Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b

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DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b cooperatively regulate cytosine methylation in CpG dinucleotides in mammalian genomes, providing an epigenetic basis for gene silencing and maintenance of genome integrity. Proper CpG methylation is required for the normal growth of various somatic cell types, indicating its essential role in the basic cellular function of mammalian cells. Previous studies using Dnmt1–/– or Dnmt3a–/–/Dnmt3b–/– ES cells, however, have shown that undifferentiated embryonic stem (ES) cells can tolerate hypomethylation for their proliferation. In an attempt to investigate the effects of the complete loss of CpG DNA methyltransferase function, we established mouse ES cells lacking all three of these enzymes by gene targeting. Despite the absence of CpG methylation, as demonstrated by genome-wide methylation analysis, these triple knockout (TKO) ES cells grew robustly and maintained their undifferentiated characteristics. TKO ES cells retained pericentromeric heterochromatin domains marked with methylation at Lys9 of histone H3 and heterochromatin protein-1, and maintained their normal chromosome numbers. Our results indicate that ES cells can maintain stem cell properties and chromosomal stability in the absence of CpG methylation and CpG DNA methyltransferases.

Introduction

Vertebrate genomes are methylated on cytosines at levels much higher than those of other eukaryotic organisms, and are almost exclusively methylated at CpG dinucleotides (Bird 2002). CpG methylation plays an important role in epigenetic gene silencing and maintenance of genome stability, and is involved in a broad range of physiological and pathological processes in mammals, including embryogenesis, genome imprinting and tumorigenesis (Jones & Baylin 2002; Li 2002; Robertson 2005). In mice, three CpG DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, coordinately regulate CpG methylation in the genome (Li et al. 1992; Okano et al. 1999). Reduction of CpG methylation by inactivating these enzymes or related molecules in various mammalian cells results in growth defects, cell death, activation of retrotransposons and genome instability (Walsh et al. 1998; Xu et al. 1999; Jackson-Grusby et al. 2001; Rhee et al. 2002; Eden et al. 2003; Gaudet et al. 2003; Bourc’his & Bestor 2004; Dodge et al. 2005; Hata et al. 2006), indicating that CpG methylation plays a fundamental role in basic cellular functions of mammalian cells. Unlike differentiated somatic cells, undifferentiated ES cells can tolerate hypomethylation caused by inactivation of Dnmt1 or inactivation of both...
Dnmt3a and Dnmt3b for their proliferation (Lei et al. 1996; Chen et al. 2003; Jackson et al. 2004), indicating that gene silencing and chromatin structures are regulated differently in ES cells.

Post-translational modifications of specific amino acid residues of histones are also important for chromatin dynamics and epigenetic gene regulation (Jenuwein & Allis 2001). Functional relationships between CpG methylation and histone modification have been demonstrated in several species (Tamaru & Selker 2001; Jackson et al. 2002; Lehnertz et al. 2003; Tariq et al. 2003; Vire et al. 2006). Biochemical analyses have also shown that methyl-CpG-binding proteins interact with either histone deacetylases or histone methyltransferases (Jones et al. 1998; Nan et al. 1998; Ng et al. 1999; Wade et al. 1999; Zhang et al. 1999; Fujita et al. 2003; Sarraf & Stancheva 2004), further supporting the mechanistic links between these epigenetic mechanisms. Interestingly, a previous study using Dnmt1–/– and Dnmt3a+/-/ Dnmt3b+/- ES cells showed that ES cells with a reduced level of CpG methylation maintain proper localization of the repressive chromatin markers histone H3 methylated at lysine 9 (H3K9) and heterochromatin protein-1 (HP1) at pericentromeric heterochromatin (Lehnertz et al. 2003), indicating that CpG methylation may contribute little to the regulation of higher-order chromatin structures in ES cells. That study, however, remains inconclusive because the mutant ES cells had residual CpG methylation and functional CpG methyltransferase activity.

