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MTHFR promotes heterochromatin maintenance

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ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the folate cycle, catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. Methionine serves as the precursor of the active methyl donor *S*-adenosylmethionine, which provides methyl groups for many biological methylations. It has been reported that MTHFR is highly phosphorylated under unperturbed conditions and T34 is the priming phosphorylation site. In this report, we generated a phospho-specific antibody that recognized T34-phosphorylated form of MTHFR and revealed that MTHFR was phosphorylated at T34 in vivo and this phosphorylation peaked during mitosis. We further demonstrated that the cyclin-dependent kinase 1 (CDK1)/Cyclin B1 complex is the kinase that mediates MTHFR phosphorylation at T34 and the MTHFR immunocomplex purified from mitotic cells exhibited lower enzymatic activity. Inhibition of MTHFR expression resulted in a decrease of H3K9me3 levels, and an increase of transcription of the centromeric heterochromatin markers. Taken together, our results demonstrated that CDK1/Cyclin B1 phosphorylates MTHFR on T34 and MTHFR plays a role in the heterochromatin maintenance at the centromeric region.

1. Introduction

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), which is a ubiquitously expressed cytoplasmic flavoenzyme involved in the folate cycle, catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-CH2-THF) to 5-methyltetrahydrofolate (5-CH3-THF). 5-CH3-THF, the most abundantly circulating form of folate, supplies methyl groups for methylation of homocysteine to methionine. Methionine is then converted into *S*-adenosylmethionine (SAM), which is the universal methyl donor for many biological methylation reactions, such as protein and nucleic acid methylation. 5,10-CH2-THF and its derivate, 10-formyl-THF, are indispensable substrates for thymidylates and purine nucleotides synthesis, respectively [1]. Thus, MTHFR couples folate cycle and methionine cycle to accomplish one-carbon metabolism in cells, regulating the balance between cellular methylation reactions and nucleic acid synthesis by channeling one-carbon unit distribution.

Genetic variation in MTHFR influences susceptibility to occlusive vascular disease [2–5], neural tube defects (NTD) [6], colon cancer [7–10] and acute leukemia [11,12]. The most extensively investigated polymorphism in MTHFR is C667T, in which its enzymatic activity decreases 30–40% in vitro [3,4]. Several retrospective clinical studies indicate that this polymorphic allele may have a protection role in colon cancer [7–9] and adult leukemia [11,12]. However, given that MTHFR deficiency both in human beings and mice leads to genome-wide DNA hypomethylation [13,14], which is a common feature of cancer and is associated with genomic and chromosomal instability, it warrants further investigation to clarify how MTHFR links to genome instability.

Heterochromatin is a highly packed and condensed DNA and protein complex. There are two types of heterochromatin, namely, facultative and constitutive heterochromatin. Facultative heterochromatin is the result of gene silencing, whereas constitutive heterochromatin usually occurs around the chromosomal centromeric and telomeric regions [15–19]. Heterochromatin, which often associates with di and tri-methylation of H3K9, mainly regulates gene expression and protects the chromosomal integrity [20–25].

It has been reported that MTHFR is phosphorylated on T34 and this phosphorylation inhibits its catalytic activity [26]. In this report, we generated a phospho-specific antibody recognizing the

Abbreviations: 5,10-CH2-THF, 5,10-methylenetetrahydrofolate; 5-CH3-THF, 5-methyltetrahydrofolate; *α-Sat*, *alpha satellite*; CDK1, cyclin-dependent kinase 1; H3K9me1, monomethylation of H3K9; H3K9me2, dimethylation of H3K9; H3K9me3, trimethylation of H3K9; *Maj-Sat*, *major satellite*; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defect; SAM, *S*-adenosyltetrahydrofolate; *Sat2*, *satellite 2*.

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T34-phosphorylated form of MTHFR and demonstrated that MTHFR is phosphorylated on T34 in vivo by CDK1/Cyclin B1 under unperturbed conditions. We also establish a functional link between MTHFR and heterochromatin maintenance at the centromeric regions.

2. Materials and methods

2.1. Cell lines, plasmids, siRNA oligos and antibodies

HEK293T, HeLa, A549, HT29 and U2OS were purchased from ATCC. All the cell lines were grown in DMEM (Thermo) containing 10% fetal bovine serum (FBS, Hyclone) and 1% standard antibiotics (Hyclone), at 37 °C in a 5% CO2 incubator (Thermo).

