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Global hypomethylation of the genome in XX embryonic stem cells

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Embryonic stem (ES) cells are important tools in the study of gene function and may also become important in cell therapy applications[1]. Establishment of stable XX ES cell lines from mouse blastocysts is relatively problematic owing to frequent loss of one of the two X chromosomes. Here we show that DNA methylation is globally reduced in XX ES cell lines and that this is attributable to the presence of two active X chromosomes. Hypomethylation affects both repetitive and unique sequences, the latter including differentially methylated regions that regulate expression of parentally imprinted genes. Methylation of differentially methylated regions can be restored coincident with elimination of an X chromosome in early-passage parthenogenetic ES cells, suggesting that selection against loss of methylation may provide the basis for X-chromosome instability. Finally, we show that hypomethylation is associated with reduced levels of the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b and that ectopic expression of these factors restores global methylation levels.

DNA methylation is thought to have evolved as a genome defense mechanism and to have acquired a role in gene or genome regulation during development[2]. In mammals, where methylation occurs symmetrically on cytosine residues of CpG dinucleotides, ~70% of available sites are methylated. High concentrations occur in repetitive sequences, but methylation is generally excluded from CpG rich islands found at the 5′ end of many genes. Exceptions to the latter include differentially methylated regions (DMRs), associated with imprinted genes and CpG islands of genes on the inactive X chromosome.

In the course of analyzing DNA methylation in an XX ES cell line, PGK12.1, we observed reduced methylation levels in pericentric major satellite repeats (**Fig. 1a**). We also observed hypomethylation in two other XX ES cell lines, LF2 (**Fig. 1a**) and TMA (data not shown). Major satellite repeats were fully methylated in XY ES cell lines (**Fig. 1b**) and differentiated XX cells (**Fig. 1c**,**d**), consistent with previous reports[3]. Major satellite repeats were also fully methylated in two different X0 ES cell lines (**Fig. 1e**), indicating that hypomethylation in XX ES cells is attributable to the presence of two (active) X chromosomes rather than to the absence of a Y chromosome.

We also observed hypomethylation in XX ES cells at repetitive C-type retroviral sequences (**Fig. 2a**), in an intragenic region located 30 kb upstream of the X-linked gene *Mtm1* (**Fig. 2b**), in an intronic region in the X-linked gene *Hprt1* (**Fig. 2c**) and, consistent

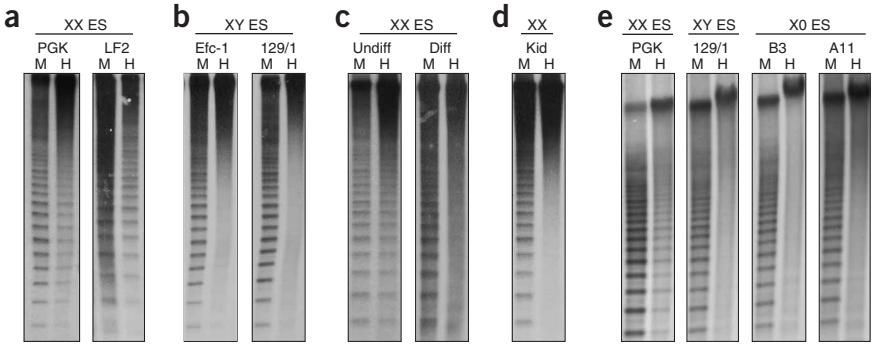

Figure 1 Hypomethylation of major satellite sequences in XX ES cells. Southern blots were analyzed using a probe specific for mouse major satellite repeats. The repeats appear as a ladder of bands using the methylation-insensitive enzyme *Msp*I (M). The methylation-sensitive isochizomer *Hpa*II (H) digests only unmethylated sites; therefore, appearance of a ladder in these samples is indicative of hypomethylation. (**a**) XX ES cell lines, PGK12.1 (PGK) and LF2. (**b**) XY ES cell lines, Efc-1 and 129/1. (**c**) Undifferentiated PGK12.1 cells (Undiff) and cells differentiated *in vitro* (Diff). (**d**) DNA from female (XX) kidney (Kid). (**e**) X0 ES cell lines, B3 and A11.