In this study, we address whether mouse ES cells can survive in the absence of epigenetic regulation by CpG methylation, and if so, whether higher-order chromatin structures can be maintained in these cells. We established ES cells deficient for all three CpG methyltransferases, Dnmt1, Dnmt3a and Dnmt3b. We show that ES cells without CpG methylation maintain stem cell properties, proliferation ability, heterochromatic domains marked with H3K9 trimethylation, and euploidy.

**Results**

**Establishment of Dnmt1/Dnmt3a/Dnmt3b triple knockout ES cells**

We investigated the effects of the complete loss of CpG DNA methyltransferase function by inactivating Dnmt1 via two rounds of gene targeting in Dnmt3a+/-/ Dnmt3b+/- ES cells (Fig. 1A). This procedure yielded five independent clones of viable TKO Dnmt1–/–/ Dnmt3a–/–/ Dnmt3b–/– ES cells. We confirmed the accuracy of the gene targeting in these five cell lines by Southern blotting analysis (Fig. 1B and data not shown). Three of the five TKO cell lines were characterized in detail by Western blotting and RT-PCR analyses, showing the loss of gene products (Fig. 1B–D), and we used these for further studies.

**Absence of CpG methylation in TKO ES cells**

We evaluated the status of genome-wide CpG methylation in the TKO cells by several independent methods. We first examined CpG methylation of repetitive sequences by Southern hybridization using methylation-sensitive restriction enzymes. The methylation of two widely dispersed retroelement sequences, C-type endogenous retrovirus and intracisternal-A particles (IAP), was much lower in TKO cells as compared with Dnmt1–/– cells or Dnmt1–/–/ Dnmt3a–/–/ Dnmt3b–/– clones (Fig. 2A). In TKO cells, the digest patterns for CpG methylation-sensitive HpaII and its CpG methylation-insensitive isoschizomer MspI were indistinguishable, suggesting that CpG methylation in TKO cells was below the detection limit (Fig. 2A). Analysis of minor satellite repeats at centromeric regions and major satellite repeats at pericentromeric regions showed similarly reduced CpG methylation states in TKO cells (Fig. 2A).

We next examined the profiles and amounts of CpG methylation in TKO cells using bisulfite sequencing analysis, which can determine the exact sites of methylcytosine (Clark et al. 1994). Pericentromeric major satellite repeats, retroelement repeats, and the single-copy imprinted genes Snrpn and Igf2r all showed extensive loss of CpG methylation (Fig. 2B). We also sequenced random fragments of bisulfite-treated genomic DNA via shotgun sequencing and compared the sequences to the mouse genome (Table 1 and <http://www.dbsh.org/>). Two experiments using TKO cells showed that almost all methylcytosines were lost in 786 CpG sites over about 75 kb (Table 1). We detected a very small amount of methylcytosine-signals both at CpG sites (0.4%) and non-CpG sites (0.1–0.4%), possibly reflecting residual methylcytosines in the genome of TKO cells resulting from mechanisms other than methylation by Dnmt1/Dnmt3a/Dnmt3b. Such low levels, however, are nearly equivalent to the loss of nucleotide misincorporation by Taq polymerase or incomplete bisulfite conversion. These data suggest that both CpG and non-CpG methylation in the TKO cells were below the detection limit in our experimental procedures.

We then investigated the loss of CpG methylation at the cellular level by focusing on two cellular responses, localization of a methyl-CpG-binding protein and silencing of retroelements. To visualize the cytological distribution
of CpG methylation in ES cells, we introduced and expressed green fluorescent protein fused to the methyl-CpG binding domain of MBD1 (GFP-MBD, Fujita et al. 1999). In interphase nuclei of wild-type and Dnmt1–/– ES cells, GFP-MBD mainly localized to pericentromeric heterochromatin regions that were densely stained by 4′,6′-diamino-2-phenylindole (DAPI). In contrast, GFP-MBD distribution was diffuse in TKO cell nuclei, and its localization was not restricted to pericentromeric heterochromatin (Fig. 2C), in agreement with our observation of extensive loss of CpG methylation at pericentromeric regions in these cells (Fig. 2A,B). We also examined the expression of two retroelements, IAP and LINE1, which are normally silenced and are de-repressed in severely hypomethylated embryos or germ cells (Walsh et al. 1998; Bourc’his & Bestor 2004; Hata et al. 2006). Consistent with the CpG methylation status of these retroelements (Fig. 2B), transcripts of...
IAP were elevated in both the TKO and the $Dnmt1^{+/−}$ ES cells, whereas transcripts of LINE1 were elevated mainly in TKO cells (Fig. 2D). These data are consistent with the absence of CpG methylation in the TKO cells.

