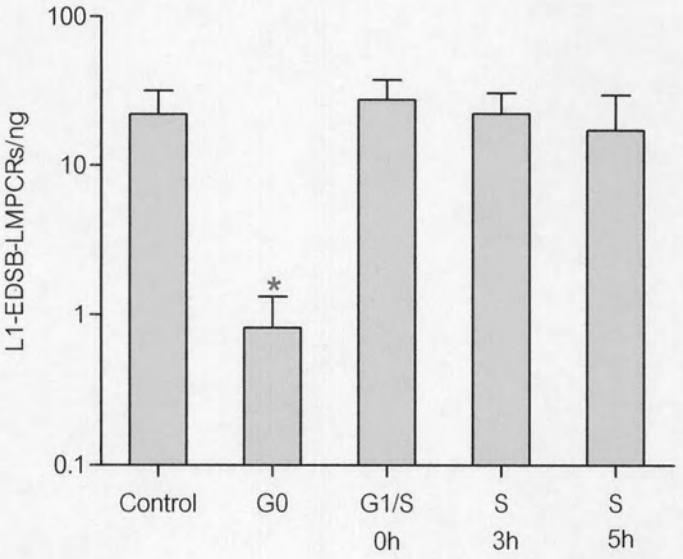

Figure 17 L1-EDSB-LMPCR quantities of HeLa cells in G0, G1/S at 0 h, and S phases at 3, and 5 h after the release into S phase from thymidine block, respectively. Control is without cell synchronization. Data represent means ±SEM. Single asterisk indicates statistical significance at $P<0.05$ (independent 1-tailed *t*-test).

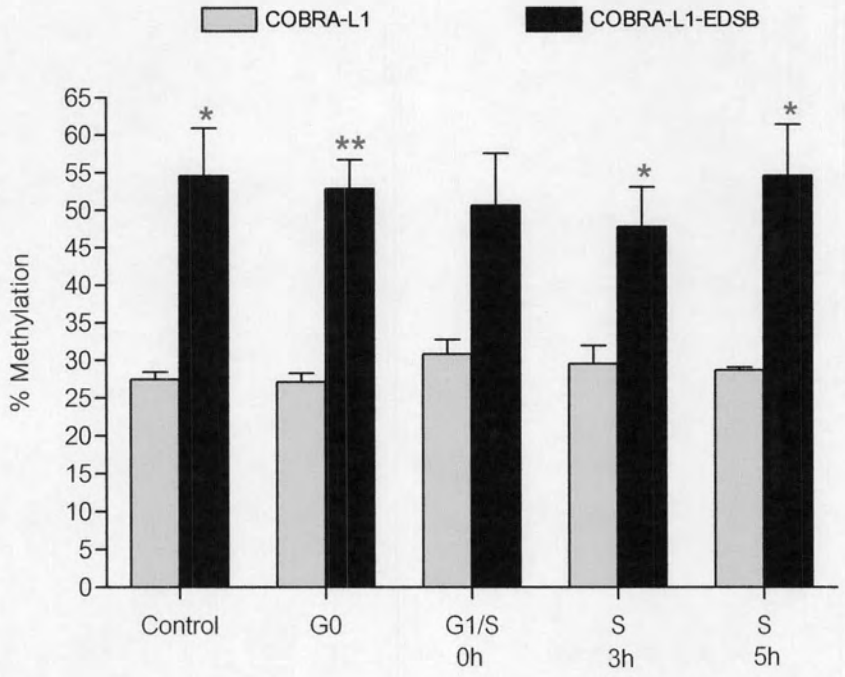

Figure 18 COBRA-L1 and COBRA-L1-EDSB levels of HeLa cells in several cell cycles. Control is without cell synchronization. Data represent means ±SEM. Single, double asterisks indicate statistical significance at $P<0.05$ and $P<0.001$ (pair 1-tailed *t*-test), respectively.

