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THE STUDY OF CORRELATION BETWEEN TEMPERATURE AND ENDOGENOUS DNA DOUBLE-STRAND BREAKS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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วัตถุประสงค์หลักของงานวิจัยในครั้งนี้ คือ ตรวจสอบว่าการเพิ่มอุณหภูมิมีผลต่อการฉีกขาดของดีเอนเอหรือไม่ ซึ่งจากการทดลองที่ผ่านมาพบว่า การให้อุณหภูมิที่สูงขึ้น จะมีผลต่อการเพิ่มขึ้นของการเติมหมู่ฟอตเฟสบริเวณ serine 139 ของ histone H2AX หรือเรียกว่า γ-H2AX ซึ่งทำหน้าที่เป็นตัวระบุบริเวณที่เกิดการฉีกขาดของดีเอนเอ แต่ไม่สามารถตรวจพบการฉีกขาดของดีเอนเอได้โดยทั่วไป การฉีกขาดของดีเอนเอจะทำให้เกิดการตายของเซลล์ แต่จากการทดลองที่ผ่านมาไม่พบว่าเซลล์มีการตายเมื่อได้รับอุณภูมิที่สูงขึ้น ดังนั้น γ-H2AX ที่เพิ่มขึ้นนี้ อาจไม่ได้เกิดจากการฉีกขาดของดีเอนเอ หรือการขาดเกิดขึ้นน้อยเกิดกว่าจะสามารถตรวจพบได้ การทดลองนี้จึงได้นำวิธี IRS-EDSB-LMPCR ซึ่งมีความไวในการวัดการฉีกขาดของดีเอนเอในปริมาณน้อยมาใช้ตรวจสอบการฉีกขาดของดีเอนเอที่เกิดขึ้นเองระหว่างเซลล์ในอุณหภูมิปกติ และ เซลล์ที่ได้รับอุณหภูมิสูง โดยได้ทดลองในเซลล์มะเร็งหลายชนิด รวมถึง เซลล์ปกติด้วย จากการทดลองพบว่า มีเซลล์สองชนิด คือ HeLa และ HN8 เมื่อให้อุณหภูมิสูง จะพบการเพิ่มขึ้นของการฉีกขาดของดีเอนเออย่างมีนัยสำคัญ ($P<0.01$และ $P<0.05$ ตามลำดับ) เซลล์ทั้งสองชนิดนี้มีระดับของหมู่เมทิลต่ำกว่าเซลล์ชนิดอื่นซึ่งจะมีผลทำให้โครงสร้างของดีเอนเอพันเกลียวกันอย่างหลวม จึงได้นำ TSA ซึ่งจะสามารถคลายเกลียวของดีเอนเอมาใช้ในการทดลองนี้ด้วย พบว่า เมื่อใส่ TSA แล้วนำไปเพิ่มอุณหภูมิ พบว่า HeLa จะมีการเพิ่มขึ้นของการฉีกขาดของดีเอนเออย่างมีนัยสำคัญ ($P<0.05$) จากการทดลองนี้ อาจกล่าวได้ว่า อุณหภูมิที่สูงขึ้นมีผลต่อการเพิ่มขึ้นของ γ-H2AX และการฉีกขาดของดีเอนเอ อาจเกี่ยวข้องกับลักษณะโครงสร้างของโครมาติน

สาขาวิชา วิทยาศาสตร์การแพทย์.....ลายมือชื่อนิสิต .....

ปีการศึกษา 2551.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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CHUTIPA PHUANGPHAIROJ : THE STUDY OF CORRELATION BETWEEN TEMPERATURE AND ENDOGENOUS DNA DOUBLE-STRAND BREAKS. ADVISOR: PROF. APIWAT MUTIRANGURA, M.D., Ph.D., 103 pp

The aim of this thesis is to evaluate if heat causes DNA double strand breaks (DSBs). There were several studies reported that heat increases of γ-H2AX foci, the serine 139 phosphorylated form of histone H2AX,, one of the earliest repair responses to DSBs. Nevertheless, there were no additional consequence of DSBs, such as apoptosis, was reported. Furthermore, heat did not demonstrate positive comet assay. Therefore, no actual evidence of DSB was detectable. Therefore, heat may induce γ-H2AX foci by other mechanisms besides DSBs or heat may induce a low number of DSBs. Consequently, the comet assay was negative and the consequences of heat induced DSBs were distinct from radiation. This thesis applied IRS-EDSB-LMPCR to compare the number of endogenous DSBs (EDSBs) between normal and heat induced genomes. Interspersed repetitive sequences (IRS) are randomly distributed. Hence, IRS-EDSB-LMPCR is a sensitive technique to measure rare and randomly distributed DSBs. We tested several epithelial and hematologic cell lines including, HeLa (cervical cancer), HN8, HN17 (Head and neck), Daudi (B lymphoblast), Molt4 (T lymphoblast) and Hacat (normal cell line) and found significant increase in EDSBs by heat in HeLa, $p < 0.001$, and WSU-HN8, $p < 0.05$. Interestingly, HeLa and WSU-HN8 possess the most genome-wide hypomethylation when compared with the others. This may suggest that heat increased EDSBs in cell may be correlate with chromatin structure. This hypothesis was proved by tricostatin A (TSA) treatment. Remarkably increased in heat induced EDSBs was found when TSA treated cells were heated, $P < 0.05$.

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Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

γ-H2AX	Phosphorylation of H2AX
ATM	Ataxia telangiectasia mutated protein
ATR	Ataxia telangiectasia related protein
AT	Ataxia telangiectasia
ATLD	Ataxia telangiectasia like disorder
BS	Bloom syndrome
BIR	Break-induced replication
CIN	Chromosomal instability
COBRA	Combined with bisulfite restriction analysis
COBRA-L1	COBRA of L1s
COBRA-L1-EDSB	COBRA of L1-EDSB
CSR	Class switch recombination
DDR	DNA damage responses
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double-strand breaks
DSBR	Double- strand break repair
EDSBs	Endogenous DNA double-strand breaks
FA	Fanconi anemia
HN	Head and Neck
HMW	High-molecular-weight
H2AX	Histone H2A variant X
HR	Homologous recombination
HSPs	Heat shock proteins
IR	Ionizing radiation
L1-EDSB-LMPCR	LINE-1(L1) human retrotransposons
LINE 1 or L1	Long Interspersed Nuclear Element type1
LMPCR	Ligation-mediated polymerase chain reaction
LN	lymph node

NHEJ	Non-homologous end-joining
NBS	Nijmegen Breakage syndrome
PIKKs	Phosphoinositide 3-kinase related protein kinases
SSA	Single-strand annealing pathway
ssDNA	Single-strand DNA
SDSA	The synthesis-dependent strand-annealing
VEGF	Vascular endothelial growth factor
UV	ultraviolet
WS	Werner syndrome

CHAPTER I

Introduction

Background and Rationale

Cancer commonly occurs from higher rate of mutation and genomic instability that cause chromosomal rearrangement and deletion of DNA, suggesting that DSBs are the intermediate products. Moreover, the mutations occur spontaneously, the DSBs should occur endogenously [1]. Vilenchik and Knudson [2] estimated the existence of endogenous double-strand breaks (EDSBs) and suggested that EDSBs could account for a substantial fraction of oncogenic events in human carcinomas. If EDSBs do not arise uniformly or are not processed at equal rates across the genome, mutation hot spots should be present [2]. Pornthanakasem *et al*, developed the new assay for detection of EDSBs, L1-EDSB-LMPCR. They found all cell types both cancer and normal could detected EDSBs level. And in G0 phase, HeLa cell has EDSBs level less than other cell cycle so that cell cycle stage is one of condition that related to the amount of EDSBs [1].

Kongruttanachok *et al.* study non-replicating cell and found consistent level of EDSBs in the same passage. It show that EDSBs not only presence in DNA replication but also presence in non-replicating genome. Moreover, after incubation time, EDSBs decreased show that the repair of EDSBs was occurring. They suggest that the rate of EDSBs repair were different in heterochromatin and euchromatin so that EDSBs in non-replicating cell (EDSB-NR) may be retained in heterochromatin and free form γ-H2AX.

γ-H2AX is phosphorylation at Serine 139 in C-terminal residue of histone H2AX. γ-H2AX is the DNA damage sensor that response to DSBs in the cell immediately and that make repaired pathway can work at a site of damage. γ-H2AX can increasing in many condition such as hyperthermia. Moreover, γ-H2AX can activate chromatin modification protein and cell cycle checkpoint protein [4-5]. γ-H2AX can increasing in many condition such as hyperthermia or radiation. Kaneko *et al.* detected increasing of γ-H2AX level when they treated cell in the high temperature but when they measured DSBs, they can't found the increasing [4].

Surprisingly, γ-H2AX, the DSBs sensor, increased in many condition but why can't detect different of DSB level. I chose hyperthermia to test this event because hyperthermia can applied in the treatment of cancer patients that make cell to sensitive to treatment. Although, many research study about hyperthermia but the mechanism for hyperthermia killing cell or cell sensitization is not clear. Therefore, I want to study correlation between high temperature and EDSBs level in the non replicating phase of cell.

Research Questions

How different temperature can effect EDSBs and γ-H2AX ?

Objectives

- to study between temperature change and EDSBs
- to study between temperature change and γ-H2AX

Hypothesis

Temperature change can make different EDSBs level

Key Words

Endogenous DNA double-strand breaks, γ-H2AX, temperature

Expected Benefit

Better understanding the mechanism about correlation between temperature and EDSBs

Research Methodology

Measured correlation between temperature and EDSB level

- corrected protein and detected γ -H2AX by western blot
- measured γ -H2AX level from ChIP assay
- detected EDSB level from HMW DNA by real time
- treated TSA and detected EDSB level
- analysis cell survival assay

Conceptual framework

How different temperature can effect EDSBs and γ -H2AX ?

↓

To explain mechanism related between temperature change and EDSBs change in G0 phase

↓

- Measure γ -H2AX change by western blot and ChIP assay
- Measure the quantity of EDSBs in G0 phase cell by using L1-EDSB-LMPCR

CHAPTER II

REVIEW OF THE RELATED LITERATURES

Hyperthermia

By the late 1980s have many literature describing heat effects on DNA, proteins, lipids and other cellular components. Hyperthermia temperature range (40–47^{o}C) in cancer therapy used to kill cell and those effects that alter the resistance of cells to radiation and/or chemotherapeutic agents [4-6]. Cancer cells are vulnerable to hyperthermia, particularly because of their high acidity caused by the inability to properly expel waste created by anaerobic metabolism. Hyperthermia can attack acidic cells by disrupting the stability of cellular proteins or act as an anti-angiogenic strategy by suppressing tumor-derived vascular endothelial growth factor (VEGF) production, thereby inhibiting endothelial cell proliferation and extracellular matrix remodeling in blood vessels. Such therapy has been used effectively for breast cancer, malignant melanoma, head and neck lymph node (LN) metastasis, glioblastoma, and cervical cancer. In addition, hyperthermia can promote immunologic host defenses in tumor tissue [7]. The combination of heat and radiation dose not result in more DNA damage than observed for radiation alone but heat shock prior, during, or immediately after ionizing radiation increase cell killing believed that hyperthermic radiosensitization is due to heat-induced inhibition of repair of radiation-induced DNA damage[4-5,8-10].

The goal of hyperthermia research is to find the molecular mechanism that heat can kill tumor or any cell and the mechanism that heat induce cell to sensitive with drug or radiation. They found that hyperthermia caused many effect in molecular changes and it can change function in all cellular compartment at temperature above 43^{o}C [6,9]. Two hypotheses have been postulated in the past to explain the thermal inhibition of DNA repair: (1) heat inactivates protein/enzyme involved in DNA repair [5,8-13] and (2) heat alters the chromatin structure rendering the damaged DNA less accessible for the repair enzyme complexes [8,11-13].

Hyperthermia has been shown to damage virtually every organelle in mammalian cells. At the level of the plasma membrane, transport is altered, presumably due to damage

to membrane transport proteins. Changes in the ionic content of the cell and the flux of ions across the membrane have been observed, and membrane receptor number and affinity are influenced by hyperthermia. There is extensive damage to the cytoplasm and cytoplasmic organelles. Cellular morphology is altered, primarily through a loss of microvilli, cell rounding and the appearance of blebs. These appear to be due to disruption of cytoskeletal structures (microtubules, microfilaments and intermediate filaments have all been shown to undergo depolymerization), which may be the most striking cellular changes occurring during hyperthermia [5,6,12,19].

At the nuclear level, there are morphological alterations in addition to inhibition of DNA and RNA synthesis, repair of DNA damage and might promote misrepair. Damage to individual cellular structures is likely to contribute differently to killing in the different phases of the cell cycle. The observation of chromosomal aberrations in cells heated in S phase, heat delayed replication and repaired resulting that increasing DNA double strand breaks (DSBs) and instability of chromosome in S-phase which appear to correlate with killing, suggests that the nucleus may be a critical target. However, when cells are heated in G1, they are less sensitive to heat-induced cell killing and no chromosome aberrations are induced and when DNA synthesis was blocked in S-phase, there was an increase in cell survival that was dependent on time that DNA synthesis was blocked until survival equaled that of a non-S phase cell [5-6,12-13,15-19].

A number of nuclear activities associated with semi-conservative replication of DNA including the incorporation of radiolabelled precursors into acid-insoluble DNA, the initiation of new replicons, the elongation of the DNA fibre at the replication fork, the synthesis and deposition of new histones into chromatin and the reorganization of nascent DNA into mature chromatin, are the most sensitive to heat. Exposure of cells to hyperthermia as they perform semi-conservative DNA replication also increases the proportion of DNA found in single stranded form and this increase is implicated in the illegitimate exchanges that lead to the formation of chromosome aberrations [13-17,19].

A decrease in DNA replication activity was observed in extracts of cells heated up to 10 min and activity reached a plateau after heating between 10–30 min at 44°C. Surprisingly, DNA replication activity recovered in extracts of cells heated for longer than 30

min at the same temperature [13,18]. Iliakis et al [18]. other heat-induced effects do not contribute to the inhibition of DNA replication in vivo. In fact, it is likely that such alterations in chromatin structure relay the initiating signal for the activation of the checkpoint response. Furthermore, the translocation of nucleolin into the nucleoplasm could explain the initial inhibition of DNA replication [13,16-18].

Respiration and protein synthesis are both sensitive to heat. Most 5–10% of the increase in protein content of isolated nuclei could be accounted for by the movement of cytoplasmic proteins into the nucleus during heating. The temperature dependence of aberrant protein binding to the nuclear matrix has two components: (1) the time- and temperature-dependent increase in protein binding to the nuclear matrix and (2) the time-dependent, post-heat shock resolution of the aberrant nuclear matrix-protein binding to pre-heat shock levels. The first order time course of the latter is dependent on the amount of post heat nuclear matrix-protein binding and the cellular levels of molecular chaperones (i.e., heat shock proteins). If both components of the time course are taken into account, the binding of proteins in excess of the amount bound to the nuclear matrix in unheated cells, correlates linearly with cell killing [16-20]. The first class of proteins shown to associate with the nucleus following heating was the heat shock proteins (Hsps). These proteins are normally present in small quantities in the nucleus, and thus migrate to the nucleus from the cytoplasm. Both Hsp70 and the constitutive form Hsp70 migrate to the nucleus in response to heat. Hyperthermia can affect the folding and aggregation of protein. Protein denaturation occur in mammalian cells at temperatures in excess of 40–41^{o}C, results in free radical production and alters cellular structures such as plasma membrane, nucleolus, centrosome and chromatin. These alterations are thought to underlie cell killing. The type of cell death induced by hyperthermia include heat induced apoptosis, mitotic catastrophe secondary to alterations in the proteins that support DNA metabolism [6,8-12,16-18,20].

The three general mechanisms of nuclear thermal damage discussed above are related to: (1) protein inactivation (either direct or indirect), (2) physical alterations to chromatin (restricted access to DNA) and (3) protein redistribution. The first two mechanisms of thermal damage are the direct consequences of protein denaturation and aggregation, which may also contribute to protein redistribution [5,8-13,17].

Although, many research study about hyperthermia but the mechanism for hyperthermia killing cell or cell sensitization is not clear.

DNA double-strand breaks (DSBs)

DNA double-strand breaks (DSBs) are probably the most dangerous of the many types of DNA damage in the cell. It can cause by exogenous agents such as ionizing radiation (IR) and/or chemical agents and by endogenous agents such as V(D)J recombination and/or DNA replication or through the action of reactive oxygen species produced as a by-product of normal metabolic processes [2,21-24]. These products introduce chemical modifications in the DNA, including modified bases and sugars,DNA-protein adducts, base-free sites and tandem lesions. From the outside, ionizing radiation (IR) from the background and ultraviolet (UV) light alter the chemical composition of the DNA backbone. A large panel of chemical agents and DNA topoisomerase inhibitors used in anti-cancer therapy also modify DNA. Non-rejoined as well as misrejoined DSB possibly give rise to chromosomal breaks, exchanges, translocations and loss of heterozygocity, which are all potentially lethal and cause chromosomal instability (CIN) that the one of carcinogenesis [12,19-20,22,25-26]

Broadly speaking, DNA lesions can be classified in two categories on the basis of their effect on chromatin integrity. The first category, which includes base and nucleotide damages as well as single interruptions of the sugar phosphate backbone, does not overly risk chromatin integrity or function, and error-free repair can be accommodated with limited, local modification of the chromatin structure using the complementary DNA strand as a template. The second category, however, which is mainly comprised of DNA double strand breaks (DSBs), but may also include some types of DNA-protein crosslinks, can bring chromatin to a state severely undermining its integrity and function [27]. This type of DNA lesion may be partly recognized by the resulting destabilization of chromatin with the resulting signaling and repair coordinated by associated modifications in chromatin structure. In comparison with other types of DNA lesions, DSBs generate the additional complication that error-free restoration is possible only through copying of lost sequence information from a different DNA molecule (or a different part of the same molecule), as the

complementary strand is also damaged. In view of the specific requirements for error-free DSB repair, as well as the immediate risks a DSB generates to chromatin stability, it is not surprising that the DSB is among the most severe DNA lesions. Unrepaired or misrepaired DSBs induced by physical agents such as ionizing radiation, chemical agents such as topoisomerase inhibitors, oxidative stress, aberrant DNA replication, aberrant V(D)J or class switch recombination, etc., can cause genomic instability and cancer if the cell escapes death altogether [19,23,27-28].

DNA repair

DNA repair is the cellular process to fix of DNA damage before it is become a mutation or chromosomal aberration, leading to cell death, tumorigenesis or aberrant germ cell formation [28]. One important form of DNA damage is the double-strand break (DSB), where the two complementary strands of the DNA double helix are broken simultaneously at sites sufficiently close to one another such that base pairing and chromatin structure are insufficiently strong to keep the two DNA ends juxtaposed for a longer period of time. DSB repair process in eukaryotes takes place in the context of chromatin structure and past decade has unraveled the modifications of chromatin itself as an early response to DNA damage. One of the best-characterized chromatin modification events in DNA damage response is the phosphorylation at serine 139 of histone H2AX, referred to as γ-H2AX and serine 129 in H2A of yeast. That is playing a critical role in their recognition, signaling and repair [3-4,28].

the phenotypes of genetic disorders involving faulty DSB repair and characterized by predisposition to cancer, such as Bloom syndrome (BS), Werner syndrome (WS), Nijmegen Breakage syndrome (NBS), Fanconi anemia (FA), Ataxia telangiectasia (AT) and Ataxia telangiectasia like disorder (ATLD) [22-23,28].

To understand the kinetics of DSB repair can described by three components, a fast, a slow repair phase and a constant component of non-repair DSB. The nature of these repair component depend on the size of DNA fragments involved [12]. In generally, DSB repair can separate into 2 type homologous recombination (HR) and non-homologous end-joining (NHEJ)

Homologous recombination (HR) rejoins DSBs using a sister homolog as a template so that this method of repair provides very high fidelity. DSB repair by HR corresponds to an exchange or a transfer of identical or quasi-identical sequences between the DNA molecule carrying the DSB and another intact DNA molecule. HR is a faithful mechanism if the DNA template used for repair is identical to the original DNA sequence present at the DSB. Otherwise, HR repair of DSBs can lead to local mutations or even more deleterious genome rearrangements. Protein related in HR such as RAD51, BRCA1 and XRCC2 [19,23]. HR has a key role in rejoining DSBs that arise as a result of replication-fork stalling. Most proteins that are required for HR are essential, probably because stalling of replication forks occurs during most cycles of replication [19]. Classical HR is mainly characterized by three successive steps: 1) resection of the 5'-ended DNA strand at break ends, followed by 2) strand invasion into a homologous DNA duplex and strand exchange, and 3) resolution of recombination intermediates. Depending on the ability of both DSB ends to perform strand invasion and on the outcome of the strand invasion intermediate, different HR pathways can complete DSB repair. These include the synthesis-dependent strand-annealing (SDSA) pathway, the classical double- strand break repair (DSBR) model for HR as defined by Szostak et al [29]. and break-induced replication (BIR). Alternatively, 5' resection of DSB ends can eventually uncover repetitive DNA that could channel repair through the single-strand annealing (SSA) pathway. All HR mechanisms are interconnected and share a large number of enzymatic steps. As described in the following section, each protein used during HR has a preponderant role in one or several of these HR pathways, which will be described together with the different HR enzymatic steps. [21-23,26,30,32].