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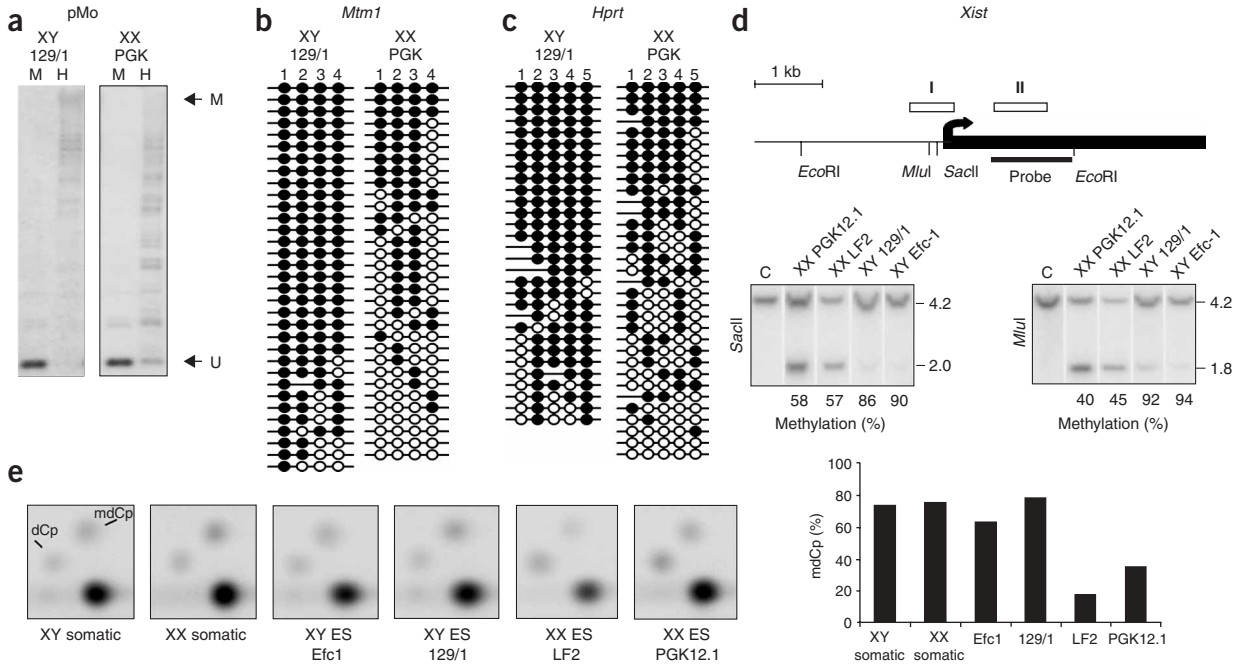


Figure 2 Global hypomethylation of the genome in XX ES cells. (**a**) Southern-blot analysis of methylation of C-type retrovirus sequences (pMo probe) after digestion with *Msp*I (M) or *Hpa*II (H), showing hypomethylation in PGK12.1 XX ES cells (PGK) compared with 129/1 XY ES cells. Bands corresponding to methylated (M) sequences and unmethylated (U) sequences are indicated. (**b**,**c**) Bisulphite sequence analysis of methylation in (**b**) a 667-bp intragenic region located 30 kb upstream of the *Mtm1* locus encompassing four 'methylatable' CpG dinucleotides (1–4) in 129/1 XY and PGK12.1 (PGK) XX ES cells and (**c**) a 380-bp region in intron 4 of the *Hprt* locus encompassing five 'methylatable' CpG sites (1–5). Methylated (filled circles) and unmethylated (open circles) CpG dinucleotides are shown for a number of independently sequenced templates (horizontal lines). (**d**) Quantitative Southern-blot analysis of *Sac*II and *Mlu*I restriction sites in promoter region I of the mouse gene *Xist* in XX and XY ES cell DNA. DNA was cut with *Eco*RI and then with either *Sac*II (left panel) or *Mlu*I (right panel). Control (C) shows sample cut with *Eco*RI alone. The percentage of methylation in each sample (shown below each panel) was quantified using analysis of phosphorimager screen exposure. (**e**) Nearest-neighbor analysis quantifying global methylation levels. The selected area shows signal for dCp and methylated dCp (mdCp). DNA was from XY and XX mouse kidney (somatic) or from ES cell lines as indicated. Quantification of methylated dCp as a percentage of the total dCp on the basis of phosphorimager analysis is shown in the graph.