Methylated EDSBs are retained in heterochromatin

Another possible mechanism results in ubiquitous presentation of methylated EDSBs may be dued to that methylated EDSBs are more innert because of the association between DNA methylation and heterochromatin. We hypothesized that heterochromatin may brace and shield EDSBs from cellular DNA repair response. Consequently, there is preferential cellular response to nonmethylated EDSBs. To explore the physiologic mechanism by which methylated EDSBs were retained, we first measured the quantity and methylation status of γ-H2AX-bound DNA. γ-H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DSB repair responses (23). γ-H2AX-bound DNA was obtained by ChIP assay (24) using a γ-H2AX antibody (85). Subsequently, precipitated γ-H2AX-bound DNA was detected the quantity by realtime PCR with 5´L1 primers, termed realtime 5´L1PCR and the methylation level by COBRA-L1, termed COBRA-L1-H2AX. To prove that ChIP assay is able to precipitate γ-H2AX-bound DNA, we performed realtime 5´L1PCR to determine precipitated DNA from HeLa cells after exposure to different doses of γ-radiation. We observed a linear correlation between the amount of precipitated DNA and the level of exposure to radiation. This result indicated that this technique could quantitatively detect the level of response to radiation-induced DSBs (Fig. 19). Further experiment, we determined the quantity of γ-H2AX-bound DNA in several cell lines and during cell cycles of HeLa cells (Fig. 20). Noteworthy, whereas leukemic cell lines possessed more L1-EDSB-LMPCR than did epithelial cells (Fig. 9), HeLa cells had more γ-H2AX-bound L1s than did Jurkat or Daudi cells. This result suggested that the amount of γ-H2AX-bound L1s may be cell-type specific and inversely related to that of L1-EDSB-LMPCR. Additionally, we also analyzed methylation status of γ-H2AX-bound DNA by using COBRA-L1-H2AX. We found that methylation status of γ-H2AX-bound DNA in all cells and most tested cell cycle phases was significantly hypomethylated, compared with methylation level of EDSBs (Fig. 21). Interestingly, methylation status of γ-H2AX-bound-DNA in Daudi cells was less than their EDSBs and genomic DNA (Fig. 21). These results showed that γ-H2AX binding may be nonrandom with bias towards nonmethylated DNA and there possibly exist methylated EDSBs free from γ-H2AX.

DNA methylation is usually associated with heterochromatin and histone deacetylation (25, 26). Furthermore, DSB repair can be regulated by histone acetylation, which can enhance phosphorylation of H2AX in the context of nucleosomes. Therefore, we hypothesized that methylated EDSBs that escape the γ-H2AX response are retained within heterochromatin. To test this hypothesis, we converted heterochromatin of HeLa cells into euchromatin with a histone deacetylase inhibitor, trichostatin A (TSA). To confirm that TSA is able to increase the acetylation level of histone H4 in HeLa cells, growing cells were treated with 100 ng/ml TSA for 2-8 hours and harvested for protein analysis by western blot. As can be observed in Figure 22, TSA treatment greatly increased the acetylation level of histone H4 in all times of treatment. Our goal was to analyze whether TSA treatment had an effect on the binding of γ-H2AX to methylated EDSBs. Thus, we evaluated the amount of γ-H2AX-bound DNA and EDSBs in treated cell lines by realtime 5′L1PCR and L1-EDSB-LMPCR, respectively. The result of L1-EDSB-LMPCR showed no significant alteration of EDSB levels in treated HeLa cells (Fig. 23). In contrast, the result of realtime 5′L1PCR revealed significant increment of the quantity of DNA binding to γ-H2AX after TSA treatment (Fig. 24). Furthermore, we determined methylaltion status of γ-H2AX-bound DNA, genomic DNA and EDSBs by using COBRA-L1-H2AX, COBRA-L1 and COBRA-L1-EDSB, respectively. We observed that the TSA treatment increased methylation level of DNA binding to γ-H2AX but did not change methylation status of both genomic DNA and EDSBs (Fig. 25). These evidences suggested that methylated EDSBs escaped the γ-H2AX response because of retained in heterochromatin.