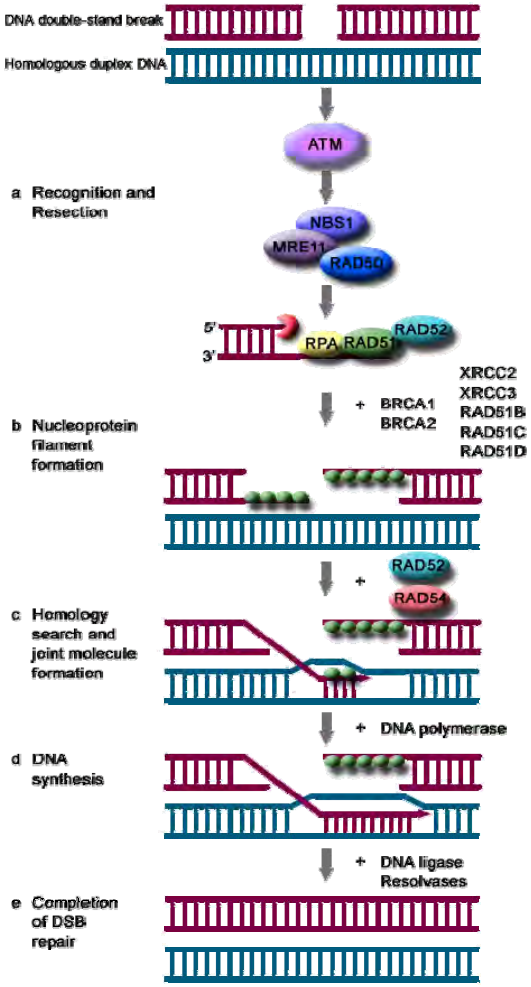


Figure 1 DSB repair via homologous recombination. (a) DSB formation triggers ATM sensor and NBS1 in MRN complex is phosphorylated by ATM. Processing of the ends occurs by the MRX complex and results in the formation of 3′ single stranded (ss) DNA overhangs. (b) The ssDNA-binding protein replication protein A (RPA) binds to the ssDNA overhangs, and RAD51 and RAD52 are recruited to the DSB. Both RPA and RAD2 help to load Rad51 onto ssDNA to form ssDNA–RAD51 nucleoprotein filaments. Other proteins implicated in the orchestration of a proper RAD51 response include BRCA1, BRCA2 and the Rad51 paralogues, XRCC2, XRCC3, RAD51B, RAD51C and RAD51D. (c) This nucleoprotein filament searches for the homologous duplex DNA in the undamaged sister chromatid. A successful search results in strand invasion, strand exchange and joint molecule formation. A reaction is stimulated by the RAD52 and RAD54 proteins. (d) DNA synthesis by DNA polymerases generates the genetic information that is required to seal the break. (e) Ligation and the resolution of the two double helices joined by strand exchange complete this error-free repair event.

(http://focosi.altervista.org/dnaturnover.html)

non-homologous end-joining (NHEJ) is a predominant pathway of DNA double-strand break repair in mammalian cells [31]. This pathway that repairs DSBs by re-ligating their two ends together. It appears to be the most powerful DSB-repair pathway because it has the potential to ligate any kind of DSB ends without the requirement for a homologous sequence, as opposed to the alternative HR DSB-repair pathway. It has frequently been considered to be the errorprone DSB-repair pathway because it was observed to generate small insertions and deletions [26]. All NHEJ reactions require the core NHEJ machinery that is composed of three complexes: MRN, Ku and the DNA ligase complexes. The order of action of these complexes has not been fully established. It is thought that MRN and Ku complexes bind DSB ends shortly after DSB formation. They appear to bridge DSB ends together and to inhibit their degradation. Ku and MRX also play crucial roles in recruiting, stabilizing and stimulating the ligase complex at DSBs. NHEJ functions throughout the cell cycle and is the predominant DSB-rejoining mechanism in G1 phase. NHEJ also rejoins DSBs that are introduced during V(D)J recombination [21-23,26,30,32].

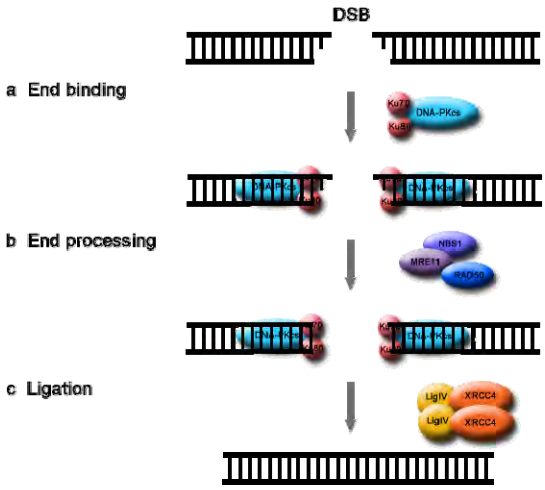


Figure 2 DSB repair via non homologous end-joining. (a) After induction of a DSB, the Ku proteins bind DNA end of DSB and recruit DNA-PKcs to this end leading to activation of DNA-PKcs. (b) The RAD50–MRE11–NBS1 (MRN) complex might be required to process broken end making it available for ligation. (c) This is followed by end-to-end ligation by the Ligase IV–XRCC4 complex. (MRE11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1; XRCC4, X-ray-repair-crosscomplementing defective repair in Chinese hamster mutant 4)(http://focosi.altervista.org/dnaturnover.html)

Both HR and NHEJ are critical in genomic stability. If there impaired, there can cause defected in hypersensitivitiy to ionizing radiation, genomic instability and replicative senescence of mouse embryonic fibroblasts (MEFs) [33].

γ-H2AX

Among the four major core histones that make up the nucleosome, H2A is known to have the largest number of variants. Two H2A variants, H2AX and H2AZ are constitutively expressed and distributed throughout the genome. H2AX represents between 2 and 25% of the total cellular H2A, whereas H2AZ accounts for 10%. H2AX variant contains a conserved SQ(E/D) motif in the extended C-terminal tail that is known to be phosphorylated at a serine residue in response to DNA damage. Both H2AX and H2AZ are important for DNA damage responses (DDR) but in contrast to H2AX, which is profusely modified in response to DDR, no post-translational modification has been reported for H2AZ. In lower eukaryotes, such as yeast, the conserved SQ motif present in the non-extended H2A proteins, and in H2Av, a variant of H2AZ in Drosophila, also show the property of phosphorylation at this motif in response to DNA damage as observed in mammalian system [5,22,27-28,34-36].

H2AX is known to be phosphorylated by members of phosphoinositide 3-kinase related protein kinases (PIKKs) such as ATM and Rad3 related (ATR) (Tel1/Mec1 in yeast), or DNA dependent protein kinase (DNA-PKcs) in response to genomic insult, although it has been recently known to occur outside the DNA damage response as well. Whereas ATM is a dominant physiological mediator of H2AX phosphorylation in response to DSB formation, ATR phosphorylates H2AX in response to single-strand breaks and during replication stress. In contrast, DNA-PKcs is known to mediate H2AX phosphorylation in hypertonically stressed cells as well as in response to DNA fragmentation during apoptosis. However, in response to DNA damage caused by ionizing radiation, all these kinases may function redundantly to phosphorylate serine 139 at C-terminal H2AX, call γ-H2AX, with half maximal amounts at 1–3 min. In response to DSB it is now known that γ-H2AX extends up to 50 kb on either side of the DSB in yeast and up to 1 megabase in mammalian cells [5,22,24,27-28,34-35].

The analysis of H2AX-deficient mice has demonstrated a role for H2AX in a variety of responses to DSBs, including DNA repair, checkpoint signaling, and immunoglobulin gene

class switching show that coating of large chromatin regions by γ-H2AX may act as a structural signal to recruit recombination and repair proteins. γ-H2AX interacts with the mediator protein MDC1, which, at the same time, interacts with ATM and MRN, thus generating further γ-H2AX and amplifying the signal. The chromatin modifications occurring around a DSB have been described as recruiting chromatin remodelling complexes that may have a role in DSB repair. Although the exact mechanism is unknown, chromatin decondensation seems to be required for efficient DSB repair [22,24,26,34-35].

HR is predominantly in S- and G2-phase while NHEJ can operate in all cell cycle phases. During HR, γ-H2AX is known to recruit an additional class of proteins, called cohesin complexes, that facilitate physical interaction between the two sister chromatids. The cohesin complexes are established during S phase and maintained until mitosis. It was also shown that H2AX regulates sister chromatid recombination in mammalian cells, promoting error-free interchromatid homologous recombination over mutagenic single-strand annealing pathway (SSA). However, functional interplay between cohesin and H2AX in mammalian cells remain to be explored. The role of γ-H2AX as an adaptor for the recruitment of chromatin modifiers to the site of damage has recently been uncovered with γ-H2AX and also the acetylation of histone H4 (H4 Ac-Lys8) in close proximity DSB sites. The elimination of the C-terminal H2A residue led to an impairment in NHEJ. No clear effect was observed in HRR, which actually appeared increased in the absence of H2A. The mouse cells lacking H2AX are radiosensitive and display deficits in DNA damage repair. Additionally, H2AX knock out mice show male-specific infertility and reduced levels of secondary immunoglobulin isotypes suggesting defects in class switch recombination (CSR). Indeed, efficient resolution of DSBs induced during CSR in lymphocytes requires H2AX, and its absence is associated with chromosome abnormalities involving the immunoglobulin locus [22,27-28,34,36].

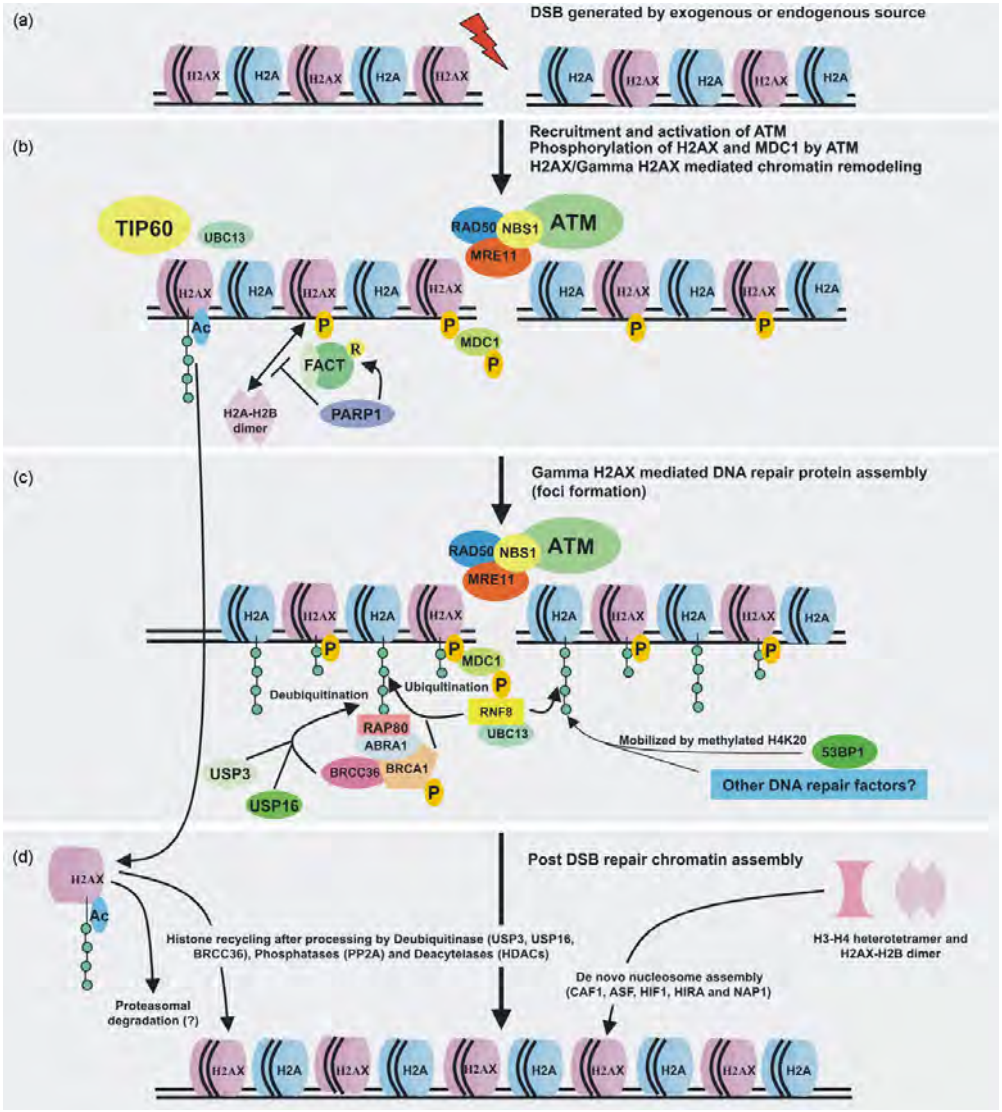
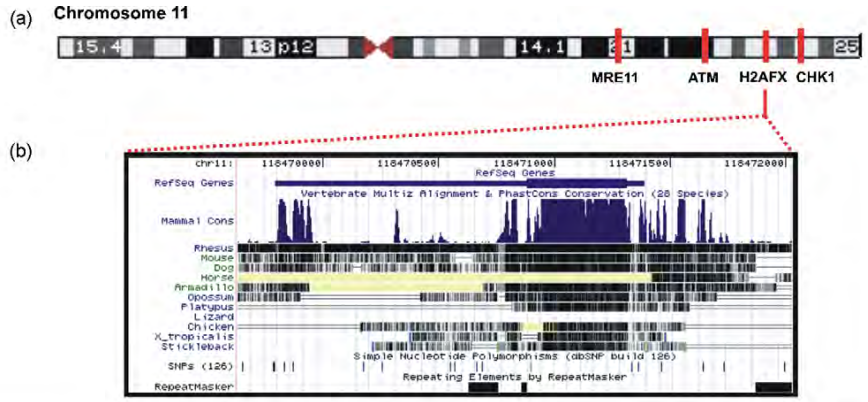


Figure 3. A proposed model of the DNA double-strand break (DSB) signaling pathway, involving post-translational modifications of histone H2AX, recruitment of chromatin remodeling and repair factors and post-DSB-repair chromatin reorganization. (a) The DSB induced by exogenous or endogenous process is the most debilitating category of DNA damage. (b) The signaling cascade is set off by ATM mediated phosphorylation of H2AX, which is known as g-H2AX. In turn, g-H2AX initiates the chromatin remodeling, exposing other modifiable histone residues and recruiting MDC1 via its interaction with BRCT domain. Also TIP-60-UBC13 mediated acetylation–ubiquitination of H2AX resulting in its eviction and/or the FACT regulated dynamic equilibrium between g-H2AX and H2AX are other possible ways of chromatin modulation involving H2AX as the key mediator. (c) Further, RNF8 relocalization to DSB is mediated through binding to phosphorylated MDC1, which also facilitates recruitment of more ATM molecules and amplification of the phosphorylation signal. Once recruited, RNF8 catalyzes ubiquitination of H2A and H2AX. At this point, the DSB-flanking chromatin becomes competent to recruit and accumulate 53BP1, BRCA1 and probably many other known/unknown repair factors by means of additional adaptor molecules having ubiquitin binding domain. RNF8 mediated ubiquitination provides a direct recognition signal for the ubiquitin-interaction motif of RAP80. This protein in turn interacts with ABRA1, a mediator protein that exists in a complex with BRCA1 as BRCA1 complex A, able to simultaneously bind to RAP80 and the BRCT domains of BRCA1.

This complex also contains an additional protein, Brcc36, with a deubiquitinase activity indicating the existence of yet another dynamic equilibrium consisting of ubiquitination–deubiquitination process in the signaling pathway. 53BP1 initially proposed to be recruited to the DSB region by interaction through its Tudor domain with exposed methylated lysine 20 residue of H4 as recently indicated could also be recruited directly or by co-operation of H2AX ubiquitination. (d) Post-DSB-repair, the post-translational marks (PTMs) may be reverted by various deubiquitinases, phosphatases, histone deacetylases, etc. and proper chromatin assembly restored either through histone recycling or by de novo histone deposition assisted by histone chaperones, CAF1, ASF1, HIF1, HIRA and NAP1. The role of proteasomal mediated degradation in this context is also hypothesized.[28]

The histone H2AX gene maps to a region (11q23.2–23.3) highly represented among those commonly deleted or translocated in several haematological malignancies or solid tumors (Fig. 4). A number of mutations and polymorphisms in DNA damage response genes have been reported in different human cancers although no significant risk association with a specific mutation or SNP has yet been established. A possible reason could be that an inactivating mutation in isolation may have a small effect on cancer risk, but when combined with additional gene mutations, it may become significant. In this context, it may be interesting to note that loss of the distal 11q arm that harbors key DDR genes; ATM, MRE11A and CHEK1 are frequently observed in many cancers (Fig. 4). H2AX and ATM double knockouts were embryonically lethal while the double deficient mouse cells displayed severe proliferation defects and remarkable degree of genomic instability. This suggested that the two proteins, ATM and H2AX, function synergistically to preserve genomic integrity and suppress tumor progression. Genetic analysis has revealed that the H2AX gene is not essential for the viability of either cells or whole animals and absence of the protein does not compromise the activation of the DSB-induced checkpoint pathway. Nevertheless, $H2AX^{-/-}$ mice are radiation sensitive, growth retarded, immune deficient and male sterile [28].



(c)

Sl. No.	Study	Findings	Reference(s)
1	Mouse knockouts in p53-/- background	dosage-dependent tumor suppressor role	Celeste et al. 2002, 2003; Bassing et al. 2002, 2003
2	Mutation analysis in 101 Familial breast cancer	No mutation detected	Monteiro et al. 2003
3	Colon and lung cancer	Activated H2AX present in precancerous lesions as anti-cancer barrier	Bartkova et al. 2005, Gorgoulis et al. 2005
4	Mutation analysis in B-cell lymphomas	No mutation detected	Walsh and rosenquist, 2005
5	Head and neck squamous cell carcinoma	Partial deletion of *H2AFX* loci	Parikh et al. 2007
6	Mouse B-cell Lymphocytes	H2AX prevents chromosoamal translocations	Franco et al. 2007
7	Sporadic breast cancer	SNPs (-1654, -1420, and -1187) in promoter region of *H2AFX* linked to increased risk	Lu et al. 2007
8	Non-Hodgkin lymphoma	SNP in promoter region (-417) of *H2AFX* associated with genetic susceptibility	Novik et al. 2007
9	Sporadic breast cancer	No mutation detected, extensive copy number alterations identified	Srivastava et al. 2008

Figure 4. Chromosome of H2AX gene. (a) Cytogenetic location of H2AFX gene on chromosome 11q along with other key DDR genes, ATM, MRE11 and CHEK1. (b) Conservation of H2AFX coding region across the species. (c) Table summarizing the studies showing involvement of H2AX in tumorigenesis.

CHAPTER III

MATERIAL AND METHOD

Cell culture

HeLa (cervical cancer), HN8, HN17 (Head and neck) and Hacat (normal) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM). Daudi (B lymphoblast) and Molt4 (T lymphoblast) cell lines were cultured in RPMI 1640. The both media were supplemented with 10% fetal bovine serum (FBS). All cells were grown in a humidified atmosphere at 37°C with 5% CO_2.

Cell synchronization

Cultured each cell line at a density of 10^6 cells per 25 cm^2 tissue culture flask for 24 hours. Cells were synchronized at G0 phase by culture in serum deprivation medium at 37°C with 5% CO_2 for 24 hours. Then, move cell to 37°C and 47°C 30 min before corrected cell. Treated cell in 100 ng/ml TSA (Sigma-Aldrich) after synchronized cultured at 37°C with 5% CO_2 for 4 hours next change cell to 37°C and 47°C 30 min before corrected cell.

High-molecular-weight (HMW) DNA preparation

To prepare HMW DNA, cell lines were embedded in 1% low-melting point agarose (LMP) at a density of 1 x 10^6 cells per plug. The plug was lysed and digested in 400 μl of digestion buffer (1 mg/ml proteinase K, 50 mM Tris, pH 8.0, 20 mM EDTA, 1% sodium lauryl sarcosine) at 37°C overnight. After, the plug was rinsed five times in TE buffer for 50 minutes. To polish cohesive-end EDSBs, T4 DNA polymerase (New England Biolabs) rinsing five times in TE buffer for 50 minutes. The modified LMPCR linkers were prepared from the two oligonucleotides 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAAGCTTACCTCGTGGACGT-3' and 5´-ACGTCCACGAG-3´. Linker (50 pmol) was ligated to HMW DNA using T4 DNA ligase (New England Biolabs) at 25°C 2 night. DNA was extracted from agarose plugs using a QIAquick gel extraction kit (QIAGEN).

L1-EDSB ligation-mediated realtime PCR (L1-EDSB-LMPCR)

The quantity of L1 - EDSBs was measured as previously described for L1 - EDSB - LMPCR with some modifications [1]. Duplicate or triplicate quantity of L1 - EDSB was measured by real - time PCR using a ABI PRISM® 7500 instrument (Applied Biosystem, Carlsbad, CA, USA) with the IRS primers including L1 primers 5´ - CTCCCAGCGTGAGCGAC - 3´ (outward), the linker primer 5´ - AGGTAACGAGTCAGACCACCGA - 3´, and the Taqman probe homologous to the 3´ linker sequence (6 - fam) ACGTCCACGAGGTAAGCTTCCGAGCGA (tamra) (phosphate). Amplification was performed with 0.5 μM of each primer, 0.3 μM Taqman probe, 0.025 U of HotStarTaq (QIAGEN, Valencia, CA, USA), 1x TaqMan® Universal PCR Master Mix (Applied Biosystem) and 30 ng of ligated DNA. Initial denaturation was at 95°C for 10 minutes, followed by denaturation at 95°C for 15 second, annealing and extension at 60°C for 2 minute for up to 60 cycles, with quantification after the extension steps. Control DNA was HeLa DNA digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers. The amounts of EDSBs were compared with the ligated control digested DNA and reported as L1 ligated *Eco*RV and *Alu*I digested genome per cell, L1 - EDSBs genome per control genome.

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was performed essentially as previously described with some modifications [38-39]. The chromatin fragments were immunoprecipitated with Anti - phospho - Histone H2AX monoclonal antibody (Upstate, Charlottesville, VA, USA) or normal mouse IgG antibody as a negative control (Santa Cruz Biotechnology). Quantification of the amount of immunoprecipitated DNA was carried out by real - time 5'L1PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland) between forward primer (L1.2HpaIIRFLPF: 5´ - CTCCCAGCGTGAGCGAC - 3´) and reverse primer (5´LIDSIP1st: 5´ - ACTCCCTAGTGAGATGAACCCG - 3´) located at 5' of L1. The amount of γ - H2AX bound L1 sequences was used to calculate the quantity of precipitated genomic DNA by relating the L1 quantity to L1s of HeLa genomic DNA. The relative quantity

unit was γ - H2AXbound genome per cell. The precipitated DNA was then subjected to COBRA - L1.