with previous findings[4–6], in the promoter region of the mouse *Xist* locus (**Fig. 2d**). Taken together, these observations indicate that DNA hypomethylation is a general feature of XX ES cells. To confirm this possibility, we quantified global methylation levels using nearest-neighbor analysis (**Fig. 2e**). We found that ~70% of CpG sites were methylated in XY and XX somatic cells and in two different XY ES cell lines, similar to previously reported estimates using this method[7]. In XX ES cells, however, 35% or less of available CpG sites were methylated.

We went on to assess methylation levels at DMRs associated with imprinted genes. The *H19* DMR, which is normally methylated on the paternal allele, was hypomethylated specifically in XX ES cells (**Fig. 3a**). Similarly, KvDMR1 located on chromosome 7 and the *Igf2r-Air* DMR located on chromosome 17, both normally methylated on the maternal allele[8,9], showed complete hypomethylation in XX ES cells (**Fig. 3b**,**c**). Methylation was not restored in XX ES cells differentiated *in vitro* (**Fig. 3b**,**c**), unlike the major satellite repeat sequences (**Fig. 1c**).

In light of our data, we considered that hypomethylation could provide the selective pressure favoring deletion of sequences from one of the two X chromosomes. Instability of the XX karyotype has previously been characterized in a series of diploid parthenogenetic ES cell lines[10]. There is also evidence that maternal imprints are unstable in parthenogenetic ES cells[11]; moreover, parthenogenetic ES cells frequently show progressive loss of methylation at the imprinted gene *U2af1-rs1* (methylated on the maternal allele) during serial passaging[12]. With these observations in mind, we analyzed methylation of the *Igf2r-Air* DMR during serial passaging in six previously described independent parthenogenetic cell lines[12] (**Fig. 4a**). We then correlated methylation of this DMR with the X:autosome ratio (**Fig. 4b**). The *Igf2r-Air* DMR is predicted to be almost fully methylated in parthenogenetic ES cells, because both alleles are of maternal origin. This was the case in the earliest passages for all six cell lines. In subsequent passages, however, there was a progressive loss of methylation, except in cell line PR1. In cell lines PR9 and PR13, methylation was lost initially but recovered in the latest passage analyzed. Overall, these results correlate well with previous analysis of the maternally methylated gene *U2af1-rs1* (ref. 12), suggesting that progressive hypomethylation in parthenogenetic ES cells occurs genome-wide.

To assess X-chromosome dosage, we quantified Southern blots hybridized with X-linked and autosomal markers (**Fig. 4b**). In cell line PR1, all passages had an X:autosome ratio similar to that seen in control XY cells, indicative of deletion of one of the two X chromosomes before passage 7. This in turn correlates with retention of methylation at the *Igf2r-Air* DMR during serial passaging. In all other cell lines, the X:autosome ratio was more similar to that of control XX cells, correlating with progressive loss of *Igf2r-Air* methylation. In PR9 cells, where restoration of methylation followed initial loss, later-passage cells had a reduced X:autosome ratio consistent with the population becoming X0 during serial passaging.