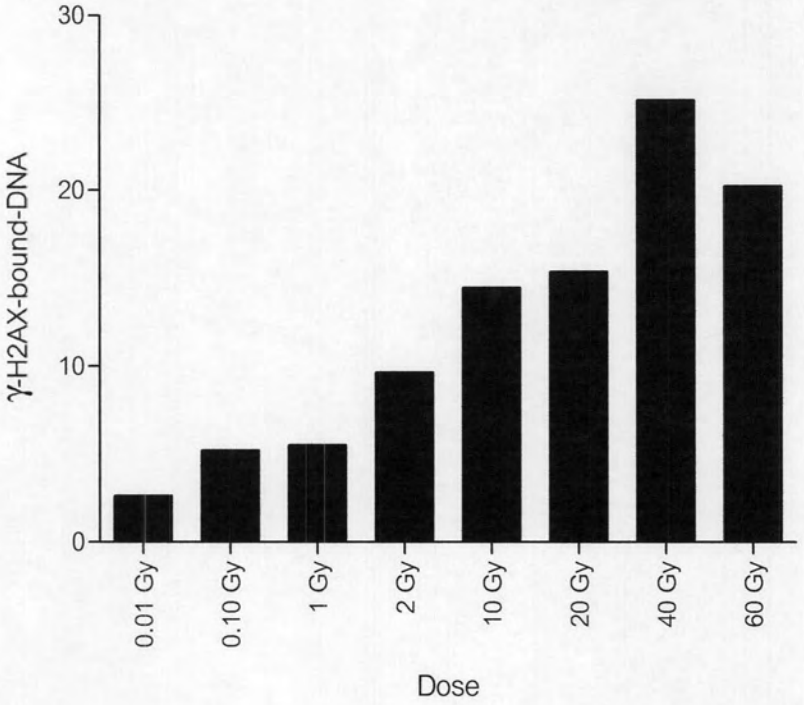

Figure 19 Level of γ-H2AX-bound DNA in HeLa cells after exposure to different doses of γ-radiation. The quantity unit is Mb per cell.

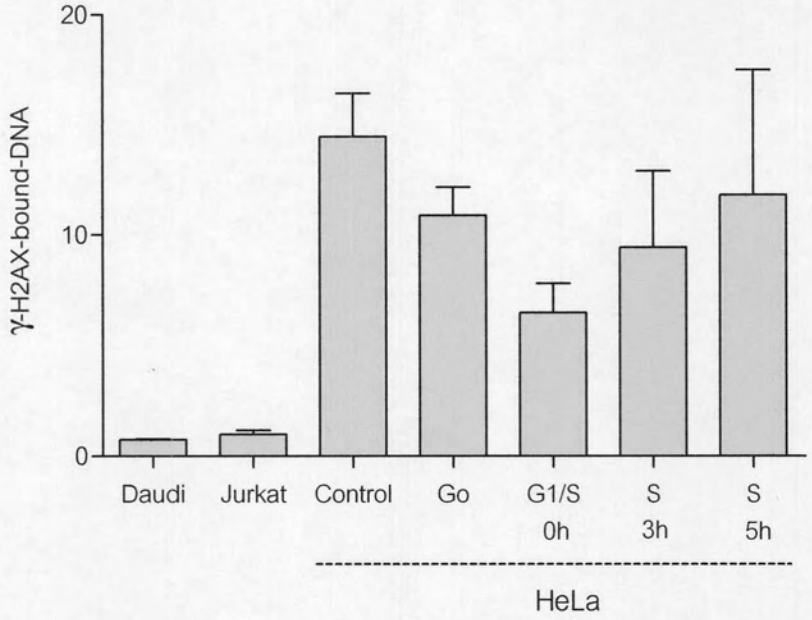

Figure 20 Level of γ-H2AX-bound DNA in several cell types and during cell cycles. The quantity unit is Mb per cell and data represent means ±SEM.