Bisulfite Treatment

Ligated HMW DNA was modified by sodium bisulfite using standard protocol [40]. Dilute 1 µg of DNA into 50 µl with dH_2O, 5.5 µl of 2 M NaOH were added then incubate for 10 minutes at 37°C to create single-stranded DNA. Then, 30 µl of 10 mM hydroquinone and freshly prepared 520 µl of 3 M sodium bisulfite at pH 5.0 were added and mixed. The sample was incubated at 50°C for 16 hours. The bisulfite-treated DNA was isolated using Wizard DNA Clean-Up System (Promega). The DNA was eluted by 50 µl of water at 95°C and 5.5 µl of 3 M NaOH were added and incubate at room temperature for 5 minutes. The DNA was precipitated by adding 17 µl of 10 M NH_4OAc, 3 volumes of ethanol and 1 µl of 20 mg/ml glycogen as a carrier then incubated at -20°C for 2 hours. After incubation, DNA was centrifuged at 14,000 rpm for 15 minutes. The DNA pellet was washed with 70% ethanol then centrifuged at 14,000 rpm for 5 minutes and air dry. The DNA pellet was resuspended in 20 µl of water. Bisulfite-treated DNA was stored at -20°C until ready for used.

COBRA-L1 and COBRA-L1-EDSB

For COBRA-L1 [41], a 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 unit of HotStarTaq (QIAGEN), 0.3 µM of B-L1-inward 5´-CGTAAGGGGTTAGGGAGTTTTT-3´, 0.3 µM of B-L1-outward 5´-RTAAAACCCTCCRAACCAAATATAAA-3´, and 2 µl of bisulfite-modified DNA. PCR was performed under the following conditions, initial denaturation at $95^{O}C$ for 15 minutes, followed by 35 cycles of denaturation at $95^{O}C$ for 1 minute, annealing at $50^{O}C$ for 1 minute, extension at $72^{O}C$ for 1 minute, and final extension at $72^{O}C$ for 7 minutes.

For COBRA-L1-EDSB, the B-L1-inward oligonucleotide was replaced with B-LMPCR oligonucleotide, 5´-GTTTGGAAGTTTATTTTGTGGAT-3´. A 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 unit of HotStarTaq, 0.3 µM of B-LMPCR, 0.3 µM of B-L1-outward, and 2 µl of bisulfite-treated DNA. PCR cycling

conditions are initial denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 7 minutes. Bisulfite-treated Daudi, Jurkat and HeLa DNA were digested with *Eco*RV and *Alu*I and ligated LMPCR linker were used as positive controls to normalize the inter-assay variation of all COBRA experiments. HeLa DNA without ligation was used as a negative control.

To prevent heteroduplex amplicons, hot-stop technique was applied in COBRA L1 and COBRA-L1-EDSB assay. ^{32}P-labeled-bisulfite-L1-outward oligonucleotides were added in the last PCR cycle. The amplicons were double-digested in a 10 μl reaction volume with 2 unit of *Taq*I and 8 unit of *Tas*I in 1x *Taq*I buffer (MBI Fermentas) then incubate at 65°C for 4 hours. This was designed to detect unmethylated and methylated sequences of 98 and 80 bp, respectively. Digested products were then electrophoresed in 6% denaturing polyacrylamide gel. The intensity of DNA fragments was measured with a PhosphorImager using Image Quant software (Molecular Dynamic). The LINE-1 methylation level was calculated as the percentage of *Taq*I intensity divided by the sum of *Taq*I- and *Tas*I-positive amplicons. HeLa DNA without linker was used as a negative control.

Western Blot

Cellular proteins are extracted with lysis buffer and then sonicated with an Ultrasonics sonicator at 70% power output 10 seconds for three times intervals on ice. Total protein was electrophoresed on 15% SDS-polyacrylamide gel at 200 V for 40 minutes and transferred to nitrocellulose at 100 V for 40 minutes for detecting γ-H2AX. To detect γ-H2AX, the nitrocellulose was blocked with 10% nonfat dry milk and 0.05% Tween 20 in Tris buffered saline for 1 hour at room temperature with constant agitation, wash by 0.05% TBS/T 4-5 time each 5 minutes and the incubated with Anti - phospho - Histone H2AX at serine 139 monoclonal antibody (Upstate, Charlottesville, VA, USA), diluted 3 μl in freshly prepared 0.05% TBS/T 15 ml for 1-2 hour.

Then, the nitrocellulose was incubated with goat anti-mouse HRP conjugated IgG, diluted 1 μl in freshly prepared 5% TBST-MLK 20 ml for 1 hour at room temperature with

agitation, wash by 0.05% TBS/T 4-5 time each 5 minutes and visualized by chemiluminescence. In brief, the two substrate components were mixed at a 1:1 ratio to prepare the substrate working solution then added to nitrocellulose blot and incubated for 5 minutes at room temperature. The blot was remove from working solution and covered with plastic wrap. The protected nitrocellulose membrane was placed in a film cassette with the protein side facing up and exposed to X-ray film (Kodak). Exposure time may be varied to achieve optimal results.

Statistical Analyses

Statistical significance was determined according to an independent sample t-test, a paired sample t-test, or ANOVA using the SPSS program version 16 as specified.

CHAPTER IV

RESULTS

γ-H2AX level in different temperature

From previous study, γ-H2AX found in DSBs site, it' function of DSBs sensor by phosphorylation at serine 139 in the C-terminal residue of histone H2AX. Phosphorylated H2AX with half maximal amounts at 1–3 min to response to DSB, it is now known that γ-H2AX extends up to 50 kb on either side of the DSB in yeast and up to 1 megabase in mammalian cells [5,22]. Therefore, you can measure occurring of DSBs by measured γ-H2AX changing.

We hypothesized that protein collected in different temperature have different level of γ-H2AX then we collected protein from HeLa incubated at 47^{O}C and 37^{O}C 30 minute then incubated at 37 ^{O}C after that for 0, 0.5, 1, 2 and 24 hr. When measured by western blot, we found increasing of γ-H2AX in heated HeLa cell more than unheated cell in earlier condition (figure 5). Moreover, when we collected cells at 24 hr, we can separate cells into 2 groups, pellets and supernatants. We detected γ-H2AX in supernatant cells more than pellets (figure 5). It shows that DSBs can increase in high temperature and can retain in several hours. However, in the normal temperature, we can found level of γ-H2AX and it can retain until 24 hours. Therefore, normal condition of cell may have DSBs from endogenous effect, called endogenous DNA double strand break (EDSB). Moreover, the supernatant cells, cell death, can observe γ-H2AX in high level DNA double-strand breaks (DSBs); therefore, these cells may cannot completely repair DNA damage because DSB level exceeds a threshold.

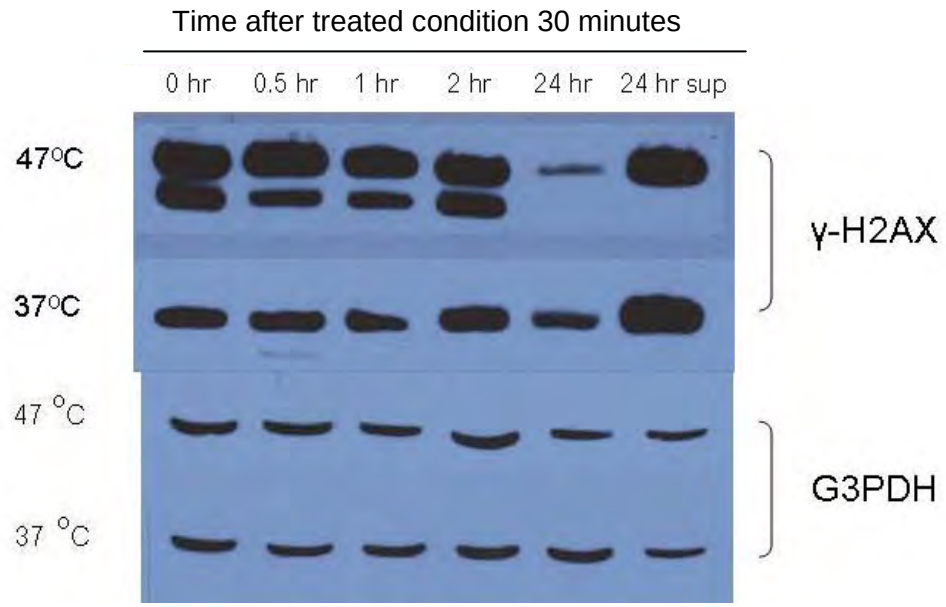


Figure 5. γ-H2AX level in different temperature by western blot. Graph shows γ-H2AX level in 47^{O}C and 37^{O}C that detected by western blot. Each lane is difference time after treated temperature.

γ-H2AX bound DNA by Chromatin Immunoprecipitation (ChIP) assay

From the previous assay, we can detected increasing of γ-H2AX in high temperature and γ-H2AX is the phosphorylated of histone H2AX so that we changed the method to Chromatin Immunoprecipitation (ChIP) for detected γ-H2AX bound DNA. γ-H2AX extends on either side of the DSB so that we should precipitated γ-H2AX in high temperature more than normal condition. We incubated non-replicating HeLa cell at 47^{O}C and 37^{O}C 30 minute, then corrected DNA immediately. The DNA was shield by sonicator and precipitated γ-H2AX by specific antibody. We selected antibody specific at serine 139 histone H2AX that have phosphorylation to respond to DSBs and normalize the result with normal mouse IgG antibody. Then precipitated γ-H2AX bound DNA was detected the quantity by real time PCR with 5'L1 primer and the methylation level by COBRA- L1, 5'L1PCR and COBRA-L1-H2AX respectively. We can measure γ-H2AX bound DNA in 47^{O}C more than 37^{O}C, the result is same from western bolt (figure 6). Therefore, this technique can be use to measured γ-H2AX bound DNA.

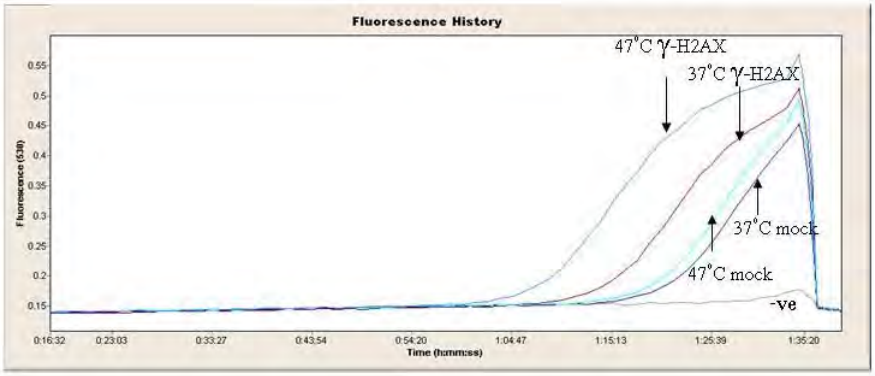


Figure 6. γ-H2AX bound DNA by ChIP assay. we measured γ-H2AX bound DNA in both temperatures by use specific antibody at serine 139 histone H2AX and detected the quantity by real time PCR with 5'L1 primer and the methylation level by COBRA- L1, 5'L1PCR and COBRA-L1-H2AX respectively.

determineDetermined quantity of EDSBs level by L1-EDSB-LMPCR

From the last assay, we found increasing of γ-H2AX bound DNA in hyperthermic cell and γ-H2AX is related with DSB therefore we hypothesis that when we treated hyperthermia in non-replicating cell, we can measured arise of EDSB level.

In order to determine quantity of EDSB, we performed a technique for the detection of EDSBs, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (Figure 7). General EDSBs are believed to occur rarely and arbitrarily throughout the genome. Using repetitive sequences that randomly scatter throughout the human genome, we can detect genome-wide EDSBs in proximity to these repetitive sequences. We therefore combined LMPCR with interspersed repetitive sequence PCR (IRSPCR) using the widely distributed L1s human retrotransposons into a new assay called "L1-EDSB-LMPCR" [1].

Figure 3. Schematic Illustration of L1-EDSB-LMLPCR. L1 sequence ligated by linker at EDSB. The white rectangle is Taqman probe complementary to LMPCR linker. Arrows are PCR primers.

Cells were prepared high molecular weight (HMW) DNA by in-gel preparation for measured EDSBs level. This technique has been reported to decrease the number of DSBs generated during DNA preparation [42] and Porntanakasem *et al.* compared between this technique and liquid DNA, they found that EDSBs level of HMW DNA less than liquid DNA. This result indicates that more DNA breaks were generated during the liquid DNA preparation process. Embedded liquid DNA into gel following by HMW DNA preparation protocol did not increase the amount of LMPCR template over liquid DNA alone [1].

When we corrected HMW DNA in G0 phase cell and measured EDSB by realtime. We found that HeLa heated cell have significant increasing EDSB more than HeLa unheated cell (P=0.001, paired T-test; n=44). When we changed cell to Head and Neck cancer cell line, HN8 and HN17, Surprisingly, we found that HN8 have significantly increasing EDSB level in high temperature (P=0.026, paired T-test; n=24) but HN17 didn't significant. However, suspension cell, Daudi and Molt4, and normal cell line, Hacat, the EDSB level are not difference between both conditions (figure 8). Therefore, heat treatment during G0 can induce EDSB and this finding may be cell type dependent.

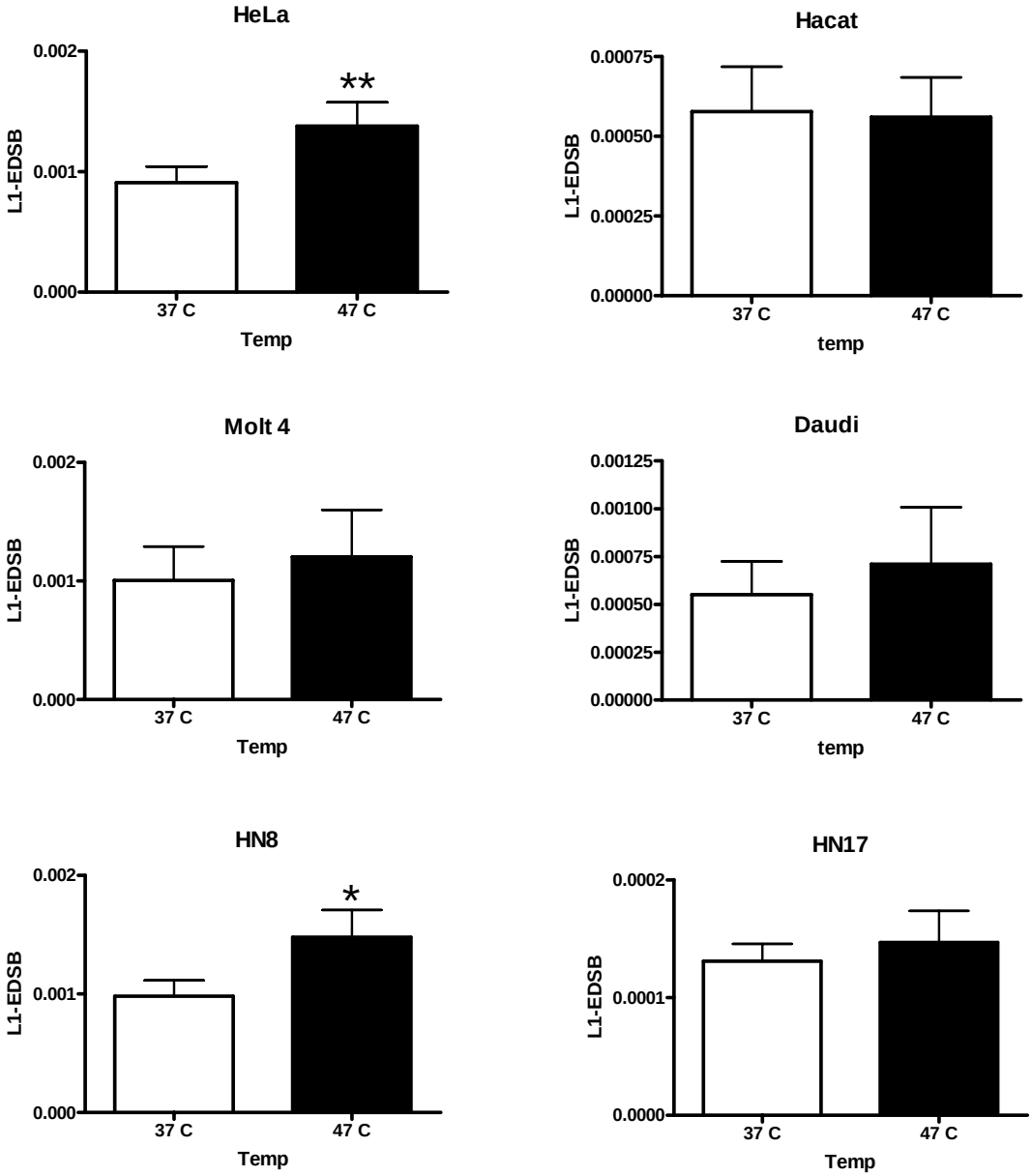


Figure 8. L1-EDSBs level by L1-EDSB-LMPCR in different cell type. In each graph show comparison of L1-EDSB level between 47^{O}C and 37^{O}C in platuea phase. (* : P< 0.05 ; ** : P <0.01)

When we spite the result in each time, we found that at 0 hr, heated HeLa cell has significantly increasing of EDSB level (P=0.023, paired T-test ; P<0.001, pearson correlation ; n=14), in addition to measured EDSB in other time we found increasing EDSB level in high temperature. Then, we changed cell to HN cell line, HN8 and HN17, we found that HN8 heated cell have significant increasing EDSB (P=0.0027, pearson correlation ; n=12). But,

HN17 didn't significant. Moreover, when we measured suspension cell, Daudi and Molt4, we still found EDSB in heated cell more than unheated cell. In contrast, we tested this condition in normal cell line, Hacat, the result didn't change (figure 9).

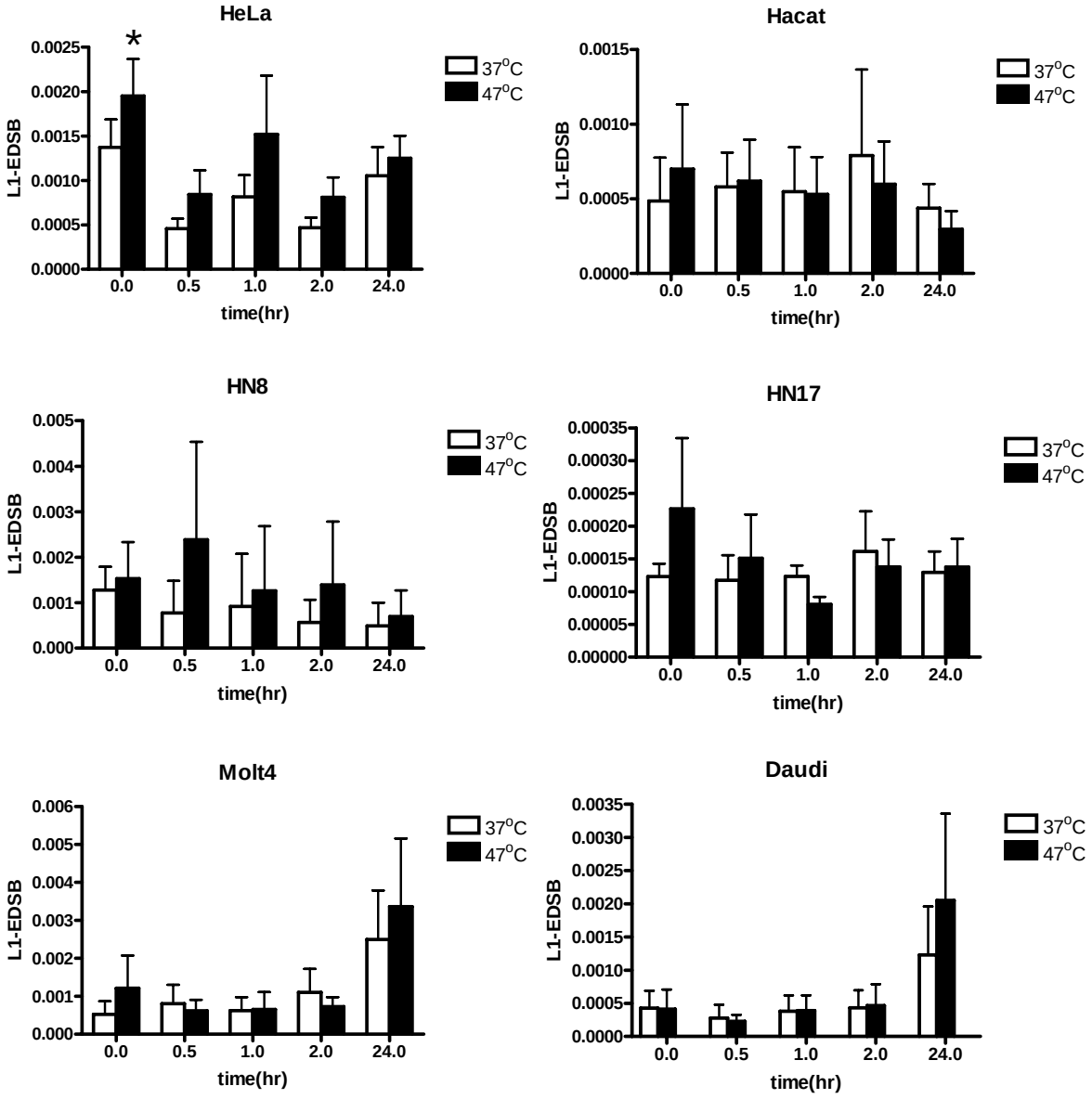


Figure 9. L1-EDSB level by L1-EDSB-LMPCR in different temperature. In each graph show EDSB level between $47^{O}C$ and $37^{O}C$ in different time, EDSB in $47^{O}C$ detected more than $37\ ^{O}C$ in almost time. (* : $P< 0.05$)

All of these result show that hyperthermia may increase EDSBs in non-replicating cell. In HeLa and HN8 at 0 hr after incubated in high temperature, we will see linear correlation between rising of EDSBs in $47^{O}C$ and $37^{O}C$ mean that if EDSB level in $37^{O}C$ was

low when we treated hyperthermia increasing of EDSBs in 47^{O}C were few. In contrast, If baseline EDSB in 37^{O}C was high, we can detect high level of EDSB in 47^{O}C (figure 10). Moreover, EDSBs in 37^{O}C of each cell were not equal, lower EDSBs in HN17 and high in HN8 and HeLa, and nearby level in suspension cell (figure 11). Because of base line EDSBs in HN17 were low, EDSBs changing was not detected significantly when we treated hyperthermia. In contrast, HN8 and HeLa had high EDSB level so that EDSB changes were more obvious.