The full-length cDNA of MTHFR (GeneBank accession number: NM_005957) was amplified by reverse-transcriptase polymerase chain reaction and subcloned into pcDNA-3HA vector, pcDNA-3FLAG vector and pTrc-HIS vector, resulting in pcDNA-3HA-MTHFR, pcDNA-3FLAG-MTHFR and pTrc-HIS-MTHFR. The point-mutant construct MTHFR(T34A) and MTHFR(T34D) were generated using the QuickChange Site Directed Mutagenesis kit (Stratagene).

All siRNA oligo duplexes (Ontarget plus option) were purchased from Dharmacon (Thermo). Control siRNA oligonucleotide duplex was siCTR: CGU ACG CGG AAU ACU UCGA. MTHFR siRNA oligonucleotide duplexes were si1-MTHFR, AGU GAG AGC UCC AAA GAUA; si2-MTHFR, GAC CAA AGA GUU ACA UCUA; si3-MTHFR, GAU CAU CAA GCC CAC CGUA; si4-MTHFR, AAA CCG GAA UGG UCA CAAA.

Antibodies against MTHFR, pH3(S10) and HA were purchased from Bethyl Laboratories Inc. Antibodies against H3K9me1 and H3K9me3 were purchased from Abcam. Antibodies against FLAG and β-actin were purchased from Sigma. Antibodies against H3 and H3K9me2 were purchased from Cell Signaling Technologies.

2.2. Cell synchronization

HeLa cells were synchronized at G2/M border by thymidine–nocodazole arrest. Firstly, the cells were blocked with 2 mM thymidine in medium for 24 h, then the thymidine-arrested cells were released into fresh medium for 3 h and 340 nM nocodazole was added. 16 h Later, the nocodazole-arrested cells were released into fresh medium and collected at 0, 2, 4, 6, 8, 10, 12, 14, and 16-h time points.

2.3. CDK1/Cyclin B1 kinase assay

For in vitro kinase assays, bacterially produced GST, GST-MyPT1(297–600aa), HIS-MTHFR or HIS-MTHFR(T34A) recombinant proteins were incubated with active baculovirus-expressed human CDK1/Cyclin B1 complex (Millipore) in the kinase buffer. The kinase assay was carried out in a 30-μl reaction, containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 0.01% Brij35 at pH 7.5, 50 mM cold ATP (or 5 μCi [γ-^{32}P]-ATP), 50 ng CDK1/Cyclin B1 complex and 1 μg purified recombinant substrates. The reactions were incubated at 30 °C for 30 min, quenched with SDS sample buffer and analyzed by SDS–PAGE followed by western blot or autoradiography.

2.4. NADPH-menadione oxidoductase assay

MTHFR enzyme activity was determined by using NADPH-menadione oxidoductase assay as described [27,28], except that the assay volume was reduced to 1 ml. The reaction was started by addition of menadione at 25 °C and absorbance changes were monitored at 340 nm.

2.5. Real-time PCR assay

Total RNA was extracted from HeLa cells with depletion of endogenous MTHFR by siRNA or mock treatment using TriZol (Invitrogen). To exclude potential contamination of DNA, extracted RNA was treated by DNase I (Takara) for 30 min at 37 °C and then reverse transcription PCR was performed by PrimeScript RT-PCR Kit (Takara). The relative expression of *α-Sat*, *Sat2*, or *Major-Sat* was measured by real-time PCR with SYBR-green dye (Bio-Rad) and CFX manager 3.0. Primers used were: *α-Sat*: CTGCACTACCTGAAGAGGAC (sense), GATGGTTCAACACTCTTACA (anti); *Sat2*: CATCGAATGGAAATGAA AGGAGTC (sense), ACCATTGGATGATTGCAGTCAA (anti); *Major-Sat*: GACGACTTGAAAAATGACGAAATC (sense), CATATTCCAGGTCCTTCA GTGTGC (anti); GAPDH: GAAGGTGAAGGTCGGAGTC (sense), GAA GATGGTGATGGGATTTC (anti) [29].