**Maintenance of stem cell properties in TKO ES cells**

Despite the absence of CpG methylation, TKO cells retained the morphological features of undifferentiated
ES cells and stained positive for alkaline phosphatase activity, indicative of a pluripotent, undifferentiated state (Fig. 3A). TKO cells also expressed four other typical markers of undifferentiated cells (Oct4, Rex1, Fgf4 and Nanog) at the same or higher levels than wild-type ES cells (Fig. 3B). Growth curve experiments suggest that proliferation of undifferentiated TKO cells was comparable to, although slightly slower than, that of wild-type ES cells (Supplementary Fig. S1). We also estimated the growth rate of TKO cells by growth competition analysis, in which wild-type and TKO cells were cocultured, and the change in the fraction of wild-type and TKO cells in the cell population during the culture period was determined by Southern hybridization. The growth of undifferentiated TKO cells was comparable to that of wild-type cells, whereas their growth was delayed upon differentiation by formation of embryoid bodies (Fig. 3C), consistent with previous findings using Dnmt1−/− ES cells (Lei et al. 1996). These results suggest that CpG methylation is not essential for the self-renewal of ES cells in an undifferentiated state.

Table 1 Random bisulfite sequencing analysis. Random fragments of bisulfite-treated genomic DNA from the ES cell lines were sequenced, and the methylation status of each cytosine was determined by alignment with the mouse genome. Methylation status of clones that mapped to multiple loci in the mouse genome was based on the highest probability alignment with the genomic sequence.

<table>
<thead>
<tr>
<th></th>
<th>No. of clones</th>
<th>Total (bp)</th>
<th>CpG</th>
<th>CpA</th>
<th>CpC</th>
<th>CpT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>119</td>
<td>16327</td>
<td>112/136 (82%)</td>
<td>7/1117</td>
<td>3/779</td>
<td>6/1142</td>
</tr>
<tr>
<td>Dnmt3a−/−;3b−/−</td>
<td>63</td>
<td>8541</td>
<td>11/72 (15%)</td>
<td>3/661</td>
<td>5/415</td>
<td>3/588</td>
</tr>
<tr>
<td>Dnmt1−/−</td>
<td>112</td>
<td>15450</td>
<td>26/143 (18%)</td>
<td>8/1106</td>
<td>2/765</td>
<td>1/1057</td>
</tr>
<tr>
<td>TKO (#19)</td>
<td>383</td>
<td>57404</td>
<td>*2/559 (0.4%)</td>
<td>8/4125</td>
<td>11/2725</td>
<td>5/4011</td>
</tr>
<tr>
<td>TKO (#121)</td>
<td>127</td>
<td>17448</td>
<td>*1/227 (0.4%)</td>
<td>3/1296</td>
<td>4/1009</td>
<td>1/1201</td>
</tr>
</tbody>
</table>

*Three cytosines at CpG sites remained unconverted to uracil after the bisulfite reaction in the TKO cells, and two of the three cytosines were derived from clones that contained more than three unconverted cytosines at non-CpG sites. In these cases, all unconverted cytosines were clustered within a 20-bp palindromic sequence, indicating the possibility of incomplete conversion by the bisulfite reaction.