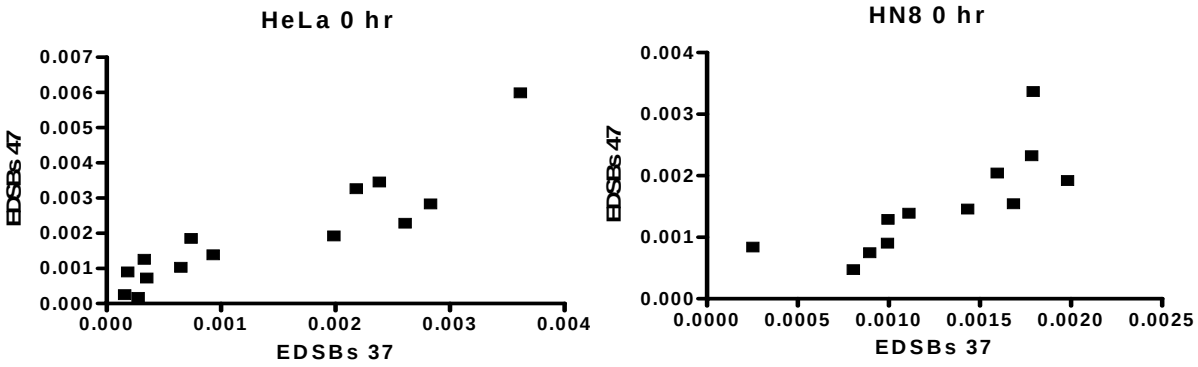


Figure 10. Correlation of EDSB between 47^{O}C and 37^{O}C. The graph shows EDSB between 47^{O}C and 37^{O}C, they have linear correlation between both temperatures.

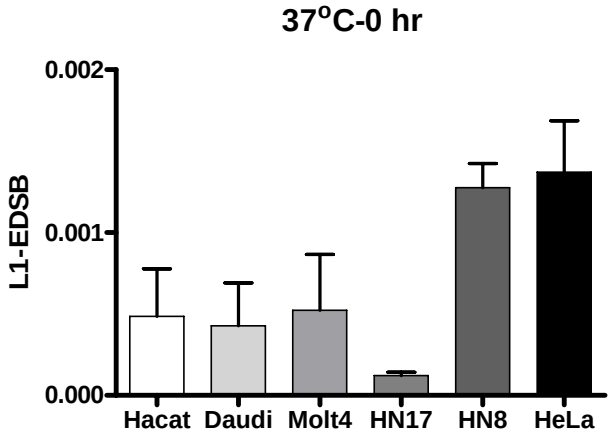


Figure 11. EDSB level in normal condition. Compared EDSB level in each cell in normal condition.

Methylation level of L1-EDSB in HeLa cells

EDSBs in G0 may be retained within heterochromatin. DNA methylation usually associates with heterochromatin. Moreover, L1 - EDSBs are hypermethylated, higher methylation levels of L1s at the sites nearby EDSBs than in the genome [1]. To analyze methyltion level in different temperature, we detected the methylation status by PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (Figure 12). This assay is based on a LMPCR, originally designed for the analysis of locus and cell-specific EDSBs. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. Therefore, bisulfite treatment generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified L1 sequences. Methylation level is then calculated and presented as a percentage of total DNA. We thus combined L1-EDSB-LMPCR with COBRA-L1 by treating linker-ligated DNA with bisulfite before PCR with L1/linker primers and restriction analysis. With this "COBRA-L1-EDSB" assay, we can measure the methylation level of L1s near EDSBs, which reflects the methylation level of EDSBs in a genome-wide fashion. The degree of methylation between genomic L1 and L1-EDSB sequences was examined by COBRA-L1 and COBRA-L1-EDSB, respectively [43].

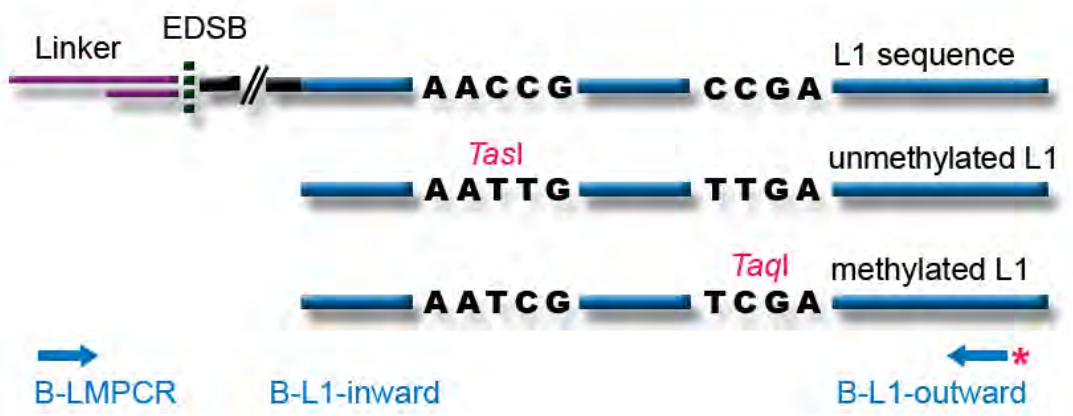


Figure 12. Schematic Illustration of COBRA-L1 and COBRA-L1-EDSB. L1 sequence ligated by linker at EDSB. Arrows are PCR primers, with star indicating 5' labeled primer with ^{32}P for COBRA. AACCG and CCGA are L1 sequences; when treated with bisulfite and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site).

The result showed that the methylation level between COBRA-L1 and COBRA-L1-EDSB were different. COBRA-L1-EDSB was higher than methylation levels of genomic L1s in both temperatures. Moreover, COBRA-L1 and COBRA-L1-EDSB didn't have different level between 47^{O}C and 37 ^{O}C (figure 13). The detected EDSB may be occurring and prevented by methylation or retained in heterochromatin. Therefore, chromatin-modified agents used to test that.

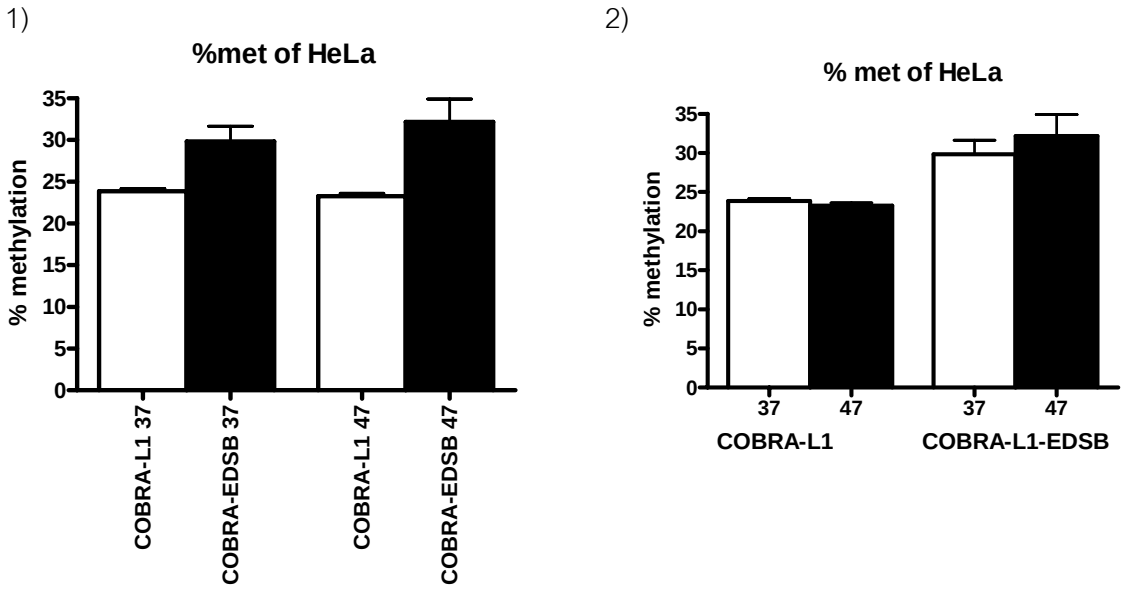


Figure 13. The methylation levels of genomic L1 and L1-EDSB. 1) Compared between Gobal methylation of genome and methylation of L1-EDSB in each condition. 2) Compare between temperature of Gobal methylation and methylation of L1-EDSB.

Hyperthermia caused increasing of EDSB

Kongruttanachok *et al.* treated cell with tricostatin A (TSA), that inhibited deacetylation make chromatin change from heterochromatin to euchromatin, they found increasing of γ-H2AX level but decreasing of EDSBs level. They suggested EDSB-NRs retained in heterochromatin and have slower repaired rate so that when they opened chromatin by a histone deacetylase inhibitor, trichostatin A (TSA), repaired complex can fix then found decreasing of EDSB level [Kongruttanachok *et al* submitted]. To test whether hyperthermia caused increasing of EDSB, I treated HeLa and HN8 in non-replicating cell with TSA to hyperacetylate histones and consequently decondense the heterochromatin.

After treated TSA 4 hrs, I incubated cell in different temperature and corrected cell by HMW DNA method. I found that heated cell have higher EDSB level more than unheated cell. Moreover, it's significant in HeLa cell (p=0.004 : paired T-test ; n=9). The delta mean the different level of EDSB in both condition, the EDSB that detected in high temperature more than normal (figure 14). When we compare delta between TSA and no TSA, the data shows that when we treated both TSA and temperature, cell can increase EDSB in high level. HeLa cell has significant, when we compare data between delta TSA and no TSA (p=0.04 : paired T-test ; n=9) (figure 14).

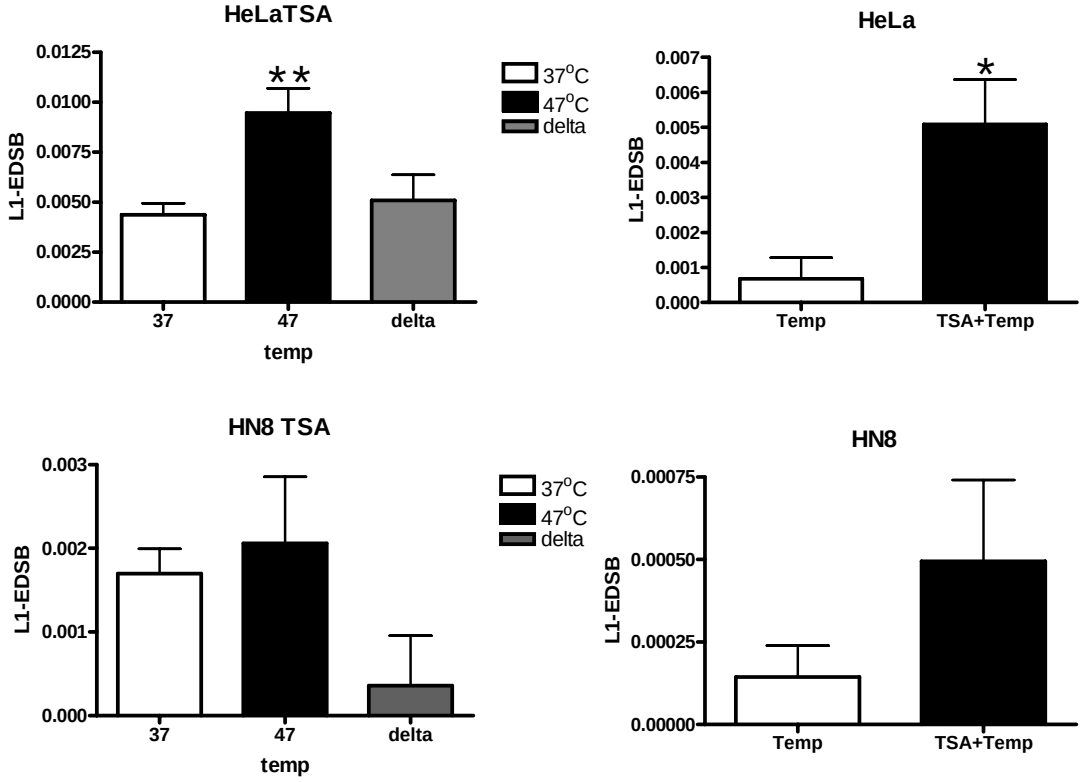


Figure 14. Hyperthermia caused increasing of EDSB. The right graph shows EDSB level when we treated cell with TSA and temperature. The left graph presents delta between temperature and temperature with TSA. The delta means difference EDSB in high temperature and room temperature. (* : P< 0.05 ; ** : P <0.01)

When we compare room temperature between treated TSA and no TSA, EDSB level didn't different and cells, only treated heat, EDSB still equal level. On the other hand, in high temperature, EDSB level in temperature with TSA is very high than cells didn't have TSA

(figure 15). Surprisingly, HeLa has more different level but didn't significantly with paired T-test.

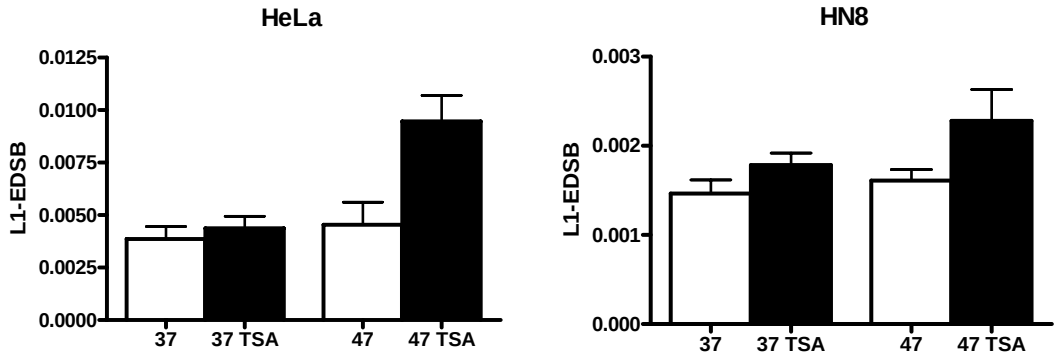
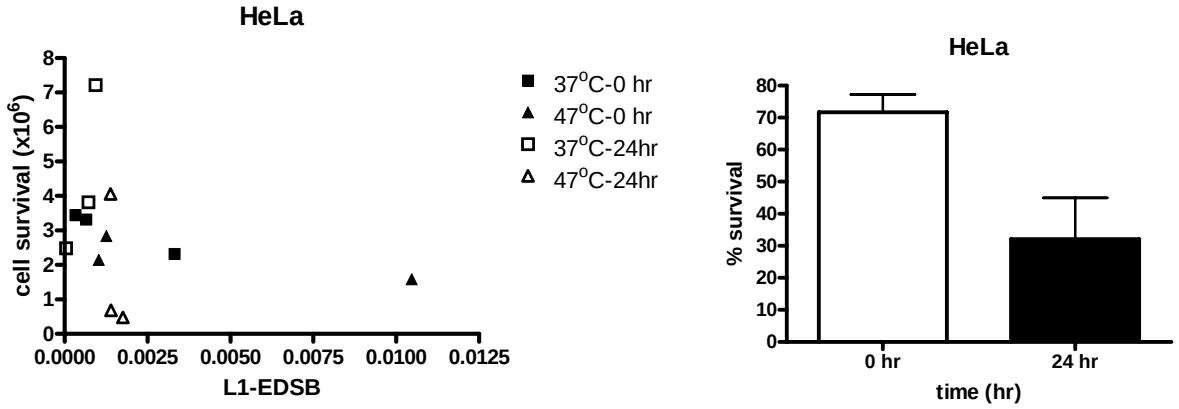


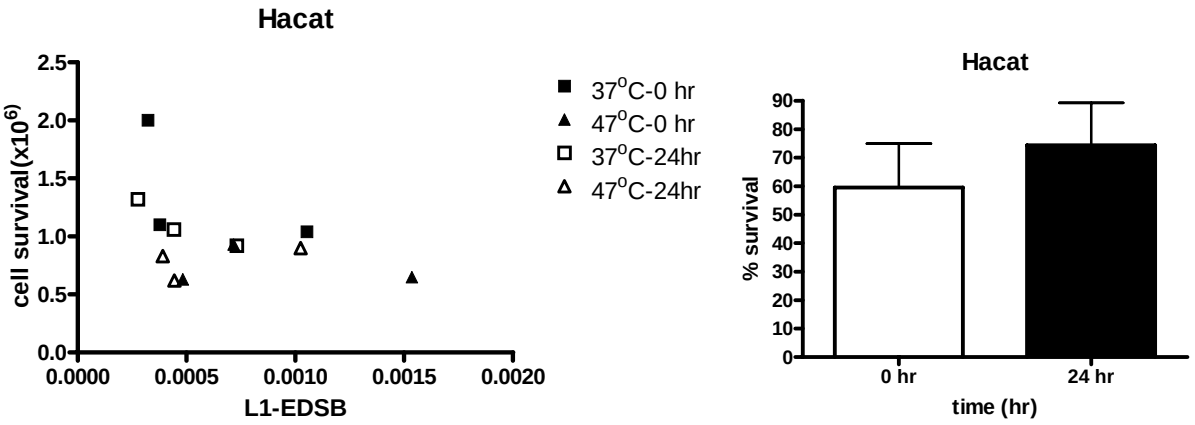
Figure 15. Different EDSB level between treated TSA and no TSA. Graph shows EDSB level between cell has TSA and no TSA, compare in the same temperatures.

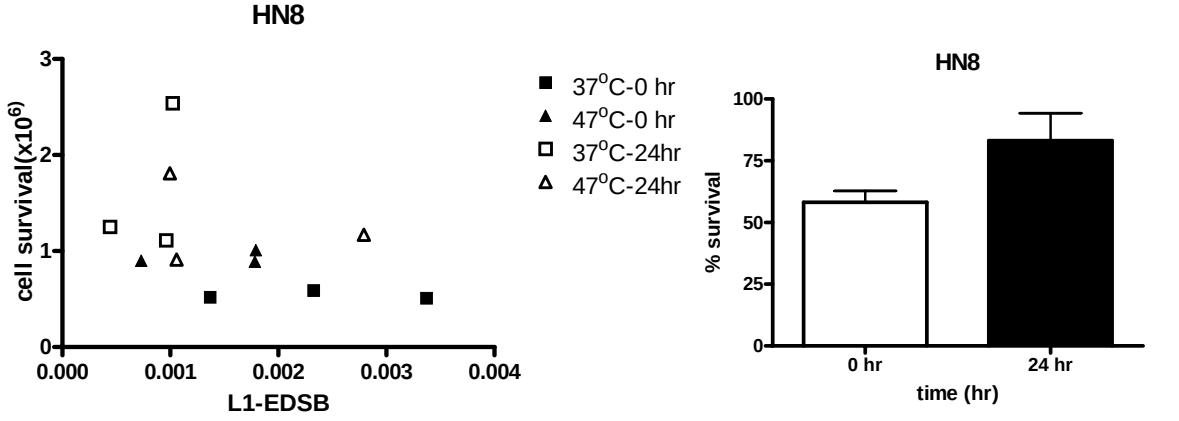
Cell survival decreased in hyperthermia

Cellular DNA can be damaged by physiological processes and environmental agents, resulting in a variety of lesions, including DNA base modifications, crosslinks and single and double-strand breaks (SSBs and DSBs). Among DNA lesions, the most harmful seems to come from the breakage of both DNA strands, since a single unrepaired DNA double-strand break (DSB) can induce cell death [23,26]. From the result above, EDSB level in heated cell was significantly higher also we treated with TSA, the result wasn't altered. Therefore, we want to test whether that when cell treated with hyperthermia, cell survival changed or not. We started cell about $1x10^6$, to synchronized cell into the G0 Phase by cultured in serum deprivation media. To analyze cell survival after treated cell 30 min in different temperature, the treated cells were trypsinized and suspended in 1ml of medium before mixed with a trypan blue stain. Viable cells can exclud the trypan blue stain from the cell so that we can separate and count. I found that the survival of all cell drop in high temperature (figure 16). Anyway, after we treated cell 30 min in both temperature then we incubated cell in 37 ^{O}C overnight, to test whether hyperthermia reduced DNA repair in cell. The result show that efficiency of DNA repair process can inhibit in high temperature in HeLa cell but other cell can be repaired so that survival of cell increase in 24 hr after treated

hyperthermia (figure 11:right). Moreover, when EDSB level was high, we will found the lower level of cell survival but no correlation between EDSB level and survival of cell (figure 11:left). Many study suggested inhibition of DNA repair was observed both heated cell in exponential phase and in plateau-phase but have 2 mechanism involved : (a) heat affected proteins/enzymes in DNA repair and (b) heat alters the chromatin structure so that DNA repair complexes less accessible to work [4-5,17].