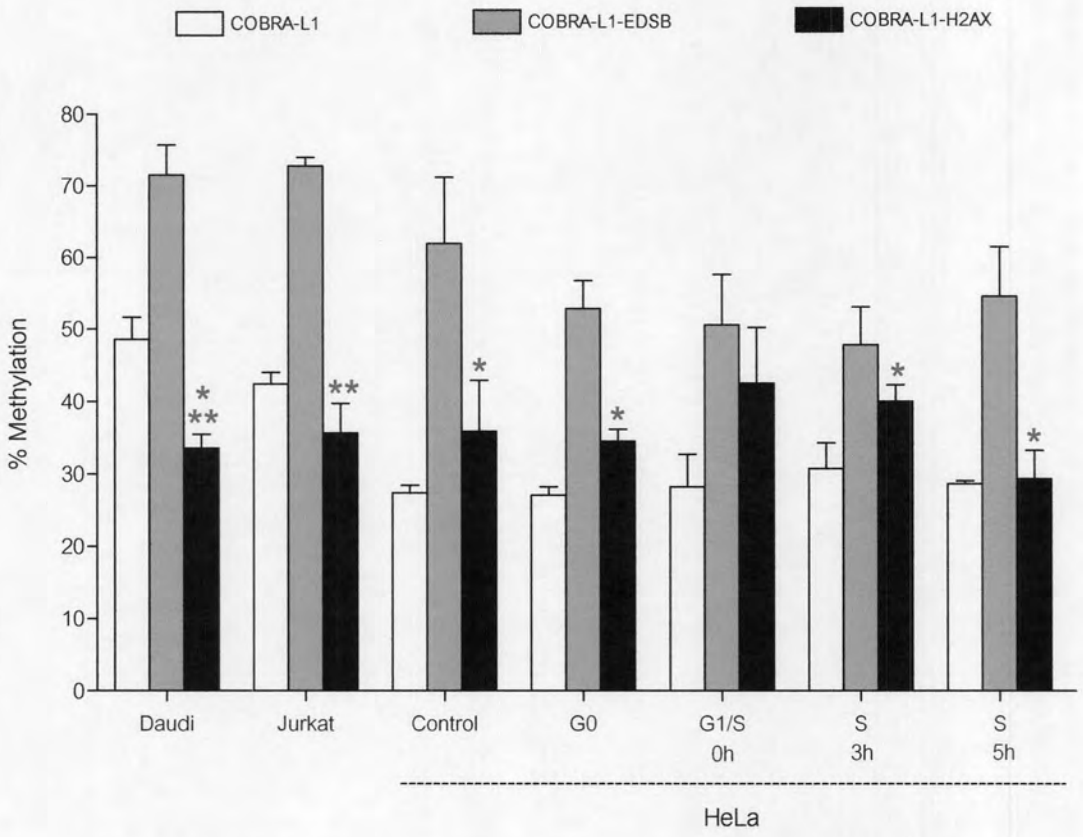

Figure 21 Methylation levels of different cell types and in different phases of the cell cycle. Data represent means ±SEM. In comparison between COBRA-L1-H2AX and COBRA-L1-EDSB, single, double and triple asterisks indicate statistical significance at $P<0.05$, $P<0.01$ and $P<0.001$ (pair 1-tailed *t*-test), respectively. Single asterisk of Daudi cells also indicates statistical significance at $P<0.01$ (pair 1-tailed *t*-test), when COBRA-L1-H2AX was compared with COBRA-L1.