3. Results

3.1. T34 is the major phosphorylation site of MTHFR

It came to our attention that the anti-MTHFR antibody was reactive to two distinct bands, of which the slower migration form was dominant over the faster migration form, in both immunoblotting analysis and immunoprecipitation/immunoblotting analysis in a variety of human cancer cell lines, including cervical cancer line HeLa, lung cancer line A549, osteosarcoma line U2OS, and colon cancer line HT29 (Fig. 1A and data not shown). When the immunoprecipitated MTHFR was treated with the calf intestinal alkaline phosphatase, most, if not all of the slower migration form of MTHFR was compressed into the faster migration form (Fig. 1A). When HA-tagged MTHFR was expressed in HeLa cells, it behaved

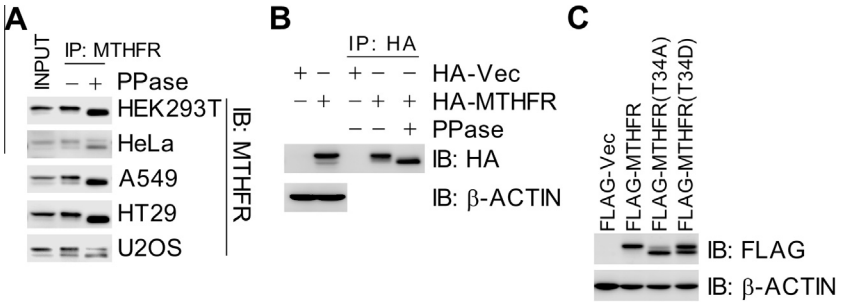

Fig. 1. The T34 residue drives MTHFR phosphorylation in vivo. (A) MTHFR is a phosphoprotein. Endogenous MTHFR was immunoprecipitated from different cancer cell lines as indicated. The MTHFR immunoprecipitates were divided into two parts for mock treatment and phosphatase (PPase) treatment respectively. The treated MTHFR immunoprecipitates were blotted with an anti-MTHFR antibody. (B) Ectopically expressed HA-MTHFR is a phosphoprotein. HA-MTHFR was transiently expressed in HeLa cells, and the HA immunoprecipitates were treated as described in (A). (C) The T34 residue drives MTHFR phosphorylation. FLAG-tagged MTHFR and its mutants were expressed in HeLa cells. Total lysates were extracted for immunoblotting with antibodies as indicated.