Figure 3 Maintenance of undifferentiated features in TKO cells. (A) Phase-contrast microscopic images (top) and alkaline phosphatase staining (bottom) of wild-type (wt) and TKO cells. (B) RT-PCR analysis of four representative undifferentiated ES cell markers, Oct4, Rex1, Fgf4 and Nanog. No signal was observed in the negative control lacking reverse transcriptase (RT). (C) Growth competition in a mixture of wt and TKO cells under culture conditions that maintain the undifferentiated state (undiff, top) or promote differentiation (diff, bottom). Changes in the fraction of wild-type and TKO cells in the cell population at the indicated days were determined by Southern analysis using the Dnmt3b probe.
We next examined whether the absence of CpG methylation affects the post-translational modification of histone termini in ES cells. We assessed global histone acetylation and methylation by Western blotting using modification- and site-specific antibodies against histones H3 and H4. Acetylation at lysines of histones H3 and H4 and methylation at Lys4 of histone H3 (H3K4) are generally associated with transcriptionally active chromatin, whereas methylation at Lys9 of histone H3 (H3K9) is associated with transcriptionally repressed chromatin. Overall, the amounts of histone acetylation and methylation in TKO cells at most sites detected by the antibodies were similar to those in wild-type cells (Fig. 4A). We did not detect a significant increase in the acetylation of histone H4 at Lys5 in TKO cells, which was observed in Dnmt3a−/− Dnmt3b−/− ES cells of high passage numbers (Jackson et al. 2004).

**Figure 4** Maintenance of global chromatin structure in TKO cells. (A) Western blot analysis of global histone acetylation and methylation levels. H3Ac, acetylated histone H3; H4Ac, acetylated histone H4; H4K5Ac, H4K8Ac, H4K12Ac and H4K16Ac, acetylation at Lys5, Lys8, Lys12, or Lys16 of histone H4, respectively; H3K4Me and H3K9Me, dimethylation at Lys4 or Lys9 of histone H3, respectively; H3K4Me and H3K9Me, trimethylation at Lys4 or Lys9 of histone H3, respectively; H4, histone H4. Anti-histone H4 was used as a loading control. (B) Immunofluorescence analysis of interphase chromatin from wild-type (wt) and TKO cells using antibodies against trimethylated H3K9, trimethylated H3K4, HP1-β and HP1-γ. DNA was visualized with DAPI. Merged images represent overlays of immunofluorescence signal (green or red) and DAPI (blue) as indicated. “Low” and “high” indicate low and high magnification, respectively.

**Maintenance of global chromatin structures in TKO ES cells**

We next examined whether the absence of CpG methylation affects the post-translational modification of histone termini in ES cells. We assessed global histone acetylation and methylation by Western blotting using modification- and site-specific antibodies against histones H3 and H4. Acetylation at lysines of histones H3 and H4 and methylation at Lys4 of histone H3 (H3K4) are generally associated with transcriptionally active chromatin, whereas methylation at Lys9 of histone H3 (H3K9) is associated with transcriptionally repressed chromatin. Overall, the amounts of histone acetylation and methylation in TKO cells at most sites detected by the antibodies were similar to those in wild-type cells (Fig. 4A). We did not detect a significant increase in the acetylation of histone H4 at Lys5 in TKO cells, which was observed in Dnmt3a−/− Dnmt3b−/− ES cells of high passage numbers (Jackson et al. 2004).
We have established the first mammalian cells deficient in this region (Lehnertz et al. 2003; Maison & Almouzni 2004). Immunofluorescence analysis of TKO cells showed that the focal staining patterns of trimethylated H3K9 and HP1 overlapped with DAPI-dense heterochromatin regions, similar to the results for wild-type cells (Fig. 4B). The diffuse speckled patterns of trimethylated H3K4 found in TKO cell nuclei were also indistinguishable from those of wild-type cells (Fig. 4B). We did not observe a redistribution of H3K4 methylation to pericentromeric heterochromatin in TKO cells, as observed in hypomethylated mouse fibroblasts deficient for Lsh (Yan et al. 2003), a member of the SNF/helicase family involved in the control of global CpG methylation. Furthermore, analysis of metaphase chromosomes showed that TKO cells retained normal chromosomal number and banding patterns (Supplementary Fig. S2 and data not shown), indicating that condensation and segregation of chromosomes during mitosis occur properly in the absence of CpG methylation in ES cells.