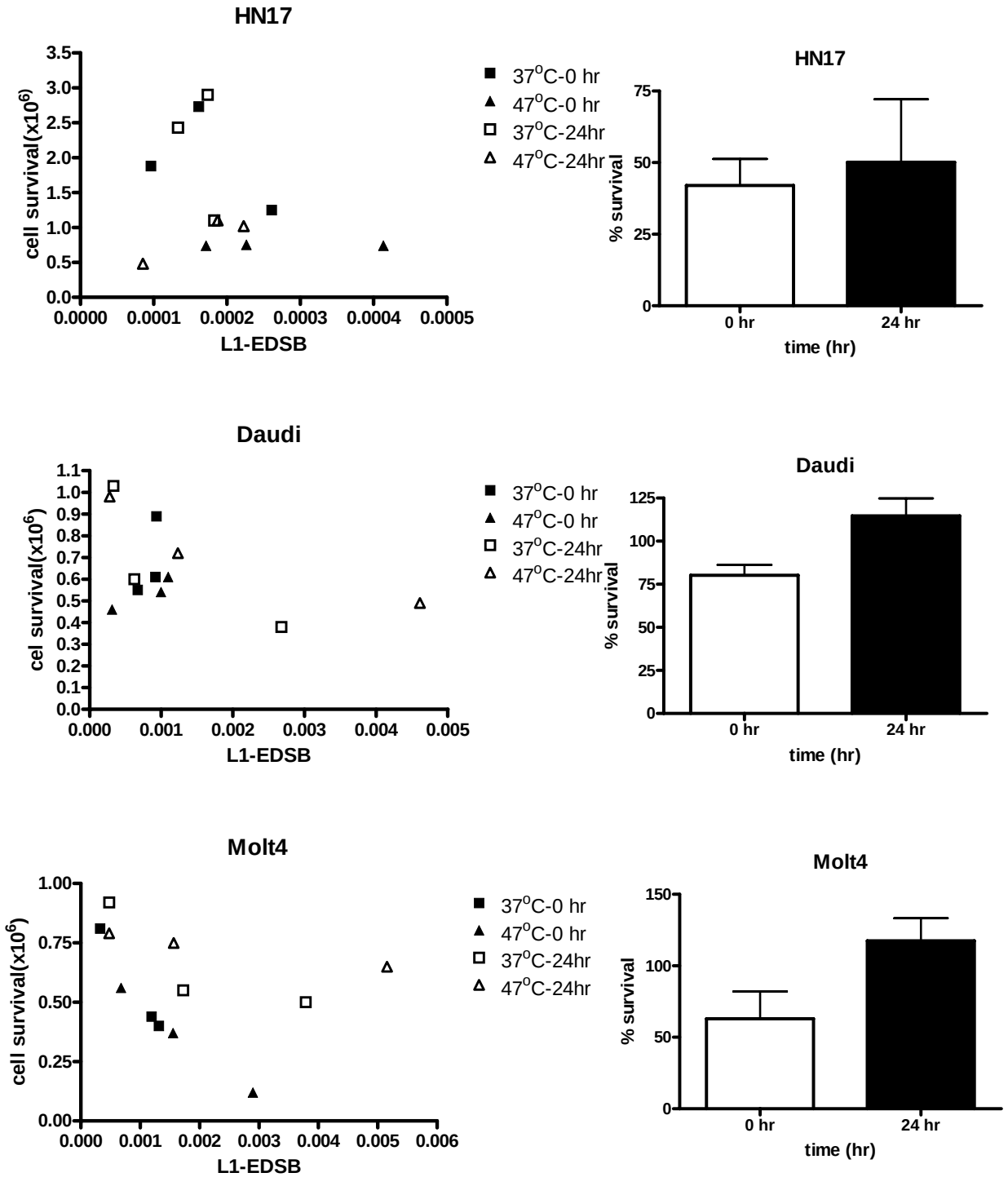


Figure 16. Cell survival assay. The left graph shows correlation between EDSB level and cell survival. The right graph presents % survival of cell incubated at 0 hr and 24 hr after treated high temperature.

CHAPTER V

Conclusion and discussion

My thesis demonstrates that heat can produce DSBs. Similar to previous studies [4-5,17,38,44], γ-H2AX bound DNA is increased when cells are incubated at 47°C. However, the increase in γ-H2AX is a cellular response and not a direct mechanical evidences. Moreover, previous studies could not demonstrate DSBs by direct measurement method such as comet assay [17,45]. Comet assay is a standard method for measuring DSBs as a number of small degraded DNA fragments that are migrated out of cells by electrophoresis [46-49]. The fact that heat produces no positive comet assay but increase γ-H2AX foci may suggest that heat may either influence γ-H2AX foci without DSBs or cause low number of DSBs and consequently, unlike radiation induced DSBs, the size of broken chromosomes may still be large. Nevertheless IRS-EDSB-LMPCR is designed to measure EDSBs that are rare and randomly distributed. My study demonstrates significant increase in EDSBs by heat in HeLa and WSU-HN8. These experiments supports that heat may produce EDSBs.

Similar to γ-H2AX foci, only some cells that heat may significant increase L1-EDSB-LMPCR product. Therefore, heat increased EDSBs is specific cell type. Interestingly, HeLa and WSU-HN8 possess the most genome-wide hypomethylation when compared with the others [50]. This may suggest that heat increased EDSBs in cell with lower genome methylation levels. Because hypomethylation is directly associated euchromatin conformation, which is increased histone acetylation [51-52], we further test how heat produced EDSBs in cells with hyperacetylation. Interestingly, I proved that hyperacetylation remarkably increased heat induced EDSBs.

The mechanism of heat induces EDSBs and euchromatin enhance this process is remained to be explored. Recently, Kongruttanachok and I proved that heterochromatin can retain EDSBs and the EDSB retention helps maintain genomic integrity (Kongruttanachok et

al submitted). When chromosome is denature in one location, because of long and continuous double helix structure, DNA double helix will be rigorously spun. We speculate that similar to topoisomerase II [53], retained EDSBs may help relax DNA tension from double helix spinning. I also speculate if heat may produce EDSBs from denaturation and spinning. Moreover, lacking of heterochromatin may also lead to lacking of retained EDSBs and consequently, hyperacetylation can dramatically enhance heat induced EDSBs.

Similar to Zeng ZC *et al,* [54], my preliminary study dose not demonstrates the correlation between heat induced DSBs and cell survival. This may be due to the number of EDSBs produced by heat is low. Consequently, other molecular physiological consequences may affect survival more significantly. Nevertheless, because heat induced DSBs in hypomethylated genome and global hypomethylation is a cardinal sign of cancer leading to genomic instability, it will be interesting to further exploration if heat can enhance genomic instability in cancer.

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APPENDICES

APPENDIX A

The Possible Roles of Endogenous DNA Double Strand Breaks under Non-replicating Conditions

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Running Title: Endogenous DSBs in Non-Replicating Cells

List of abbreviations

EDSBs, endogenous DNA double strand breaks; EDSB-NRs, EDSBs in non-replicating genomes; NHEJ, non-homologous end joining repair; ; DNA-PKcs, DNA-dependent protein kinase catalytic subunit;ATM, ataxia telangiectasia mutated ; DSBs, double strand breaks; EDSB-R, DSBs from replicative DNA polymerase encountering single-strand DNA breaks; LMPCR, ligation-mediated polymerase chain reaction; IRSPCR, interspersed repetitive sequence PCR; PFGE, pulse field gel electrophoresis; L1 or LINE-1, long interspersed element-1; HMWDNA, High molecular weight DNA; Chromatin immunoprecipitation, ChIP.

Abstract

Recently, we invented a new PCR technique to measure endogenous DNA double strand breaks (EDSBs). Methylation levels of long interspersed element-1 at sites close to EDSBs are higher than in the genome. The most significant differences were observed in non-replicating cells. For a better understanding of EDSBs and their epigenetic modification, we evaluated EDSBs in non-replicating genomes (EDSB-NRs). Variable EDSB-NR levels were identified from all tests and were significantly reduced after prolonged cultured in serum-deprived conditions. Higher temperatures increased EDSB-NRs. Vanillin, an inhibitor of the DNA-dependent protein kinase catalytic subunit, a non-homologous end joining repair (NHEJ) enzyme, sporadically increased unmethylated EDSBs. EDSB-NRs normally possess higher methylation levels than γ-H2AX-bound DNA, one of the earliest repair responses to DSBs. The histone deacetylase inhibitor, trichostatin A, loosens heterochromatin, decreases EDSB-NRs and increases methylated γ-H2AX-bound DNA. Cells that are depleted in ataxia telangiectasia mutated (ATM) demonstrated higher EDSB-NR methylation levels. The role of retained EDSB-NRs may be the opposite of the role of radiation induced DSBs. Cells with higher numbers of EDSB-NRs demonstrated a higher rate of cell survival upon treatment with vanillin. In conclusion, EDSB-NRs are ubiquitously present and may be produced under normal physiologic conditions or during benign physical stress. Heterochromatin may prevent immediate cellular response to methylated EDSBs. Therefore, in contrast to unmethylated EDSBs, methylated EDSB-NRs may be delayed to be repaired, retained within heterochromatin, devoid of γ-H2AX and are repaired by the ATM dependent NHEJ pathway. In contrast to radiation-induced DSBs, retained EDSB-NRs may benefit genome stability.

Key Words: DNA methylation, Endogenous DNA double strand breaks, γ-H2AX, heterochromatin, long interspersed nuclear element-1

Introduction

Hazardous chemical agents and ionizing radiation produce large numbers of DNA double strand breaks (DSBs), which can be observed as fragmented DNA [1; 2]. This breakage can trigger apoptosis and error repair may lead to mutation [3]. Nevertheless, low levels of DSBs can occur spontaneously and are known as endogenous DSBs (EDSBs). Several mechanisms, including replicative DNA polymerase encountering single-strand DNA breaks (EDSB-R), temperature, osmolarity, oxidative DNA damage and endonucleases [4; 5; 6; 7; 8; 9; 10; 11; 12], produce γ-H2AX foci. γ-H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DSB repair responses [13; 14]. The roles of EDSBs induced by different causes are different. In 2003, Vilenchik and Knudson proposed that the number of EDSB-Rs [4] should be enough to play a key role in genomic instability in cancer, as they can be intermediates in DNA or chromosomal rearrangements [4]. DSBs, however, do not play a role in heat or hypertonicity-induced cell killing [9; 15]. V(D)J recombination is important in lymphocyte development [16], and topoisomerase II helps maintaining genomic integrity [17].

Recently, we developed a novel PCR technique, called IRS-EDSB-LMPCR, to measure the number of EDSBs [18] by combining ligation-mediated polymerase chain reaction (LMPCR) [16] and interspersed repetitive sequence PCR (IRSPCR) [19]. IRS-EDSB-LMPCR measures DSBs differently from comet assay [1; 2], pulse field gel electrophoresis (PFGE) [20] and γ-H2AX foci [13; 14]. While γ-H2AX foci represent one of the cellular responses to DSBs, comet assay, PFGE and IRS-EDSB-LMPCR measure exact number of DSBs. High dose radiation can produce positive comet assay and PFGE as multiple small fragmented DNA. However, comet assay and PFGE cannot detect small number of DSBs, occurring randomly, because DNA is remained large in size and majority of the chromosomes, including telomere and centromere, are intact. In contrast, due to the interspersed distribution

and large number of IRSs, IRS-EDSB-LMPCR could identify and measure the minute number of randomly distributed EDSBs. We identified EDSBs in all tested cells and cell phases. The majority of EDSB ends, blunt and 5' phosphorylated [18], are similar to the signal ends of V(D)J recombination [16] and hypermutation [21].

We chose to extensively evaluate the long interspersed element-1 (L1 or LINE-1) because the methylation status of these retrotransposable elements has been extensively studied [22; 23; 24]. The number and methylation state of EDSBs were analyzed at the L1 sequences near EDSBs of L1-EDSB-LMPCR templates [18]. L1-EDSBs of almost all tested cells were hypermethylated, having higher methylation levels in L1s at the sites nearest EDSBs than at other sites in the genome [18]. Moreover, while EDSBs were hypermethylated in most examined cell phases, G0 was the most significant phase [18]. In contrast, EDSB-Rs possess lower methylation levels [18], produced from abnormal DNA lesions, and they can cause mutations in cancer [4]. Therefore the roles of EDSB-NRs may be unique.

Materials and Methods

Cell culture and survival assay

The cell lines used, including HeLa (cervical cancer), Daudi (B lymphoblast), HCT116 (colon cancer) and Jurkat (T cell leukemia), were purchased from ATCC (Manassas, VA, USA). To synchronized the cells in the G0 phase, the cells were cultured in serum deprivation medium for 48 hr. HeLa cells in G1/S and S phase were synchronized by the thymidine block method and were cultured with 2 mM thymidine (Sigma-Aldrich, St. Louis, MO, USA) to obtain cells in the G1/S phase [25]. Flow cytometry was used to determine the stages of the cell cycle as well as to identify fragmented and apoptotic cells. To observe the

effect of temperature, cells were cultured at 47°C for 30 min. To evaluate the consequences of histone hyperacetylation, a single dose of 100 ng/ml trichostatin A (TSA) (Sigma-Aldrich), an inhibitor of histone deacetylase, was added to synchronized G0 HeLa cells. Fostriecin (100 nM; Sigma-Aldrich) was added to the cells at 37°C for 30 min prior to TSA to observe the γ-H2AX-bound DNA. To inhibit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia mutated (ATM) and DNA-PKcs simultaneously with ATM, HeLa cells were treated with 2.5 mM vanillin, 1-5 mM caffeine and 40 μM LY294002 (Sigma-Aldrich), respectively. Significant apoptosis was observed in the LY294002 and caffeine experiments, respectively; however, these experiments were not used in further analyses. HCT116 cells were treated with 2.5 mM vanillin. To analyze survival, triplicate treated cells were trypsinized and suspended in 1 ml of medium before testing their viability with a trypan blue stain. Viable cells that excluded the trypan blue stain were counted. For radiation treatment, the medium of the HeLa cells was replaced with ice-cold medium, and the cells were exposed to 0.01, 0.1, 1.0, 2.0, 10, 20, 40, and 60 Gy γ-rays at a rate of 6.22 cGy/min with a ^{60}Co source (Eldorado78).

High molecular weight DNA preparation

HMW DNA was prepared as described previously [18]. To prepare High molecular weight (HMW) DNA, 1×10^6 cells were embedded in 1% low-melting-point agarose, lysed, and digested in 400 μl of 1 mg/ml proteinase K, 50 mM Tris, pH 8.0, 20 mM EDTA, 1% sodium lauryl sarcosine. The plugs were rinsed four times in TE buffer for 20 min. To polish cohesive-end EDSBs, T4 DNA polymerase (New England Biolabs, Beverly, MA, USA) was added, followed by rinsing four times in TE buffer for 20 min. The modified LMPCR linkers

were prepared from the oligonucleotides 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAAGCTTACCTCGTGGACGT-3' and 5´-ACGTCCACGAG-3´. The linkers (50 pmol) were ligated to HMW DNA using T4 DNA ligase (New England Biolabs) at 25°C overnight. DNA was extracted from the agarose plugs using a QIAquick gel extraction kit (QIAGEN, Basel, Switzerland).

Detection and quantification of L1-EDSBs

After the LMPCR linkers, 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAA GCTTACCTCGTGGACGT-3' and 5´-ACGTCCACGAG-3´, were ligated to HMW DNA, the number of L1-EDSBs was measured as previously described for L1-EDSB-LMPCR with some modifications [18]**.** A duplicate or triplicate number of L1-EDSBs was measured by real-time PCR using a ABI PRISM® 7500 instrument (Applied Biosystems, Carlsbad, CA, USA) with the IRS primers, including the L1 primers 5´-CTCCCAGCGTGAGCGAC-3´ (outward), the linker primer 5´-AGGTAACGA GTCAGACCACCGA-3´, and the Taqman probe homologous to the 3´ linker sequence (6-fam) ACGTCCACGAGGTAAGCTTCCGAGCGA (tamra) (phosphate). Amplification was performed with 0.5 μM of each primer, 0.3 μM Taqman probe, 0.025 U of HotStarTaq (QIAGEN, Valencia, CA, USA), 1x TaqMan® Universal PCR Master Mix (Applied Biosystem) and 30 ng of ligated DNA for up to 60 cycles, with quantification after the extension steps. Control HeLa DNA was digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers. The numbers of EDSBs were compared with the ligated control digested DNA and reported as L1 ligated *Eco*RV and *Alu*I digested genome per cell and L1-EDSBs genome per control genome.

Study of L1 and L1-EDSB methylation

L1 and L1-EDSB, COBRA-L1, and COBRA-L1-EDSB were measured by combined bisulfate restriction analysis as previously described [18]. Ligated HMW DNA was modified with bisulfite. Bisulfite-modified DNA was recovered using a Wizard DNA clean-up kit (Promega, Madison, WI, USA) and desulfonated before PCR amplification. For COBRA-L-1, bisulfite-treated DNA was subjected to 35 cycles of PCR with two primers, B-L1-inward 5´-CGTAAGGGGTTAGGGAGTTTTT-3´ and B-L1-outward 5´-RTAAAACCCTCCRAACC AAATATAAA-3´. Applying a hot-stop technique to prevent heteroduplex amplicons, the α^{32}P-labeled-bisulfite-L1-outward oligo was added in the last PCR cycle. The amplicons were doubly digested in a 10 μl reaction volume with 2 U of *Taq*I and 8 U of *Tas*I in 1× *Taq*I buffer (MBI Fermentas, Vilnius, Lithuania) at 65°C for 4 hr. This method was designed to detect unmethylated and methylated sequences of 98 and 80 bp, respectively. The intensity of DNA fragments was measured with a PhosphorImager using Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). The LINE-1 methylation level was calculated as the percentage of *Taq*I intensity divided by the sum of *Taq*I- and *Tas*I-positive amplicons. For COBRA-L1-EDSB, the B-L1-inward oligo was replaced with the B-LMPCR oligo, 5´-GTTTGGAAGTTTATTTTGTGGAT-3´, and 40 PCR cycles were carried out according to the same protocol. Bisulfite-treated Daudi, Jurkat, and HeLa DNA digested with *Eco*RV and *Alu*I and ligated LMPCR linker were used as positive controls to normalize the inter-assay variation of all COBRA experiments. HeLa DNA without ligation was used as a negative control.

ChIP

The ChIP assay was performed essentially as previously described with some modifications [7; 26]. The chromatin fragments were immunoprecipitated with anti-phospho-Histone H2AX monoclonal antibody (Upstate, Charlottesville, VA, USA) or normal mouse IgG antibody as a negative control (Santa Cruz Biotechnology). Quantification of the amount of immunoprecipitated DNA was carried out by real-time 5'L1PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland) between the forward primer (L1.2HpaIIRFLPF: 5´-CTCCCAGCGTGAGCGAC-3´) and reverse primer (5´LIDSIP1st: 5´-ACTCCCTAGTGAGATGAACCCG-3´) located at the 5' end of L1. The amount of γ-H2AX-bound L1 sequences was used to calculate the quantity of precipitated genomic DNA by relating the L1 quantity to the L1s quantity of HeLa genomic DNA. The relative quantity unit was γ-H2AX-bound genome per cell. The precipitated DNA was then subjected to COBRA-L1.

siRNA

The oligonucleotide sequences of siRNA targeting ATM was as previously described by Zhang, et al [27]. These oligonucleotides were inserted into the PsilencerTM 3.1 vector (Ambion, Austin, Texas, USA) and transfection was mediated by siPORTTM *XP-1* (Ambion, Austin, Texas, USA).

Western blot analysis

Antibodies used for Western blot included an anti-G3PDH antibody (Trevigen, Gaithersburg, MD, USA) as a control; an antibody against acetylated-histone H4 which recognizes histone H4 acetylated at lysines 5, 8, 12, or 16 (Upstate, Charlottesville, VA, USA) for analyzing histone acetylation in TSA-treated cells; ATM (2C1) (GeneTex, San Antonia, Tx, USA), for analyzing ATM, in siRNA experiments; and horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) conjugate (Zymed® Laboratories, San Francisco, CA, USA) for GAPDH and acetylated-histone H4 and goat anti-mouse IgG-HRP sc-2005 HRP conjugated (Santa Cruz Biotechnology) for ATM. Signals were developed using the Supersignal west chemiluminescent substrate optimization kit (Pierce, Rockford, IL, USA).

Statistical analyses

Statistical significance was determined according to a paired sample *t*-test, Spearman or Pearson rank correlation statistics when appropriate.

Results

EDSBs in non-replicating cells

First, we measured L1-EDSBs under serum deprivation conditions, and EDSB-NRs were detectable in all samples (Figure 1A). When cells from the same passage were separated and simultaneously cultured, we observed consistent levels of EDSBs in each experiment, suggesting that we were able to make precise and reproducible measurements (Figure 1A). There was no statistical difference between 48 and 72 hrs (n=12, pair two-tailed t test, p=0.0926) (Figure 1B); however, the levels of L1-EDSBs at 48 hrs were significantly lower

than those at 24 hrs (n=12, pair two-tailed t test, p=0.031) (Figure 1B). This may indicate that EDSBs were commonly identifiable and that the repair of EDSB-NRs was occurring. The number of EDSBs can be varied due to tissue culture conditions. An increase in the number of EDSBs was observed when cells were cultured at higher temperature (n=14, pair two-tailed t test, p=0.009) (Figure 1C). The increased numbers of EDSBs are still hypermethylated (Figure 1D). Heat increased the number of EDSBs, which may support previous findings of heat-induced γ-H2AX in mammalian cells [5; 6; 7]. Vanillin is an inhibitor of phosphatidylinositol-3-kinases that is selective for DNA–PKcs, a non-homologous end joining (NHEJ) repair enzyme, and it was reported to cause cell death under certain conditions [28; 29; 30]. Sporadic increases in the number of unmethylated L1-EDSBs were found in cells treated with vanillin (Figure 1E). Therefore, production and repair of EDSB-NRs can be observed. However, unmethylated EDSBs may occur transiently and are immediately repaired.