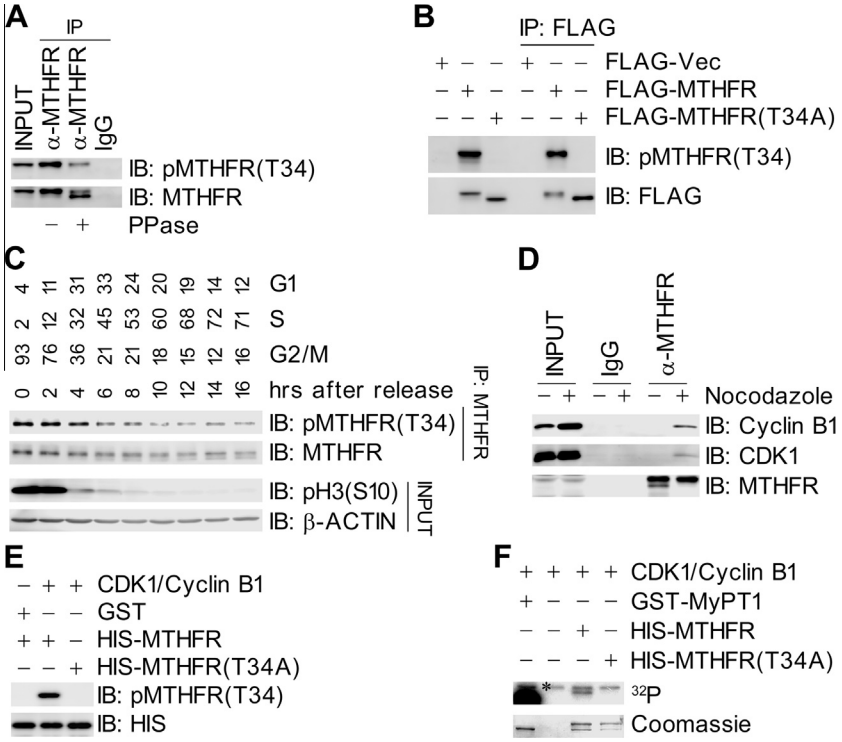

Fig. 2. CDK1/Cyclin B1 phosphorylates MTHFR on T34. (A) and (B) Specificity of MTHFR T34 phospho-specific antibody. (A) Endogenous MTHFR immunoprecipitates were either mock-treated or treated with lambda phosphatase (PPase) and detected with the phospho-specific antibody pMTHFR(T34) we generated or the MTHFR antibody. (B) Ectopically expressed FLAG-MTHFR or FLAG-MTHFR(T34A) in HeLa cells was immunoprecipitated with an anti-FLAG antibody and probed with antibodies as indicated. (C) pMTHFR(T34) levels peak during mitosis. HeLa cells were synchronized at G2/M phase by thymidine–nocodazole arrest. The arrested cells were released into fresh medium and harvested at indicated time points for cell cycle determination and immunoprecipitation/immunoblotting analysis. (D) MTHFR interacts with CDK1/Cyclin B1 upon nocodazole treatment. HeLa cells were mock-treated or treated with nocodazole overnight and harvested for immunoprecipitation with an anti-MTHFR or control IgG. The MTHFR immunocomplex was blotted with antibodies as indicated. (E) and (F) In vitro CDK1/Cyclin B1 kinase assays. Insect-cell-produced active CDK1/Cyclin B1 was incubated with bacterially produced MTHFR proteins as indicated in the absence (E) or the presence (F) of ^{32}P-ATP. GST-MyPT1(297–600) fragment in (F) served as a positive control. *: Non-specific signal.

exactly as the endogenous MTHFR (Fig. 1B). These results indicated that MTHFR is phosphorylated in vivo under unperturbed conditions. When T34 of MTHFR was mutated to alanine (A), the faster migration form in HeLa cells became dominant; whereas when T34 was mutated to asparagine (D), the protein levels of the slower migration form in HeLa cells became similar to those of the faster migration form (Fig. 1C). These observations are consistent with earlier report by Yamada et al [26], confirming that T34 is the priming phosphorylation site in MTHFR under unperturbed conditions.

3.2. MTHFR T34 is phosphorylated by the CDK1/Cyclin B1 kinase complex

To further understand the biological consequence of MTHFR T34 phosphorylation, we attempted to generate a MTHFR T34

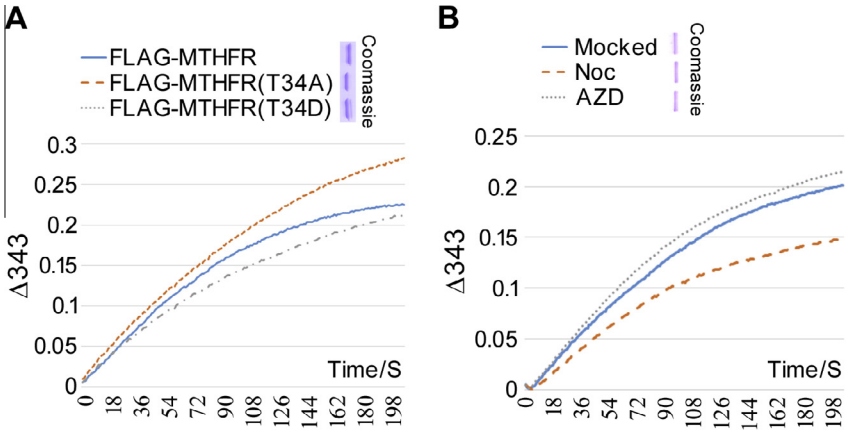

Fig. 3. The MTHFR enzymatic activity is low during mitosis. The NADPH-menadione oxidoreductase assay was used to measure MTHFR enzymatic activity. (A) FLAG-MTHFR, FLAG-MTHFR(T34A) and FLAG-MTHFR(T34D) were transiently expressed in HEK293T cells, total cell lysates were harvested for immunoprecipitation with the anti-FLAG (M2) beads and enzymatic assays were performed on beads. (B) HEK293T cells transiently expressing FLAG-MTHFR were mock-treated, treated with nocodazole (Noc, 340 nM, 16 h) or AZD5438 (AZD, 2 μM, 16 h). The anti-FLAG immunoprecipitated beads were proceeded for enzymatic activity measurement.

phospho-specific antibody. As shown in Fig. 2A, this antibody was able to recognize the immunoprecipitated endogenous MTHFR from HeLa cells in the immunoblotting analysis, while this signal was significantly reduced when the immunoprecipitated MTHFR was pretreated with lambda phosphatase. Furthermore, this antibody recognized FLAG-MTHFR, but not FLAG-MTHFR(T34A), expressed in HeLa cells (Fig. 2B). Taken together, these data demonstrated that the MTHFR T34 phospho-antibody specifically recognizes T34 phosphorylated form of MTHFR (pMTHFR(T34)) and MTHFR is phosphorylated in vivo on T34 under unperturbed conditions.