Discussion

We have established the first mammalian cells deficient for the three known CpG DNA methyltransferases; CpG methylation is apparently absent in these cells. Our results provide direct genetic evidence that CpG methylation is dispensable for ES cell growth in an undifferentiated state, as suggested previously (Lei et al. 1996; Chen et al. 2003; Jackson et al. 2004). This stands in clear contrast to cases involving other cell types. Loss of Dnmt1 in mouse embryonic fibroblasts leads to growth arrest associated with increased expression of p21Waf1/Cip1 and p57Kip2, and p53-dependent cell death (Jackson–Grusby et al. 2001); moreover, the loss of both DNMT1 and DNMT3B in human colon cancer cell lines causes growth impairment that is dependent on re-expression of p16INK4A (Rhee et al. 2002; Bachman et al. 2003). The unique cell cycle regulation of mouse ES cells (Hong & Stambrook 2004) may explain the robust growth and viability of our TKO cells.

Reduction of Dnmt1 activity in mice has been shown to induce tumors and chromosomal instability (Eden et al. 2003; Gaudet et al. 2003), and global hypomethylation in male germ cells results in meiotic abnormality (Bourc’his & Bestor 2004; Hata et al. 2006). Furthermore, inactivation of Dnmt3b in primary mouse embryonic fibroblast cells causes aneuploidy and chromosomal breaks and fusions (Dodge et al. 2005). These results suggest that proper CpG methylation level is important for chromosomal stability, at least in certain cell types. It remains unknown why undifferentiated ES cells retain chromosomal stability without CpG methylation and CpG DNA methyltransferases. One possibility is that ES cells maintain stable heterochromatin and chromosomes by an epigenetic mechanism that is independent of CpG methylation. Previous studies have shown that ES cells have both unique global chromatin modifications and heterochromatin arrangement, and that chromatin proteins bind more loosely to chromatin in ES cells than in differentiated cells, indicating the existence of specific epigenetic regulatory mechanisms in ES cells (Kimura et al. 2004; Meshorer et al. 2006). The absence or reduction of such mechanisms may be attributed to the chromosomal abnormality in differentiated cell types with decreased levels of CpG methylation. Taken together, these results suggest that the relative contribution of CpG methylation to both chromosomal stability and epigenetic gene silencing varies in a cell type-specific manner.

Our results suggest that at least some functional properties of heterochromatic domains are retained in the absence of CpG methylation in ES cells. However, it is possible that some unrecognized changes in heterochromatin occur in TKO cells. Indeed, the redistribution of the histone variant macroH2A into pericentromeric heterochromatin in hypomethylated Dnmt1−/− ES cells has recently been reported (Ma et al. 2005), indicating that CpG methylation modulates some properties of heterochromatic domains. Although the apparent contribution of CpG methylation to the steady-state heterochromatin structure in ES cells may be small, it is possible that CpG methylation might serve as a marker for anchoring heterochromatin during dynamic chromatin remodeling or during multiple replication processes. Further studies in this line are required for understanding the exact role of CpG methylation in higher-order chromatin structures.