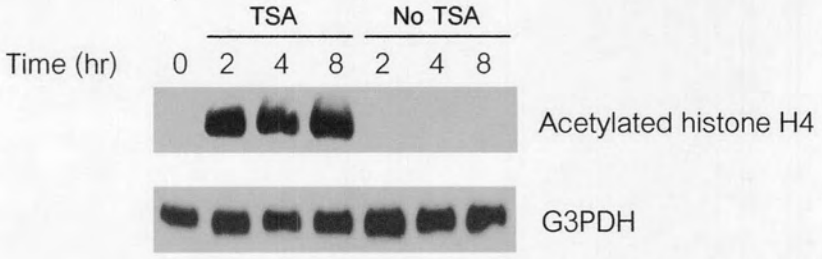

Figure 22 Western blots. Total proteins were extracted from HeLa cells after treated with 100 ng/ml TSA for the indicated number of hours. 50 μg of proteins was load on a 15% SDS-PAGE and actylated histone H4 was detected. G3PDH was used as a control for loading.

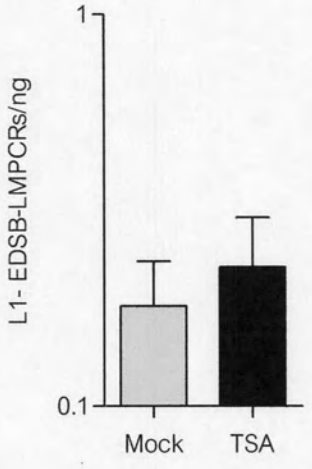

Figure 23 Quantity of L1-EDSB-LMPCR in HeLa cells after TSA treatment. Mock was untreated cells as control. Data represent means ±SEM.

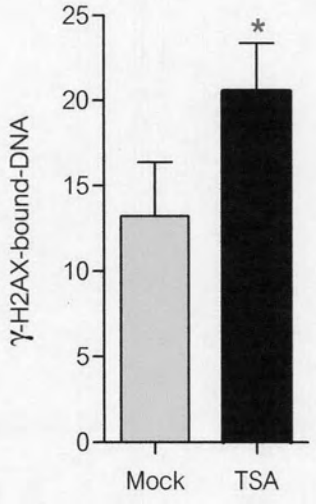

Figure 24 Level of γ-H2AX-bound DNA in HeLa cells after TSA treatment. Mock was untreated cells as control. The quantity unit is Mb per cell and data represent means ±SEM. Single asterisk indicates statistical significance at $P<0.01$ (pair 1-tailed *t*-test).

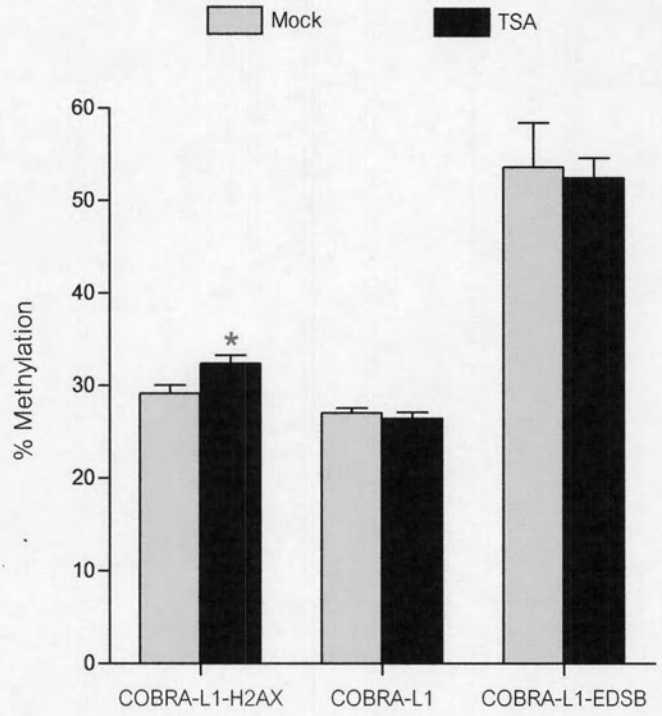

Figure 25 Comparative EDSB methylation levels of γ-H2AX-bound DNA, L1s, and EDSBs between mock and TSA. (D) *P<0.01, and, (F) * *P*<0.001. Data represent means ±SEM. Single asterisk indicates statistical significance at *P*<0.001 (paired 1-tailed *t*-test.).