With the phospho-specific antibody in hand, we examined if the pMTHFR(T34) levels fluctuate throughout the cell cycle. HeLa cells were synchronized at the G2/M phase by single thymidine block followed by nocodazole arrest, cell cycle profiles were determined by flow cytometric analysis at different time-points before and after release from the nocodazole arrest, and total lysates were harvested and immunoprecipitated with an anti-MTHFR antibody followed by immunoblotting with the pMTHFR(T34) antibody and other antibodies as indicated in Fig. 2C. The pMTHFR(T34) levels were peaked at the G2/M (Fig. 2C).

We took a close look of the surrounding amino acid sequence of MTHFR T34 and found that it matches with the substrate consensus motif for the CDKs. Co-immunoprecipitation assays revealed that both CDK1 and Cyclin B1 were present in the MTHFR immunocomplex only after nocodazole treatment (Fig. 2D). This indicated that CDK1 could be the candidate kinase for MTHFR(T34). In vitro kinase assays demonstrated that CDK1/Cyclin B complex efficiently phosphorylated HIS-tagged wild type MTHFR, but not MTHFR(T34A) mutant, when detected with the pMTHFR(T34) antibody (Fig. 2E) or autoradiography (Fig. 2F). Taken together, these results demonstrated that CDK/Cyclin B1 complex phosphorylates MTHFR at T34 both in vitro and in vivo.

3.3. The enzymatic activity of MTHFR decreases during mitosis

MTHFR is a flavoenzyme responsible for the conversion of 5,10-CH2-THF to 5-CH3-THF, it was reported that MTHFR T34 phosphorylation inhibited its catalytic activity using the NADPH-menadione oxidoreductase assay [27,28]. Indeed, the in vitro NADPH-menadione oxidoreductase assay revealed that the phosphorylation-deficient mutant MTHFR(T34A) exhibited higher activity than the wild type MTHFR, whereas the phosphorylation-mimic mutant MTHFR(T34D) had a lower enzymatic activity than the wild type MTHFR (Fig. 3A). Given that pMTHFR(T34) peaked during mitosis, we reasoned that MTHFR might have lower catalytic activity during mitosis. Indeed, nocodazole treatment, which arrested cells at G2/M, led to a decrease of MTHFR catalytic activity (Fig. 3B), whereas treatment with AZD5438, a specific inhibitor for CDK1/Cyclin B1 complex, resulted in an increase of the MTHFR catalytic activity (Fig. 3B). Thus, our results demonstrated that the enzymatic activity of MTHFR decreases during mitosis.

3.4. MTHFR plays an important role in maintaining the heterochromatin structure at the centromeric region

As we have shown that pMTHFR(T34) levels inversely correlate with its catalytic activity, it would be very interesting to determine the biological consequences upon inhibition of MTHFR expression in cells. We tested the knockdown efficiency by transfecting 4 individual siRNA oligos specific for MTHFR or the control siRNA oligo (siCTR) in HeLa cells. Both si1-MTHFR and si4-MTHFR achieved a knockdown efficiency over 85%, better than si2-MTHFR or si3-MTHFR (Fig. 4A). Genomic DNA extracted from HeLa cells depleted of MTHFR either with si1-MTHFR or with si4-MTHFR was more sensitive to the micrococcal nuclease digestion than that in siCTR-depleted cells in the micrococcal nuclease assays (Fig. 4B). This indicated that depletion of MTHFR might result in relaxation of heterochromatin. To exclude the possibility of the siRNA off-target effects, we engineered HeLa cells to express the silent mutant FLAG-MTHFRres, whose expression was specifically resistant to inhibition by si1-MTHFR (Fig. 4C). We further examined the status of several heterochromatin markers. Depletion of MTHFR expression led to a decrease of trimethylation of H3K9 (H3K9me3) levels, but not monomethylation of H3K9 (H3K9me1) or dimethylation of H3K9 (H3K9me2) (Fig. 4D), and an increase of the centromeric transcripts including *alpha-satellite* (*α-Sat*), *major-satellite* (*Maj-Sat*), and *satellite 2* (*Sat2*) (Fig. 4E), whereas re-introduction of FLAG-MTHFRres into the MTHFR-depleted cells restored these changes (Fig. 4D and E). Taken together, these results demonstrated that MTHFR promotes heterochromatin maintenance under unperturbed conditions, especially at the centromeric region.