EDSB-NR reduction by trichostatin A

EDSB-NRs may be processed depending on their chromatin conformation. Detectable EDSBs are hypermethylated because methylated and unmethylated EDSBs may be processed differently [18]. DNA methylation usually associates with heterochromatin [31]. The L1-EDSB methylation exists in the genome prior to the DNA breakages [18]. The methylation levels of genomic L1s are distinct among loci [23]. Consequently, methylated EDSB-NRs may be located specifically in heterochromatin. To test whether EDSB-NRs are really associated with heterochromatin, we treated HeLa cells with a histone deacetylase inhibitor, TSA, to hyperacetylate histones and consequently decondense the heterochromatin. TSA treatment of G0 stage HeLa cells significantly reduced the number of L1-EDSBs (paired two tailed t-test, n=18, p=0.0049) (Figure 2A). We further compared the correlation between the reduced,

control minus TSA, L1-EDSBs and L1-EDSBs of control or TSA-treated samples (Figure 2B). We observed a strong direct correlation with EDSBs of control cells (n=14, Pearson r=0.8471, P value (one-tailed) <0.0001) (Figure 2B). The more control cells possessed EDSB-NRs, the more the reduction in EDSB-NRs by TSA can be observed. In contrast, there was no correlation between the reduced L1-EDSBs and the L1-EDSBs of TSA-treated samples (r -0.2733, p=0.1722) (Figure 2B). This result may suggest that EDSB-NRs may be retained within chromatin having limited acetylated histone. In addition, TSA treatment, which converts heterochromatin into euchromatin, causes immediate EDSB repair (or end modification), and the degree of this process depends on the amount of L1-EDSB substrates.

EDSB-NRs and γ-H2AX

γ-H2AX foci are one of the earliest DSB repair responses [13]. However, γ-H2AX foci form preferentially in euchromatin over heterochromatin after ionizing-radiation [32]. To evaluate the correlation between EDSB-NRs and γ-H2AX, γ-H2AX-bound DNA was obtained by Chromatin immunoprecipitation (ChIP) [26] using a γ-H2AX antibody, and bound L1 sequences were quantitated with real-time PCR using 5´L1 primers [7] (Figure 3). All cells, including leukemic cell lines and HeLa cells, in both the non-replicating and replicating cell cycle phases possess both L1-EDSBs and γ-H2AX-bound L1s (Figure 4A-D). One characteristic of L1-EDSBs is L1 hypermethylation [18]. When we analyzed the methylation status of γ-H2AX-bound L1s, we found that γ-H2AX-bound L1s in all cells were significantly less methylated than L1-EDSBs (paired two-tailed t-test; n=3; p=0.008, 0.0193, 0.0243 for Daudi, Jurkat and HeLa cells, respectively) (Figure 4E). The γ-H2AX-bound L1s in Daudi cells were also significantly less methylated than genomic L1s (p=0.0179) (Figure

4E). Moreover, in G0, there was a more prominent distinction between the methylation levels of L1-EDSBs and γ-H2AX-bound L1s (paired two-tailed t-test, n=6, p=0.0024) than in the S phase (p=0.026) (Figure 4F). Therefore, a significant number of methylated EDSB-NRs may be free from γ-H2AX.

γ-H2AX-bound DNA and histone acetylation

TSA also increased the number of γ-H2AX-bound L1s (paired t test, n=16, p=0.0189) (Figure 5A). The increase in γ-H2AX-bound L1s was directly correlated with the number of L1-EDSBs that existed prior to the beginning of the experiment (n=10, Spearman r=0.7576, P value (two-tailed) = 0.0149) (Figure 5B). TSA treated samples with increased γ-H2AX-bound L1s possessed higher levels of γ-H2AX-bound L1 methylation than the control (paired two-tailed t-test, n=8, p=0.0007) (Figure 5C). This higher methylation level may be due to this process producing new γ-H2AXs at methylated genomes. The methylation levels of increased γ-H2AX-bound L1, Δγ-H2AX, were also higher than in control (p=0.019) and TSA treated samples (p=0.0447) (Figure 5C). These data indicate that EDSB-NRs may stay within heterochromatin and remain free from γ-H2AX. Histone acetylation may allow the cells to respond to and repair these EDSBs. This EDSB-NRs retention suggests that there may be different rates of repair between heterochromatin- and euchromatin-associated EDSBs. This finding is supported by the fact that H2AX foci form preferentially in euchromatin but not in heterochromatin after ionizing-radiation [32]. Moreover, heterochromatin radiation induces a slow repair of DSBs and requires ATM, a DNA repair gene [33]. Similarly, the down-regulation of ATM results in the higher methylation levels of L1-EDSBs, especially during G0 (Figure 6).

EDSB-NRs, cell survival and TSA

We next evaluated the biological roles of EDSB-NRs. Radiation-induced DSBs reduce cell survival in a dosage dependent manner. First, we compared the survival of cells possessing different levels of EDSB-NRs and found no correlation between the number of L1-EDSBs and cell survival after 4 hrs of incubation (n=9, Spearman r=-0.1667, P value (two-tailed)=0.6777) or 24 hrs (n=9, Spearman r=0.1000, P value (two-tailed)=0.798) (Figure 7A). Second, we tested if TSA could expose EDSB-NRs hidden within heterochromatin and, consequently, decrease cell survival. Synchronized G0 cells were treated with TSA, and cell survival was measured at 4 and 24 hrs. No significant change was observed at 4 hrs, but a decrease in survival could be observed at 24 hrs after TSA treatment (paired two-tailed t-test, n=9, p=0.0002) (Figure 7B). No correlation was observed between the number of L1-EDSBs before TSA treatment and cell survival (n=9, Spearman r=0.25 and -0.0500 and P value (two-tailed) = 0.5206 and 0.9116 for 4 and 24 hrs, respectively) (Figure 7C and D). Therefore, unlike the case of radiation-induced DSBs, we found no evidence of EDSB-NRs being pathological lesions.

EDSB-NRs, cell survival and vanillin

Finally, we further tested how EDSB-NRs are related to vanillin treatment. After 4 hrs of incubation, no change in survival was observed (n=9, Spearman r=0.3167, P value (two-tailed)=0.4101) (Figure 7B and E). Nevertheless, a significant decrease in cell survival was observed at 24 hrs after vanillin addition (paired two-tailed t-test, n=9, p<0.0001) (Figure 7B). Interestingly, at 24 hrs after vanillin was added to the culture medium, fewer cells survived if they possessed fewer EBSD-NRs (n=9, Spearman r=0.7833, P value (two-

tailed)=0.0125) (Figure 7F). Similar findings were observed when HCT116, colon cancer, cells were treated with vanillin (n=11, Spearman r=0.7091, P value (two-tailed)=0.0146) (Figure 8A and B). Therefore, these G0 cells with fewer L1-EDSBs were more sensitive to vanillin than cells with higher L1-EDSB levels.

Discussion

EDSB-NR retention and repair

EDSB-NRs are hypermethylated because methylated and unmethylated EDSBs are not equally processed [18]. In this study, we showed that histone hyperacetylation reduced EDSB-NRs and increased methylated γ-H2AX-bound DNA. Therefore, methylated EDSB-NRs may be retained in heterochromatin and histone acetylation may facilitate their repair. EDSB retention may be opposite to what is generally believed. DSBs are known to be hazardous events. A single DSB, if unrepaired, will induce lethality [34]. However, it is reasonable to discover EDSB retention when the DSB ends are prevented from general cellular response to DSBs. For example, signal EDSB ends can persist within V(D)J recombination complex and do not normally activate the DNA-damage-dependent cell cycle checkpoint [35]. We speculate that cellular responses to methylated EDSBs may be delayed by chromatin conformation. DNA methylation usually associates with heterochromatin [31], whose tightly packed structure may brace the broken chromosome. Recently, Cowell *et al*. found that H2AX foci form preferentially in euchromatin but not in heterochromatin after ionizing-radiation [32]. EDSB retention may help explain how TSA increased methylated γ-H2AX-bound DNA. When TSA relaxed chromatin conformation, retained EDSB-NRs might be exposed to the immediate cellular DSB responses. In the S phase, EDSBs are still

hypermethylated, albeit with less significance than in the G0 phase [18]. Because DNA replication does not occur simultaneously throughout the genome, heterochromatin may still capture the methylated EDSBs that locate away from replication forks.

The mechanical and consequent DNA repair processes between EDSBs associated with heterochromatin and those associated with euchromatin should be different. In general, DSB repair pathways are redundant and interchangeable [36], but the reduction of ATM expression led to increased methylation of EDSB-NRs (Figure 6). EDSB-NRs may be similar to radiation-induced DSBs in heterochromatin that is slowly repaired by ATM [33]. In contrast to other NHEJ pathways, the ATM-dependent repair pathway has been proposed to be more precise [37; 38]. Therefore, methylated EDSB-NRs may be able to avoid error-prone NHEJ repair [39; 40; 41; 42; 43]. Consequently, the rate of spontaneous mutations from methylated EDSB-NRs may be limited.

L1-EDSB-NRs and γ-H2AX bound L1 methylation

Methylation statuses between L1-EDSB-NRs and γ-H2AX bound L1 EDSB-NRs may provide an additional suppport that EDSB-NRs may be devoid of γ-H2AX. Each L1 locus has distinctive DNA methylation levels, and the DNA methylation levels of L1s located within the same gene are closely correlated [23]. The methylation levels of L1-EDSB-NRs are higher than γ-H2AX-bound-L1s. Moreover, the γ-H2AX-bound L1s of some cells were also less methylated than genomic L1s (Figure 4). This finding argues against the possibility that L1-EDSB-NR and γ-H2AX-bound-L1 methylations are different due to the expansion of γ-H2AX. Moreover, both methylated of L1-EDSB-NRs and γ-H2AX-bound-L1s may be

differently distributed. Consequently, significant numbers of EDSB-NRs may devoid of γ-H2AX.

EDSB-NR production

Radiation-induced DSBs are hazardous to cells and can lead to faulty DNA recombination. Therefore, the production of EDSB-NR in all cells without strong environmental insults needs explanation. Even though EDSB-NRs were significantly reduced during prolong cell culture in G0, increased levels of EDSB-NRs from matched samples were sometime observed (Figure 1B). Moreover, new EDSB-NRs can be detected by heat treatment (Figure 1C). This suggests that EDSB-NRs can be produced without DNA breakage chemical agents or radiation. When vanillin was added, the levels of unmethylated EDSBs were sporadically increased (Figure 1E). Therefore, EDSB-NRs can be produced from both unmethylated and methylated DNA. The precise mechanism producing EDSB-NRs is unknown. Nevertheless, L1-EDSB-NRs ends are different from topoisomerase II EDSB ends if there is no further enzymatic modification. Topoisomerase II lesions occur transiently and form a covalent phosphotyrosyl bond at the 5′-terminus, with four-base 5′-single-stranded cohesive ends [44; 45; 46; 47]. The modified 5'- terminus should prevent LMPCR linker ligation, and the majority of EDSB-NR ends are blunt [18]. It remains to be explored how EDSB-NRs are produced. Speculative hypotheses of the mechanism include endonucleases, oxidative stress or the consequence of increased DNA tension from DNA unwinding by transcription, cellular movement, altered osmolarity or increased temperature.

EDSB-NRs versus EDSB-Rs and radiation induced DSBs

EDSB-NRs are unique in term of epigenetic modification, cellular response and cell survival. The difference in methylation levels between L1-EDSBs and γ-H2AX-bound L1s or genomic L1s in G0 was more prominent than in the S phase (Figure 4). Therefore, EDSB-NRs are different from EDSB-Rs [4] in that EDSB-Rs are less methylation dependent [18] and may not be free from γ-H2AX [4]. Retained methylated EDSB-NRs, unlike radiation-induced DSBs, may not induce cell death. EDSB-NRs are present in all cells and do not correlate with decreased cell survival. Furthermore, whereas heat can produce EDSB-NRs (Figure 1C), DSBs do not have a role in heat-induced cell killing [15]. In contrast, EDSB-NRs may help the cell to survive. Vanillin not only induces apoptosis [37] but also prevents mutations [48; 49]. One of the mechanisms may be due to the fact that vanillin, a DNA-PKcs inhibitor, selectively kills genomically unstable cells, such as irradiated cells. In contrast, we demonstrated that vanillin may selectively do less harm to cells that possess more EDSB-NRs. Therefore, this study reveals an unprecedented characteristic of EDSBs. Whereas radiation-induced DSBs are lethal to vanillin treated cells in a dose-dependent manner [28], EDSB-NRs may help the cell survive vanillin toxicity A higher number of EDSB-NRs may be relate to a more stable genome as if an immune to genomic stress. These findings, while surprising, are reasonable. EDSB-NRs that are retained in heterochromatin may possess a similar role as topoisomerase lesions that are important in maintaining DNA integrity by reducing the tension of DNA [17; 50].

EDSB retention may help explain several scientific findings.

There are several scientific findings that have not yet been explained and EDSB retention may help provide a possible clue. For example, single cell PCR is an important method for preimplantation diagnosis [51]. However, allele drop out has been an unexplained major drawback [52]. If an EDSB is retained or occurred in the PCR template of one allele, allele drop out will occur. Second, histone deacetylase inhibitors have been found to induce γH2AX in several cancers, especially leukemia [53]. It would be interesting to evaluate EDSB retention in leukemia. Interestingly, in 2005, Yaneva *et al*. reported high cellular toxicity when NHEJ inhibitor and TSA were combined [30]. Interestingly, here, we demonstrated that TSA reduced EDSB-NRs and cells with lower EDSB-NRs survived less from vanillin treatment.

Finally, this study may help speculate the connection how vanillin prevents mutation and how global hypomethylation leads to genomic instability. Genomic hypomethylation is a common molecular event in cancer [22; 54; 55], and it leads to chromosomal instability [56; 57; 58; 59]. The methylation levels of EDSB-NRs are in direct correlation with the genome. The EDSB methylation levels of hypermethylated genomes are higher than genomes that possess lower methylation levels [18]. Interestingly, vanillin not only inhibits NHEJ repair, increases unmethylated EDSBs, induces apoptosis, and prevents mutation, but also cells with lower EDSB-NRs are more sensitive to vanillin treatment. It will be interesting to further explore whether retained methylated EDSB-NRs contribute to genomic stability and whether lower levels of the EDSB-NRs are directly associated with hypomethylation-related instability.

Conclusions

DSBs are lethal unless repaired; however, limited numbers of EDSB-NRs are always detectable. Therefore, EDSBs may be produced under normal physiologic conditions or during benign physical stress. Heterochromatin may brace methylated EDSBs and prevent immediate cellular response. In contrast to unmethylated EDSBs, methylated EDSB-NRs may be delayed to be repaired, retained within heterochromatin and devoid of γ-H2AX. Nonetheless, methylated EDSBs are repaired by the ATM dependent NHEJ pathway. In contrast to general DSBs, retained methylated EDSB-NRs may benefit genome stability.

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Figure Legends

Figure 1 Levels of L1-EDSB-NRs. L1-EDSBs are the number of L1-EDSB genomes per genome digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers or L1-EDSB genomes per control genome. (A) Duplicates or triplicates of L1-EDSBs from different passages and incubation times in serum deprivation media. Each dot of the same experiment (exp) marks HeLa and HCT116 cells from the same passages but derived from different tissue culture flasks. Dots within the same drawing mark cells from different experiments but whose DNA and PCR experiments were prepared simultaneously. (B) L1-EDSBs incubated for different amounts of time in serum deprivation media.. (C) L1-EDSB-LMPCR and (D) COBRA-L1 and COBRA-L1-EDSB of HeLa cells at 37ºC and 47ºC. Data represent means ±SEM. (E) HeLa cells were incubated for 24 hours with vanillin. Sporadic accumulation of hypomethylated but not hypermethylated EDSBs can be observed. The Δ-L1-EDSBs axis and the Δ-%methylation axis are values of EDSB quantity and methylation levels of vanillin-treated cells subtracted by the means of mock experiments, respectively.

Figure 2 L1-EDSB-NRs and TSA. (A) L1-EDSBs of HeLa cells treated with TSA and the control. (B) A comparison between decreased L1-EDSBs, ΔL1-EDSBs, delta, L1-EDSBs of control minus TSA, and L1-EDSBs of control, ◊, and TSA,•.

Figure 3 γ-H2AX-bound L1s and radiation. γ-H2AX-bound L1 genomes per cell of HeLa cell in ice-cold-media after exposure to radiation. An increase in γ-H2AX-bound DNA with increasing doses of radiation was observed. Data represent means ±SEM.

Figure 4 Methylation status of γH2AX-bound-L1s. (A, B) L1-EDSB genomes per control genome and (C, D) γ-H2AX bound L1 genomes per cell. (A, C) Daudi, Jurkat and HeLa and (B, D) HeLa in the G0, G1/S and S phases. (E, F) L1 methylation levels of L1, L1-EDSB, and γ-H2AX-bound L1 in (E) Daudi, Jurkat and control HeLa and (F) HeLa in the G0, G1/S and S phases. Data represent means ±SEM.

Figure 5 γH2AX-bound-L1s in cells with and without TSA. (A) γ-H2AX-bound L1 genomes per cell treated with TSA and control. (B) Correlation between the increased levels of γ-H2AX-bound L1s, ΔγH2AX-bound-L1s, γ-H2AX-bound L1s of TSA minus control, and L1-EDSB genomes per control genome of HeLa prior to TSA treatment. (C) Methylation levels of control, TSA-treated HeLa and increased γH2AX-bound L1s after TSA treatment, ΔγH2AX. % methylation of ΔγH2AX was calculated using the following formula: ((%methylation X γH2AX-bound L1s of TSA) - (%methylation X γH2AX-bound L1s of control)) / ((γH2AX-bound L1s of TSA) – (γH2AX-bound L1s of control)). The control group was cells treated with solvent lacking TSA. (A, C) Data represent means ±SEM.

Figure 6 COBRA-L1-EDSB and ATMsi. COBRA-L1 and COBRA-L1-EDSB of stable ATM and control siRNA-transfected HeLa cells. (A) Data represent means ±SEM, with statistical significance between L1-EDSB determined by the 2-tailed paired *t*-test. The level of EDSB methylation of ATMsi was higher than EDSBs of the control, especially in G0. (B) L1 and L1-EDSB methylation from all tests in a previous study [18]. Each circle represents an individual test result of COBRA-L1 and COBRA-L1-EDSB.

Figure 7 EDSB-NRs and survival. (A) A comparison between 10^6 surviving HeLa cells after continued culturing at G0 for 4 and 24 hrs. (B) % survival of cells treated with vanillin or TSA at 4 and 24 hrs, respectively. (C-F) Comparison between % survival and L1-EDSB genomes per control genome of cells prior to be treated with (C) TSA for 4 hrs, (D) TSA for 24 hrs, (E) vanillin for 4 hrs, or (F) vanillin for 24 hrs.

Figure 8 EDSB-NRs and survival of HCT116. (A) % survival of HCT116 cells treated with vanillin for 24 hours. Data represent means ±SEM. (B) Comparison between % survival and mean L1-EDSB genomes per control genome of cells prior to be treated with vanillin. A similar trend as HeLa cells can be observed: fewer cells survived if they possessed fewer EBSD-NRs.

Figure 1 Levels of L1-EDSB-NRs

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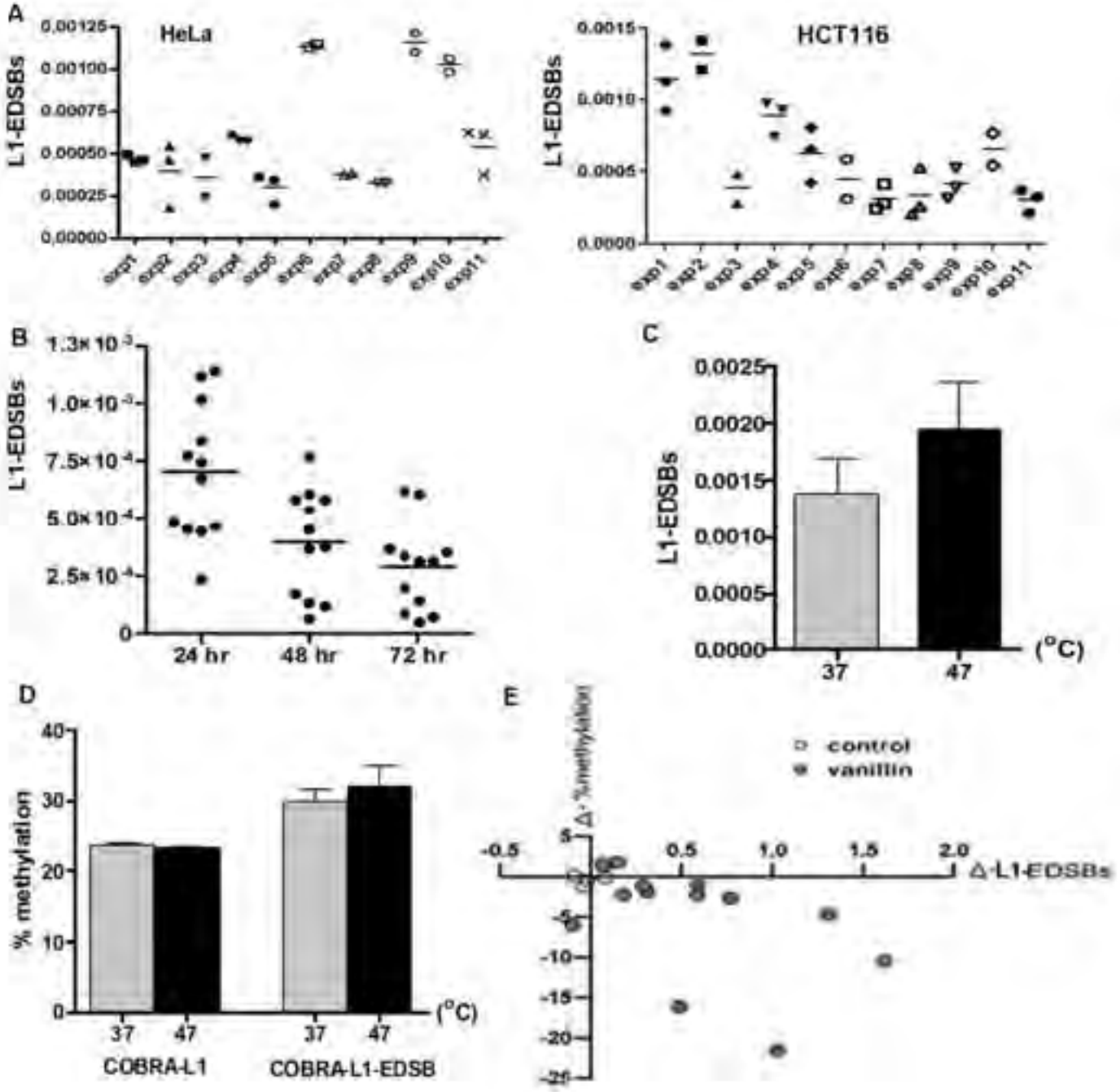