The genomes of primordial germ cells and preimplantation embryos are extensively hypomethylated during normal embryogenesis (Hajkova et al. 2002; Li 2002), and global reduction of CpG methylation is a common feature of neoplasia (Feinberg et al. 2006). The generation of these triple methyltransferase-deficient ES cells will be useful in studying the role of CpG methylation in cell type-specific epigenetic regulation during normal and pathological processes. These cells will be also useful in investigating the function of chromatin modifications and remodeling in the absence of CpG methylation and endogenous CpG DNA methyltransferases in mammalian cellular conditions.
Experimental procedures

**ES cell culture**

ES cells were maintained in Glasgow modified Eagle medium (Sigma) supplemented with 15% foetal bovine serum, 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and 2000 U/mL of leukemia inhibitory factor (LIF) and were grown on gelatinized culture dishes without feeder cells. For growth curve experiments, 0.9 × 10^5 ES cells were plated on gelatinized 10-cm culture dishes on the first day. Then, ES cells were passaged every 2 days with 6- to 12-fold dilution. For induction of differentiation by embryoid body formation, ES cells were seeded on low cell-binding dishes (Nunc) in medium without LIF. For growth competition analysis, wild-type and TKO cells were mixed at a 1 : 1 ratio and cultured either as undifferentiated ES cells or as embryoid bodies, as previously described (Lei et al. 1996). The change in cell population between wild-type and TKO cells was determined by Southern analysis using the Dnmt3b probe, which distinguishes between wild-type and TKO cells.

**Generation of Dnmt1−/−Dnmt3a−/−Dnmt3b−/− ES cells**

The following nucleotide positions of Dnmt1 genomic DNA all refer to AC073775.2. The Dnmt1 targeting vector, pTA009, was constructed by subcloning the 3-kb 5′ arm (nt 66903–70025) and a splicing acceptor–internal ribosomal entry site–blasticidin-resistant gene cassette flanked by losP sites (losP-SAiresBlas-losP) into pBluescript II SK. The Dnmt1 genomic fragments were amplified by PCR, from genomic DNA of ES cells (Supplementary Table S1). For targeting of the first Dnmt1 allele, the Dnmt1 targeting vector was transfected in Dnmt3a−/−Dnmt3b−/− ES cells (Okano et al. 1999) via electroporation, and transfected cells were selected with 5 µg/mL blasticidin. Drug-resistant clones were screened by Southern hybridization using a 5′ probe (nt 66500–66790) and a 3′ probe (nt 76647–77192) external to the targeting construct (Fig. 1A). The targeting frequency was 14% (3/22). To generate blasticidin-sensitive ES cells, Cre recombinase was transiently expressed in blasticidin-resistant Dnmt1−/−Dnmt3a−/− Dnmt3b−/− ES cells (clone 18) by lipofection of a circular Cre expression plasmid. For targeting of the second wild-type Dnmt1 allele, the same targeting vector was transfected into blasticidin-sensitive Dnmt1−/−Dnmt3a−/− Dnmt3b−/− ES cells (clone 67), and the transfected cells were selected with 3.5 µg/mL blasticidin. Targeting frequency was 4% (5/113).

**DNA methylation analysis**

For Southern analysis, genomic DNA was digested with the CpG methylation-sensitive restriction enzymes HpaII or MspI (Roche), blotted and hybridized with probes specific for C-type endogenous retroviruses (pMOS), IAP, minor satellite repeats, or major satellite repeats (Okano et al. 1999). For bisulfite sequencing analysis of specific sequences, 2 µg of genomic DNA digested with HindIII was treated with bisulfite followed by deamination (Clark et al. 1994). Deaminated fragments were amplified with primers for IAP, LINE1, major satellite repeats, Igf2r or Surr2 (Supplementary Table S1). We sequenced 15–30 clones for repetitive sequences and 9–15 clones for single-copy genes. Bisulfite sequencing of random genomic fragments was carried out as previously described (Ramsahoye et al. 2000). The sequences were aligned to the mouse genome sequence, NCBI Mouse Build 33 (Y.K. and H.R.U., unpublished data). Sequenced clones containing five non-CpG cytosines per sequenced clone that remained unconverted to uracil by the bisulfite reaction were excluded from the analysis. Overall, 80% of the sequenced clones longer than 51 bp could be mapped to the mouse genome. For localization of the methyl-CpG-binding protein, the GFP-MBD1(MBD+NLS) plasmid, which encodes an enhanced green fluorescent protein (EGFP) fused to the amino terminus of human MBD1 containing both a methyl-CpG-binding domain and a nuclear localization signal (Fujita et al. 1999; gift from M. Nakao), was transfected into the ES cells by Lipofectamine (Invitrogen). After 16 h of culture, the transfected cells were fixed with 4% paraformaldehyde and 0.5% [v/v] Triton X-100 in phosphate buffered saline (PBS) and were stained with DAPI.