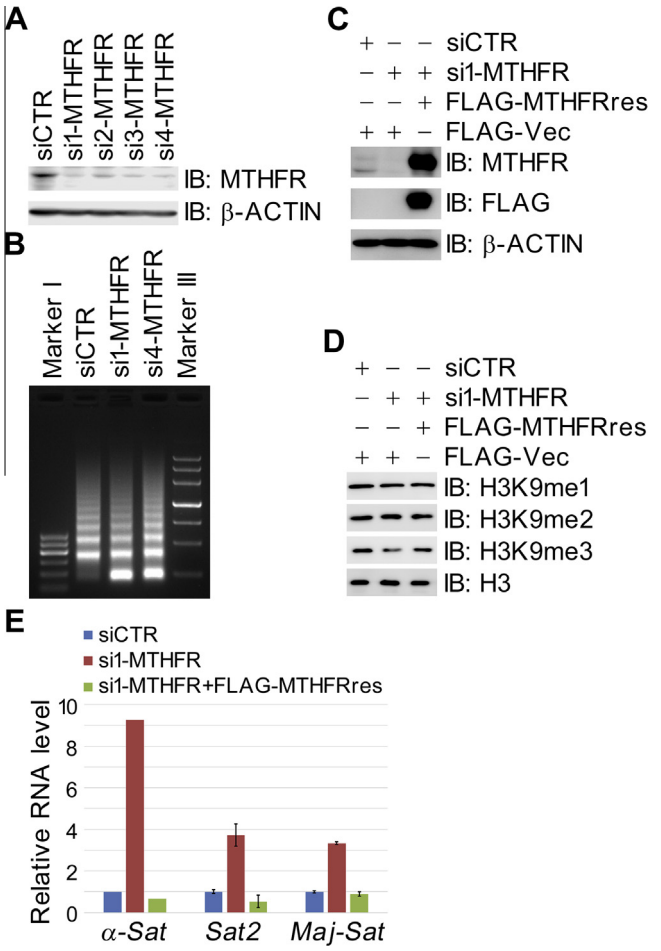

Fig. 4. MTHFR promotes heterochromatin maintenance at the centromeric region. (A) HeLa cells were transfected with siCTR, or individual siRNA oligos specific for MTHFR. Total cell lysates were harvested 48 h later for immunoblotting with antibodies as indicated. (B) Genomic DNA from MTHFR-depleted cells are sensitive to micrococcal nuclease digestion. HeLa cells were transfected with siCTR, si1-MTHFR, or si4-MTHFR. Nuclear extracts were extracted 48 h after transfection and treated with micrococcal nuclease. Genomic DNA was subsequently extracted and separated by a 1.2% agarose gel. (C–E) Depletion of MTHFR compromises the heterochromatin structure at the centromeric region. HeLa cells were transfected with siCTR or si1-MTHFR (duplicate), and the si1-MTHFR transfectants were further transfected with FLAG-Vec or FLAG-MTHFRres (a silent mutant resistant to the inhibition by si1-MTHFR) 24 h after siRNA transfection. Total cell lysates were harvested 48 h after the second transfection for immunoblotting with antibodies as indicated (C), histone extracts were prepared for immunoblotting with antibodies as indicated (D), and total RNAs were extracted for real time-PCR analysis to determine the relative transcript levels of *α-Sat*, *Sat2* and *Major-Sat* (*Maj-Sat*) (E).

4. Discussion

We have demonstrated that MTHFR is phosphorylated on T34 by CDK1/Cyclin B1 in vivo and this phosphorylation peaks during mitosis and decreases its enzymatic activity. This is consistent with the previous report that H3K9me3 levels are reduced when entering S phase and maintained at relatively low levels through G2/M phase [30,31]. This is further supported by that inhibition of MTHFR expression, which is, to some extent, equivalent to reduction of MTHFR activity, decreased H3K9me3 levels (Fig. 4D). We thus speculate that CDK1/Cyclin B1-mediated MTHFR phosphorylation and the resulting decreased enzymatic activity may contribute to the maintenance of H3K9me3 levels during mitosis.

Both the *Mthfr* knockout mice [14] and carriers of variant alleles for *MTHFR677* and/or *MTHFR1298* [13] exhibit genome-wide DNA hypomethylation, which is a characteristic of genome instability. However, molecular epidemiological studies have reached conflicting conclusions if these genetic variants associate with incidence of certain type of cancer [1]. Our results demonstrated that depletion of MTHFR leads to relaxation of heterochromatin, a decrease of H3K9me3 levels that often associate with heterochromatin at the centromeric and telomeric regions, and an increase of certain transcripts at the centromeric region (Fig. 4). This uncovers a novel function of MTHFR in promoting heterochromatin maintenance and ultimately genome stability. Its detailed mechanism warrants further investigation.

Acknowledgments

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