Figure 2 L1-EDSB-NRs and TSA
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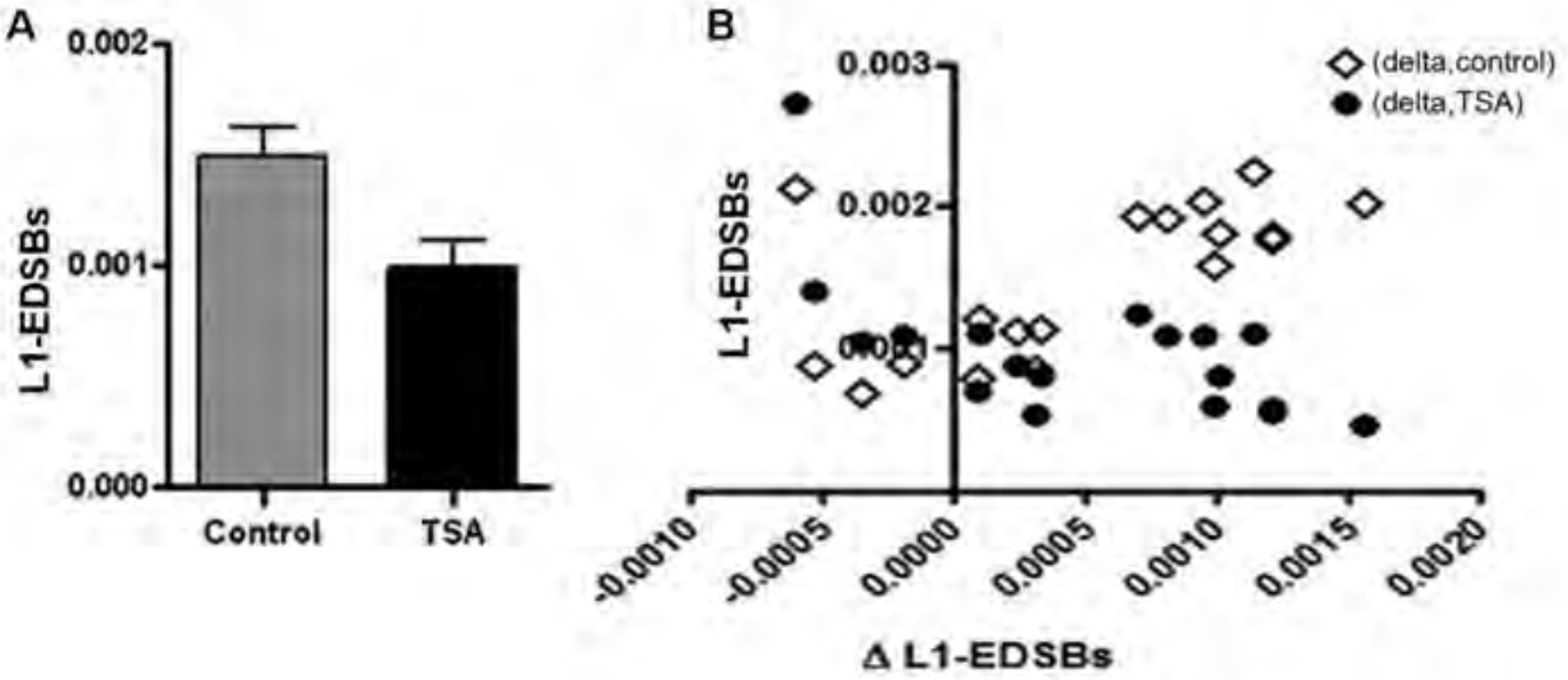

Figure 3 gamma-H2AX-bound L1s and radiation
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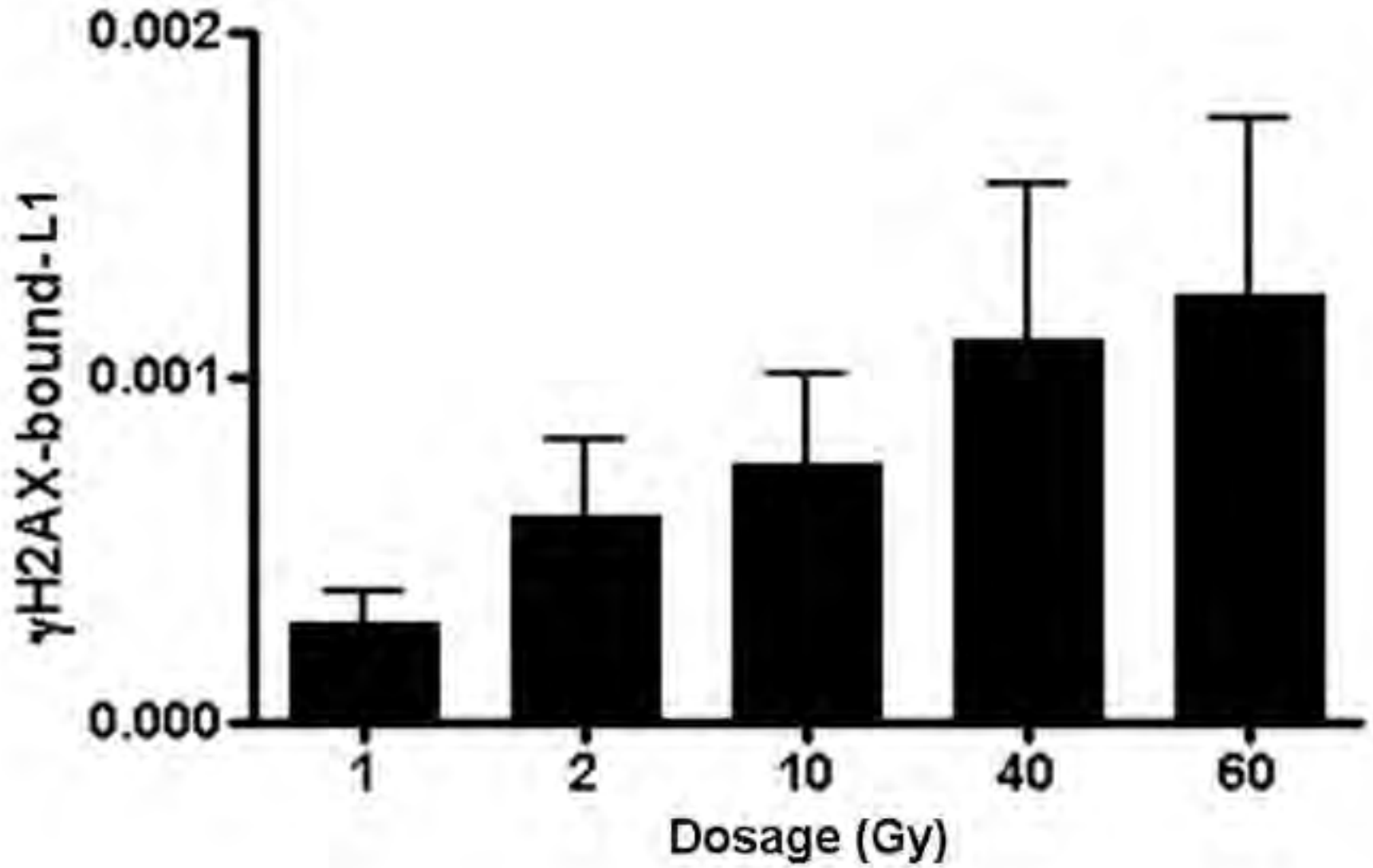

Figure 4 Methylation status of gamma-H2AX-bound-L1s
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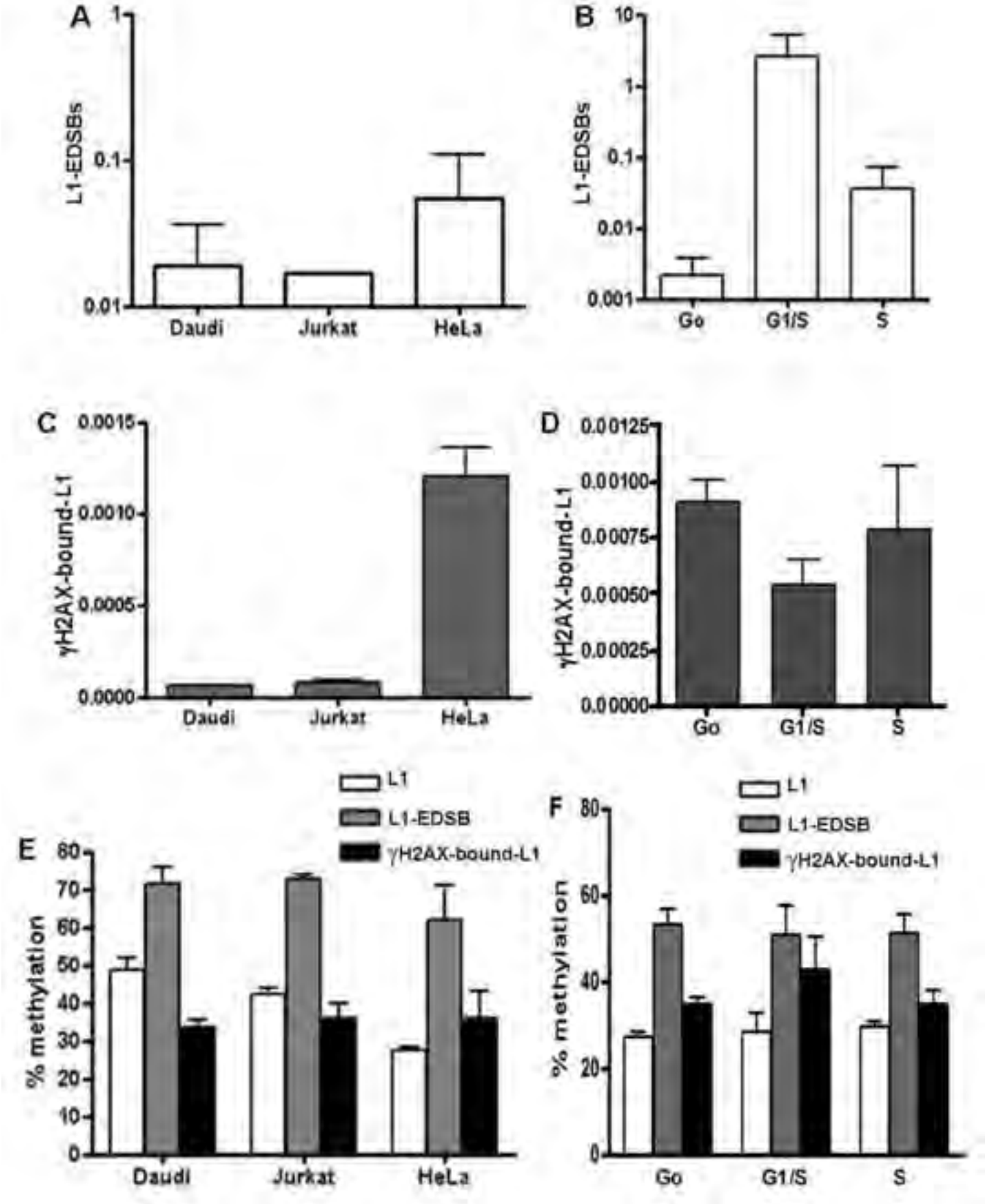

Figure 5 gamma-H2AX-bound-L1s in cells with and without TSA
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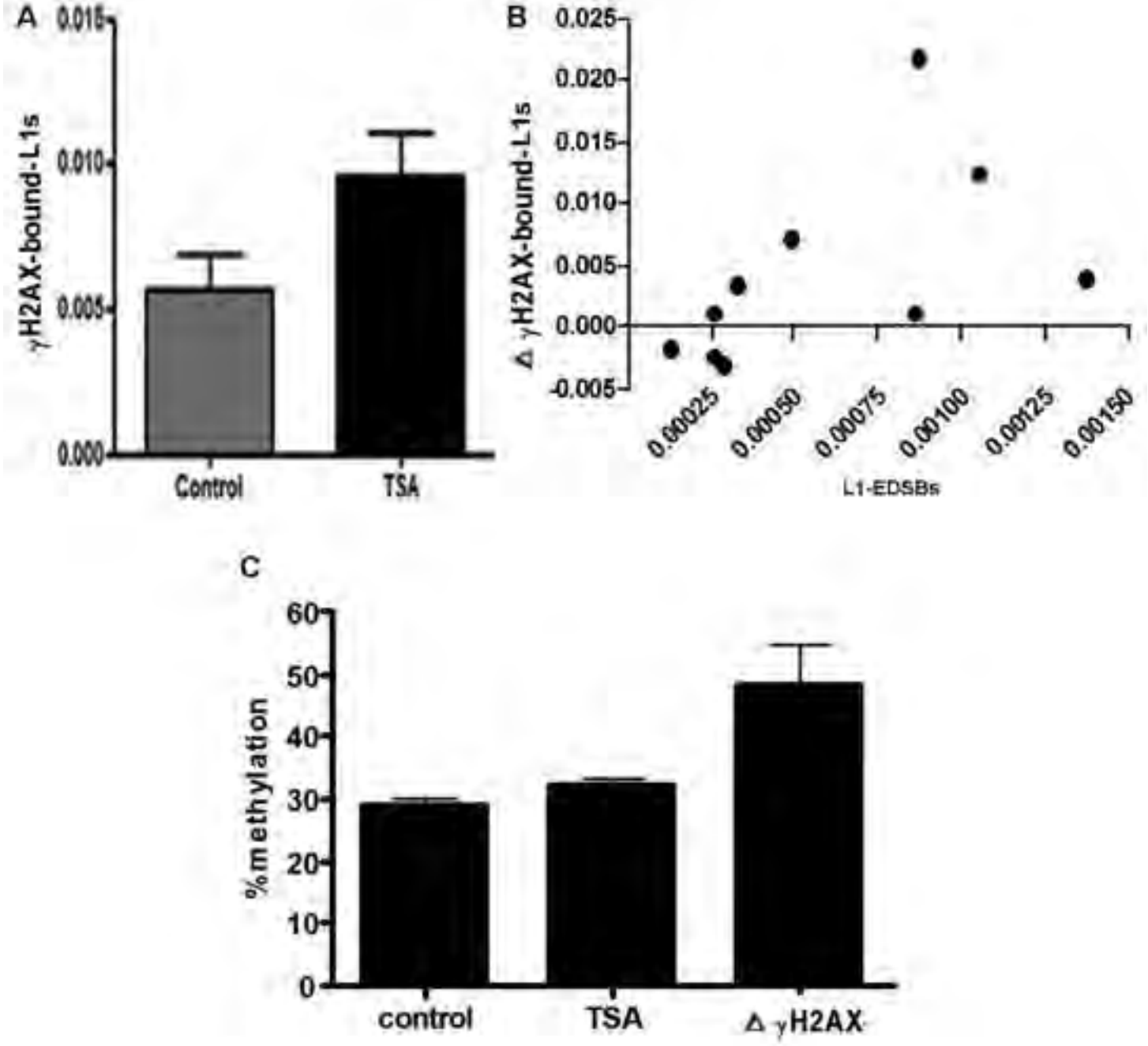

Figure 6 COBRA-L1-EDSB and ATMsi
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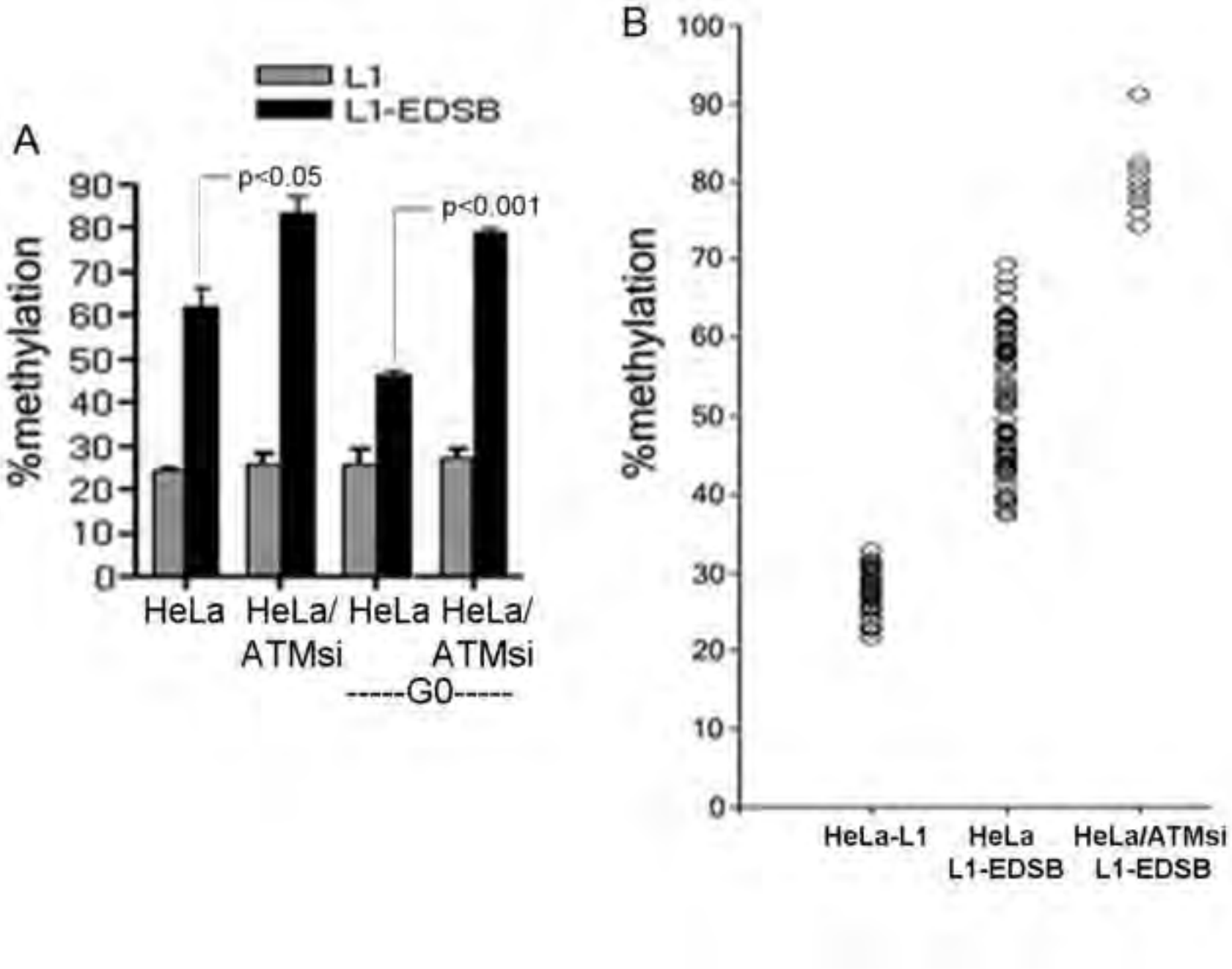

Figure 7 EDSB-NRs and survival

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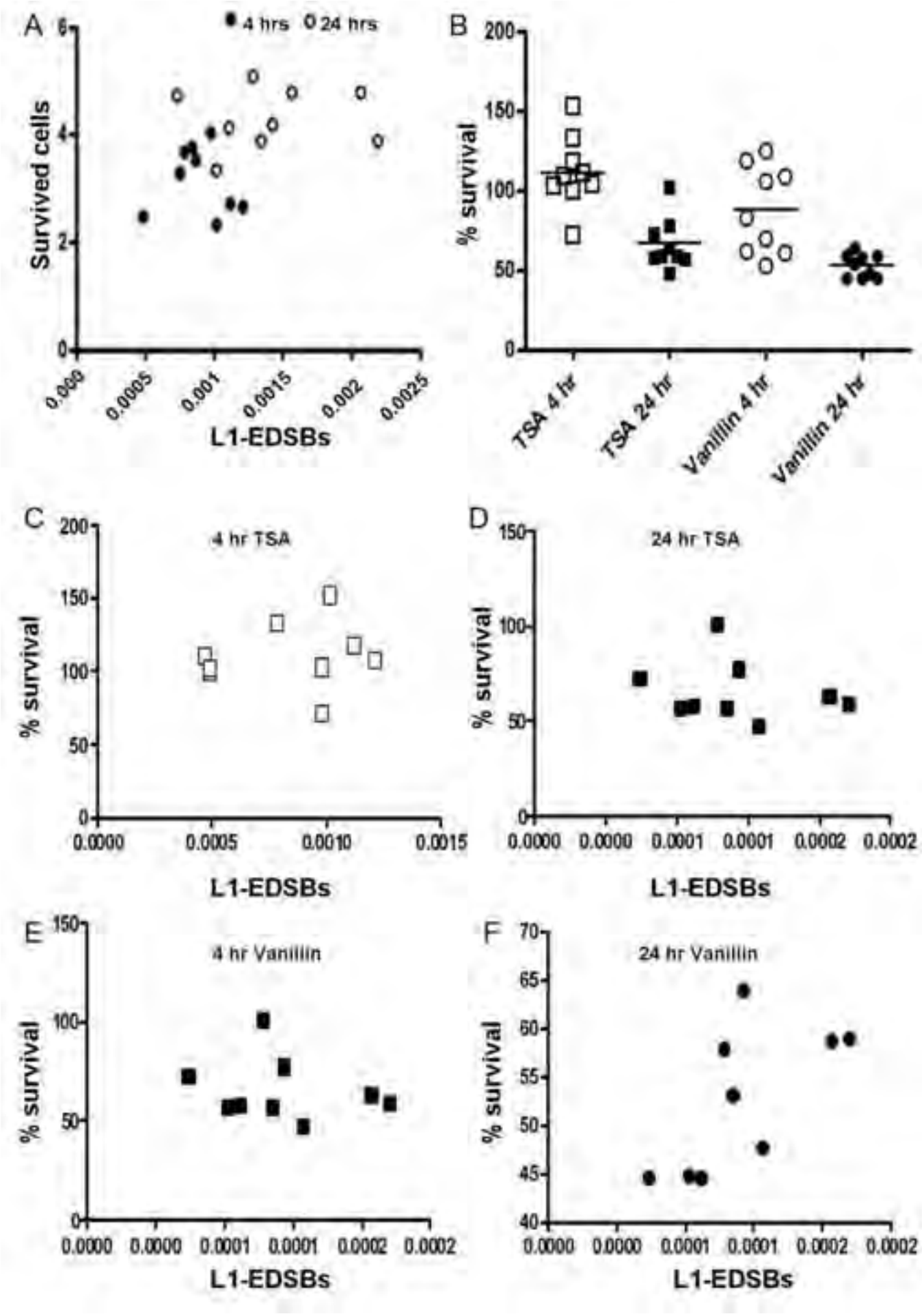