**RT-PCR and Northern blotting**

RNA samples were isolated from subconfluent cells using the Trizol RNA extraction reagent (Invitrogen). Primer sets for Dnmt1, Dnmt3a, Dnmt3b, Oct3/4, Rex1, Nanog, Fgf4 and Gapdh were used for RT-PCR (Supplementary Table S1). Poly(A)+ RNA was prepared using the Poly(A) Tract purification system (Promega). Poly(A)+ RNA (1 µg) was used for Northern analysis. The LINE1 probe was amplified by PCR (Supplementary Table S1).

**Antibodies, Western blotting and immunofluorescence analysis**

The following antibodies were used: anti-Dnmt1 (sc-20701) and anti-[acetyl-Lys5]H4 (sc8659-R) from Santa Cruz Biotechnology; anti-Dnmt3a (IMG-268) and anti-Dnmt3b (IMG-184) from IMGENEX; anti-β-tubulin (CP06) from Oncogene; anti-acylated histone H3 (Lys9, Lys14; 06–599), anti-acetylated histone H4 (Lys5, Lys8, Lys12, Lys16; 06–866), anti-[acetyl-Lys8]H4 (17–211), anti-[acetyl-Lys12]H4 (17–211), anti-[acetyl-Lys14]H4 (17–211), anti-[acetyl-Lys12]H4 (17–211), anti-[acetyl-Lys12]H4 (06–762) and (07–329), anti-[dimethyl-Lys4]H3 (07–030), anti-[dimethyl-Lys4]H3 (07–212) and anti-histone H3 (06–755) from Upstate; anti-[acetyl-Lys16]H4 (ab1762), anti-[trimethyl-Lys4]H3 (ab8580) and anti-[trimethyl-Lys9]H3 (ab8898) from Abcam; and anti-HP1γ (1MOD 1A9) and anti-HP1γ (2MOD 1G6) from EUROMEDEX. Alexa 488- and Alexa 546-conjugated secondary antibodies were purchased from Molecular Probes. A polyclonal antibody for Dnmt1 was raised against a modified linear peptide, CRESEAAAVKAKAEEATKQ, corresponding to the carboxy terminus of mouse Dnmt1. For Western blot analysis, whole-cell extracts were prepared by sonication in SDS-PAGE sample buffer, fractionated on an 8% SDS-PAGE gel and blotted using standard...
glycerol in PBS containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane were processed sequentially with 20%, 40%, 60% and 80% [v/v] several washes with PBS, 0.1% [v/v] Tween-20, the stained cells aldehyde in PBS and then incubated with secondary antibody. After washing with PBS, 0.25% BSA, 0.1% [v/v] Tween-20, cells were re-fixed with 2% formaldehyde in PBS and then incubated with secondary antibody. After several washes with PBS, 0.1% [v/v] Tween-20, the stained cells were processed sequentially with 20%, 40%, 60% and 80% [v/v] glycerol in PBS containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane (DABCO) for mounting. The 20% and 40% glycerol contained 0.5 μg/mL DAPI. Finally, cells were mounted in 90% glycerol in distilled water for microscopic observation. Fluorescence microscope images were acquired using a DeltaVision microscope system (Applied Precision). Three-dimensional optical section images were taken at 0.5-μm focus intervals and computationally processed using SoftWoRx software (Applied Precision).

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References


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Supplementary material

The following supplementary material is available for this article online:

**Figure S1** Growth curve analysis for TKO cells.

**Figure S2** Chromosome analysis by Giemsa staining for TKO cells.

**Table S1** Oligonucleotides used in this study.

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