Figure 8 EDSB-NRs and survival of HCT116
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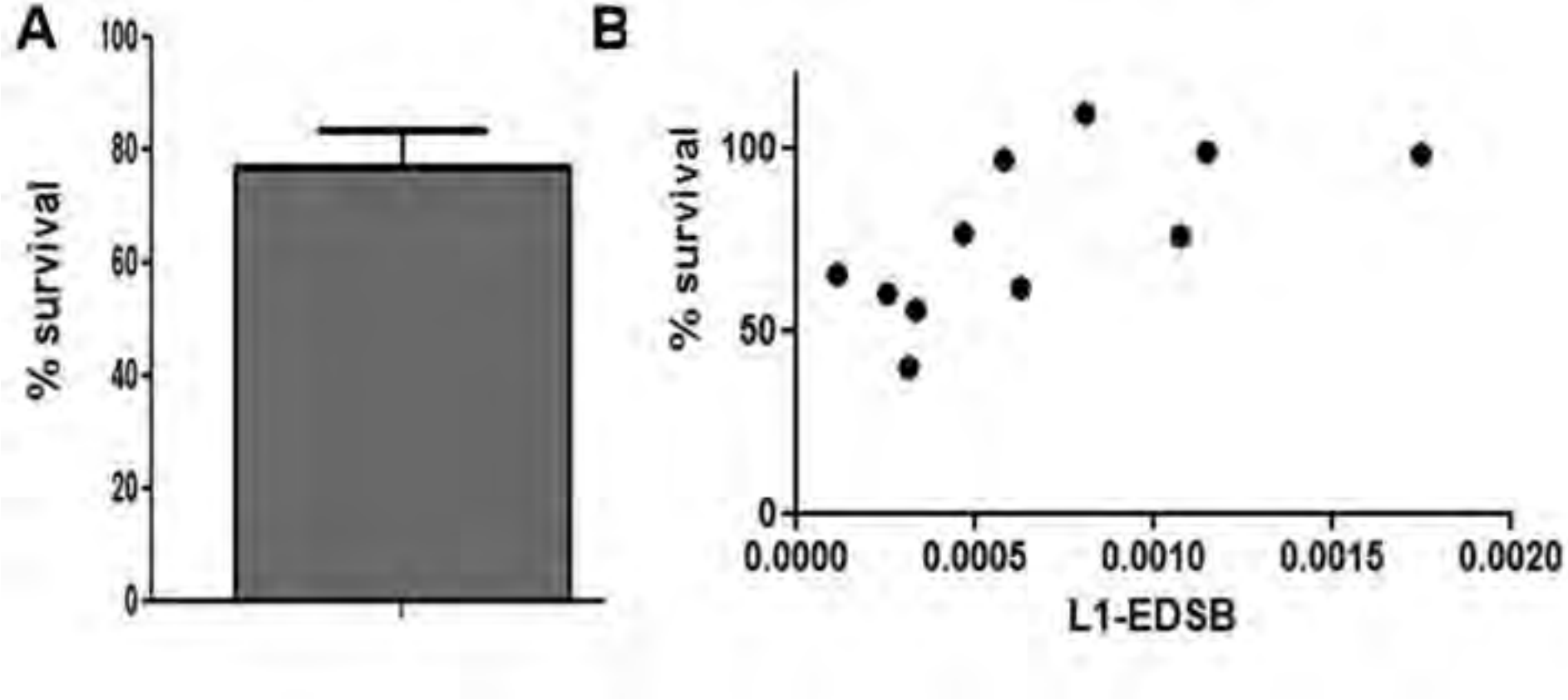

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Temperature dependent γ-H2AX binding to DNA

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ABSTRACT: γ-H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DNA double-strand breaks repair responses. Therefore, the quantity of γ-H2AX-bound DNA usually reflects the extent of DNA damage and consequently, mutagenic potential. The purpose of this study was to assess the cellular evidence regarding whether and how temperature is related to mutation. This study applies our novel assay technique to measure the quantity of γ-H2AX-bound DNA by PCR. Chromatin immunoprecipitation technique was applied to select for the γ-H2AX-bound DNA complex. Then, the amount of precipitated DNA was measured by long interspersed element-1 (LINE-1) repetitive sequences real-time PCR. We observed a positive correlation between the amount of precipitated LINE-1 sequences and the exposure to temperature. The quantity of γ-H2AX-bound DNA increased significantly when HeLa was incubated in 47 °C for 30 min. In contrast, incubation at 4 °C for 30 min reduced the γ-H2AX-bound DNA quantity. Precise quantification of γ-H2AX-bound DNA will be a tool to prove whether there is a linkage between temperature and mutagenic effect.

KEYWORDS: γ-H2AX, DNA double-strand breaks, DSBs, ChIP, LINE-1, real-time PCR, carcinogenic potential

INTRODUCTION

DNA double-strand breaks (DSBs) are biologically significant lesions leading to mutations which are a cause of cancer and hereditary disease[1]. DSBs are induced by several environmental agents and also occur spontaneously from cellular processes[2]. In mammalian cells, DSBs can be repaired *via* non-homologous end joining or homologous recombination depending on the cell cycle[3-5]. γ-H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DSB repair responses[6,7]. Therefore, a technique for γ-H2AX-bound DNA detection and quantification is a crucial tool to understand the role of DSBs and DSB repair in human diseases, particularly hereditary and cancer developmental process[8]. To detect the phosphorylated form of H2AX as foci corresponding to each DSB site in nuclei, an immunofluorescence staining method with antibodies specific for γ-H2AX has been successfully used[9-13]. γ-H2AX increases in a variety of conditions in relation to DSBs generation processes, including radiation[14,15] and high temperature[16,17]. Although the exact role of heat-directed DSBs has been argued over[18], the fact that heat can induce γ-H2AX foci leads to the possibility of association between temperature and carcinogenesis[19]. γ-H2AX not only marks DSB lesions but also may play an important role in DSB repair. Depletion in H2AX or phosphorylation of H2AX has been demonstrated to cause genome instability and consequently carcinogenesis. For example, H2AX-deficient mice and mouse embryonic stem cells were hypersensitive to ionizing radiation (IR) and showed elevated levels of chromosome instability[20–24]. Additionally, H2AX knock out mice were prone to develop tumours[20,22,25].

In this study, we developed a novel technique to quantify the γ-H2AX bound DNA by PCR and called this new assay "H2AX qPCR." We used the chromatin immunoprecipitation technique (ChIP)[26] to select the γ-H2AX-bound DNA complex and combined it with interspersed repetitive sequence PCR[27–30]. We used real-time quantitative PCR[31,32] to detect and to quantify the γ-H2AX-bound DNA that occurs randomly throughout the genome. The interspersed repetitive sequences in this protocol were LINE-1 repetitive sequences[33]. This technique improved quantitative accuracy compared to the conventional immuno-fluorescence staining method. Therefore, we applied the technique to evaluate the γ-H2AX-bound DNA under different temperatures.

MATERIALS AND METHODS

Cell culture

HeLa (cervical cancer) cells were grown in DMEM containing 10% FBS. Before further study, cells were synchronized at G 0 phase by culturing in

a serum-deprived medium for 24 h. The cells were maintained at 37 °C in a conventional humidified CO_2 incubator. For temperature treatments, HeLa cells were incubated at 4 °C or 47 °C for 30 min.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed essentially as previously described with some modifications[34]. Histone cross-linking to DNA was induced by adding formaldehyde directly to the culture medium at a final concentration of 1%, with incubation at 37 °C for 10 min. After stopping the reaction with glycine (0.125 M final concentration) for 5 min at room temperature, adherent cells were washed twice with ice-cold PBS, and then scraped into ice-cold PBS containing Halt protease inhibitor cocktail (Pierce, Rockford, IL, USA). Nuclei were isolated by resuspending the cell pellet in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, containing Halt protease inhibitor cocktail) and incubated on ice for 20 min. Intact nuclei were collected by centrifugation at 3210 *g* for 5 min at 4 °C, resuspended in nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM EDTA, containing Halt protease inhibitor cocktail), and incubated on ice for 10 min. Chromatin was sheared with an ultrasonic sonicator at 30% power output for four 30 s intervals on ice to an average size of 500–1 000 bp. After centrifugation at 21,720 *g* for 10 min at 4 °C, the chromatin solution was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, containing Halt™ Protease Inhibitor Cocktail kit) and then precleared for 30 min at 4 °C with protein G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with rotation. The agarose beads were pelleted for 1 min at 180 *g* and the chromatin fragments were immunoprecipitated overnight at 4 °C with Anti-phospho-histone H2AX monoclonal antibody (Upstate, Charlottesville, VA, USA) or normal mouse IgG antibody as a negative control (Santa Cruz Biotechnology) on a rotator. Protein-DNA-antibody complexes were isolated by the addition of protein G Plus-Agarose. After 2 h, agarose beads were collected by centrifugation at 120 *g* for 1 min, washed once each in 500 mM, 550 mM and 600 mM high-salt wash buffers (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 500–600 mM NaCl), and twice in wash buffer (100 mM Tris-HCl pH 8.0, 500 mM 1% Nonidet P-40, 1% deoxycholic acid). Complexes were eluted with elution buffer (50 mM $NaHCO_3$,1% SDS) for 15 min at room temperature. Cross-links were reversed by adding NaCl (200 mM final concentration) and RNA was removed by adding 10 mg/ml of RNase A, followed by incubation for 4 h at 65 °C, and then precipitated overnight with ethanol. Samples were deproteinized with proteinase K. After phenol/chloroform extraction, the DNA was precipitated with ethanol. The precipitated sample was subjected to real-time 5´LINE-1 PCR to measure the quantity of LINE-1 sequence

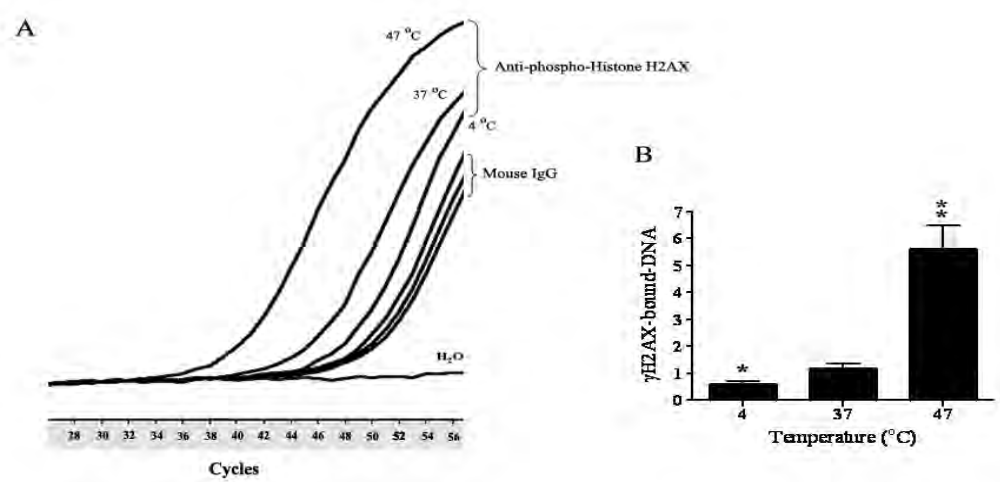

Fig. 1 The quantity of γ-H2AX-bound DNA. (A) An example of real-time LINE-1s PCR from ChIP of anti-phospho-histone H2AX monoclonal antibody or normal mouse IgG antibody bound DNA and water. (B) γ-H2AX-bound DNA from HeLa cells in 4 °C, 37 °C, and 47 °C media. The quantity of γ-H2AX-bound-LINE-1 sequences was used to calculate the relative amount of γ-H2AX-bound genomic DNA. The units are Mb per cell. **P*<0.05, ***P*<0.001 (paired 2-tailed t-test).

Real-time PCR

Quantification of the amount of immuno-precipitated 5′LINE-1 sequences was carried out by real-time PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 1× Quanti Tect SYBR Green PCR Master Mix, 0.2 μM forward primer (L1.2HpaIIRFLPF:5′-CTCCCAGCGTGAGC GAC-3′), and 0.2 μM reverse primer (5′LIDSIP1st-AC TCCCTAGTGAGATGAACCC G-3′) were used for each PCR assay. The PCR program was initiated at 95 °C for 15 min to activate the HotStarTaq DNA polymerase, followed by 50 thermal cycles of 15 s at 95 °C, 20 s at 57 °C, and 20 s at 72 °C. A melting curve test (68 °C) was always carried out after the final reaction step to confirm that appropriate amplification products were obtained. Each sample was analysed in triplicate PCR reactions. All reactions were run on a Lightcycler™ instrument (Roche Applied Science). Control HeLa genomic DNA was used as a positive control and for calculating the precipitated DNA quantity from real-time PCR.

Quantification of the γ-H2AX-bound DNA

From real-time PCR, the amount of γ-H2AX-bound DNA was calculated using:

$$\text{Amount of γ-H2AX-bound DNA per cell (Mb per cell)} = \frac{N(A-B)}{C},$$

where N is the amount of mega base pairs (Mb) in one cell (which corresponds to the whole genome), A is the quantity of γ-H2AX -bound DNA, B is the quantity of mouse IgG antibody-bound DNA, and C is the total starting DNA input per one reaction of real-time PCR. The γ-H2AX -bound DNA, measured mouse IgG antibody-bound DNA, and total starting DNA input per one reaction are measured in the same units. For practical purposes, this unit is a genome. The units of γ-H2AX-bound DNA is Mb per cell.

RESULTS

We investigated the association between temperatures and γ-H2AX-bound DNA by using our novel method called H2AX qPCR. In this assay, γ-H2AX-bound DNA was obtained by ChIP using a γ-H2AX antibody. Subsequently, the bound LINE-1 sequences were detected and quantificated by real-time PCR with both primers located at the 5′ region of LINE-1. The result of H2AX qPCR is shown in Fig. 1A and indicates that the amount of γ-H2AX-bound DNA was measured by PCR. Both γ-H2AX and interspersed repetitive sequences are randomly distributed in our genome[33,35,36]. Therefore, the amount of γ-H2AX-bound LINE-1 should be represented in direct proportion to the γ-H2AX-bound DNA. Nevertheless, there are thousands more copies of LINE-1s than unique DNA sequences. Consequently, nonspecific binding of repeat sequences cannot be completely excluded from the experiments. To estimate the extent of this nonspecific binding, we included a nonspecific antibody, normal mouse IgG antibody, as a mock experiment to compare with the test anti-γ-H2AX monoclonal antibody. Our results indicate that the mock experiments usually yielded a significantly lower quantity of precipitated DNA than the test (Fig. 1A). We did not apply unphosphorylated H2AX antibody as a negative control. First, the anti-γ-H2AX monoclonal antibody was specific to the phosphorylated form of H2AX[9–13]. Second, the aim of this technique was to measure γ-H2AX-bound DNA that represents the quantity of DSBs in a genome or a cell. Since there is no information regarding the total amount of unphosphorylated H2AX per cell, the measurement of γ-H2AX-bound DNA in relation to the unphosphorylated H2AX bound DNA may provide biased information regarding the extent of DSBs. Finally, H2AX is a subclass of eukaryotic histone proteins, densely distributed throughout the genome. Immuno-precipitating the proteins may yield a significant quantity of the genome, a million times larger than γ-H2AX-bound DNA. This would lead to a very wide margin of error.

Previous studies[16,17] demonstrated increased γ-H2AX foci in cells under hyperthermic conditions (45–47 °C) and suggested that heat may induce DSBs. However, the possibility that heat alters the cellular repair response to endogenous DSBs is another reasonable hypothesis. Here we applied our new established technique to evaluate whether we could measure the quantity of temperature-induced γ-H2AX-bound DNA alteration. Our qPCR technique yields precise quantities. Because there is a significant amount of γ-H2AX-bound DNA under physiological conditions (37 °C), we investigated how a lower temperature would affect the quantity of γ-H2AX-bound DNA. We incubated Hela cells at 4 °C, 37 °C and 47 °C for 30 min and evaluated the quantity of γ-H2AX-bound DNA by using H2AX qPCR. We observed a significant increase in H2AX-bound DNA when the temperature increased (Fig. 1B). Therefore, this technique successfully identified the increased γ-H2AX-bound DNA from hyperthermia. In contrast, a smaller amount of γ-H2AX-bound DNA from hypothermia was observed (Fig. 1B).

DISCUSSION

DSBs are important DNA lesions. Failure to repair the lesions will lead to apoptosis, and errors in DNA repairs can lead to mutations. The most crucial biological lesions lead to hereditary diseases and cancer. Therefore, it is important to evaluate the causes of DSBs and the conditions that determine cellular effectiveness in DSB repair. Alteration of γ-H2AX-bound DNA at higher and lower temperatures is an important finding. Even though our physiological condition usually maintains cells at 37 °C, many cells such as skin and the upper gastrointestinal tract are often exposed to lower and higher temperatures. Consequently, our finding could be important in explaining the natural consequences of abnormal temperature to our cells. This study confirmed that heat can increase γ-H2AX-bound DNA. Moreover, we are the first to demonstrate that reduced temperature can decrease γ-H2AX-bound DNA. There are several possible mechanisms by which lower temperatures can reduce endogenous H2AX bound DNA. The lower temperature may reduce either heat-induced DSBs or the enzymatic activity of DSB repair proteins. Additionally, temperature may alter the chromatin configuration and consequently change the DNA repair response. Further investigation to prove these mechanisms is crucial since each possibility leads to a different mutation potential. If lower temperature reduces endogenous DSBs, lower temperature may play a role in mutation prevention. On the contrary, if lower temperature reduces DNA repair activity, low temperature may contribute to genomic instability and consequently alter the mutation rate.

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APPENDIX B

BUFFER AND REAGENT

1. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

2. Proteinase K

Proteinase K	20	mg
Distilled water to volume	1	ml

Mix the solution and store at -20^{o}C

3. 1 M Tris-HCL (pH 7.5)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

4. 0.5 M EDTA (pH 8.0)

Disodium ethyleneidamine tetraacetate	18.66	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

5. 1 M $MgCl_2$

$MgCL_2 . 6H_2O$	20.33	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 5 M NaCl

NaCl	29.25	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 10X TBE buffer

Tris-base	108	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml
Distilled water to volume	1,000	ml

Mix the solution and store at room temperature.

8. 6X loading dye

Ficoll 400	15	g
Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
1 M Tris (pH 8.0)	1	ml
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

9. TE buffer (pH 8.0)

1 M Tris (pH 8.0)	10	ml
0.5 M EDTA (pH 8.0)	2	ml
Distilled water to volume	1,000	ml

Mix the solution and store at room temperature.

10. HMW digestion buffer

1 M Tris (pH 8.0)	10	µl
20 mg/ml proteinase K	50	µl
10% Sodium lauryl sarcosine	100	µl
0.2 M EDTA (pH 8.0)	100	µl

Distilled water to volume	500	µl

Mix the solution and use 400 µl per agarose plug.

11. 10% Sodium lauryl sarcosine

Sodium lauryl sarcosine	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

12. 10X TBS

Tris base	61	g
NaCl	90	g
Distilled water to volume	1,000	m

Mix to dissolve and adjusted pH to 7.6

Sterilize the solution by autoclaving and store at room temperature.

13. 1X SDS Buffer (Lysis Buffer)

1 M Tris-HCl pH 8.8	6.25	ml
10% SDS	20	ml
Glycerol (87%)	11.5	ml
Distilled water to volume	100	ml

14. 20 mg/ml glycogen

Glycogen	200	mg
Distilled water to volume	10	ml

Sterilize the solution by filter through 0.2 µm membrane, aliquot and store at -20^{o}C

15. 10M NH_4OAc

NH_4OAc	77.08	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

16. 6X Sample loading buffer

1 M Tris-HCl pH 6.8	3	ml
SDS	1.2	g
Glycerol (100%)	6	ml
Bromphenol blue	30	mg
Distilled water to volume	10	ml

Add 24% β-mercaptoethanol before using (stock 14.2 M and working 864 nM)

17. 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel (10 ml)

40% Acrylamide:Bis (37.5:1)	3.75	ml
1 M Tris-HCl (pH 8.8)	2.5	ml
10% SDS	100	µl
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	5	µl
Distilled water	3.75	ml

Stacking gel (4 ml)

40% Acrylamide:Bis (37.5:1)	0.4	ml
0.5 M Tris-HCl (pH 6.8)	1	ml
10% SDS	40	µl
10% $(NH_4)_2S_2O_8$	20	µl
TEMED	4	µl
Distilled water	2.6	ml

18. 1 M Tris-HCl (pH 8.8)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 8.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

19. 0.5 M Tris-HCl (pH 6.8)

Tris base	6.055	g

Dissolve in distilled water and adjusted pH to 6.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

20. 10X Tris-glycine (pH 8.3)

Tris base	6.055	g
Glycine	147.1372	g

Dissolve in distilled water and adjusted pH to 8.3

Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

21. Running Buffer

10X Tris-glycine (pH 8.3)	100	ml
10% SDS	10	ml
Distilled water to volume	890	ml

Mix the solution and store at room temperature.

22. Transfer Buffer

10X Tris-glycine (pH 8.3)	100	ml
Methanol	200	ml
Distilled water to volume	800	ml

Mix the solution and store at room temperature.

BIOGRAPHY

Miss Chutipa Phuangphairoj was born in Bangkok in 1983. In 2005, she graduated from faculty of Science in genetic program from Chulalongkorn University and then attended to study in Medical Science program in faculty of Medicine for her master degree.