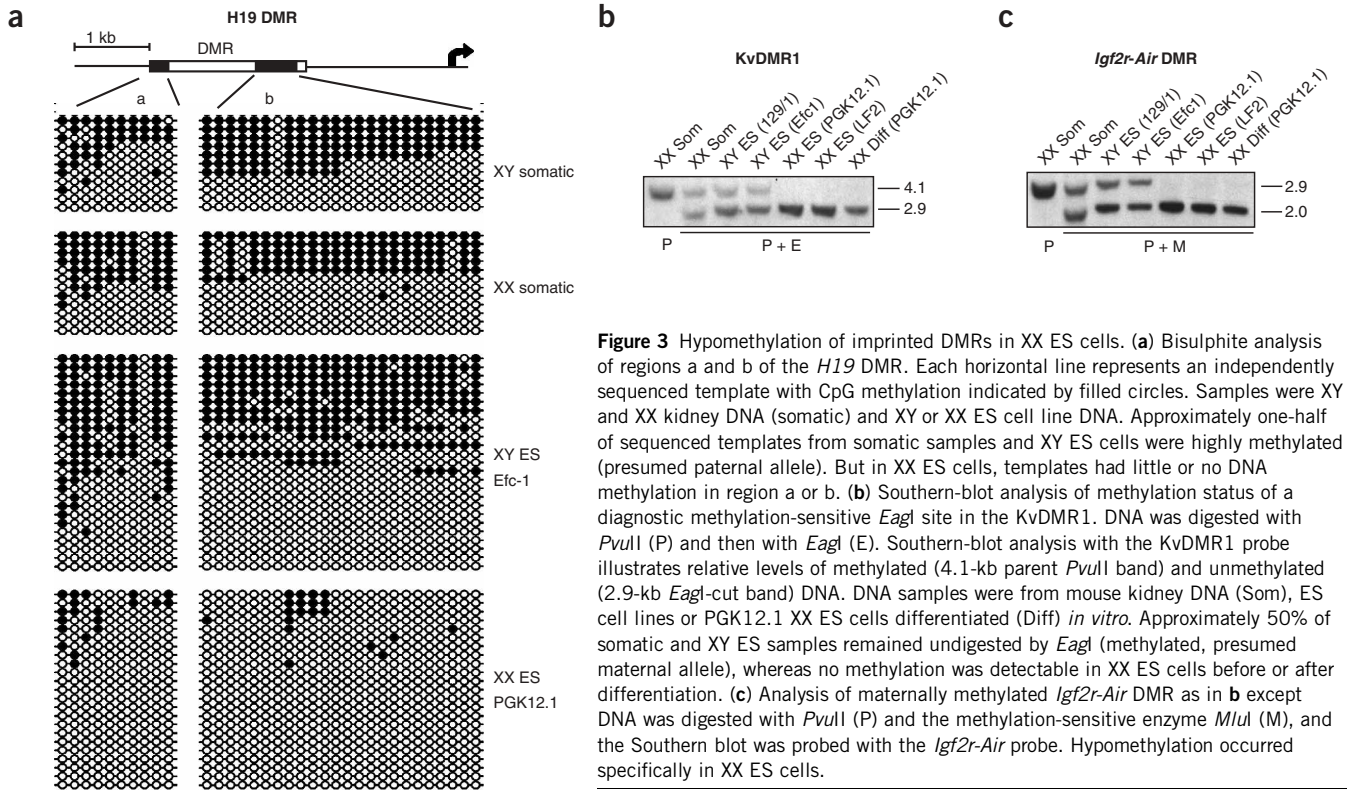


Figure 3 Hypomethylation of imprinted DMRs in XX ES cells. (**a**) Bisulphite analysis of regions a and b of the *H19* DMR. Each horizontal line represents an independently sequenced template with CpG methylation indicated by filled circles. Samples were XY and XX kidney DNA (somatic) and XY or XX ES cell line DNA. Approximately one-half of sequenced templates from somatic samples and XY ES cells were highly methylated (presumed paternal allele). But in XX ES cells, templates had little or no DNA methylation in region a or b. (**b**) Southern-blot analysis of methylation status of a diagnostic methylation-sensitive *Eag*I site in the KvDMR1. DNA was digested with *Pvu*II (P) and then with *Eag*I (E). Southern-blot analysis with the KvDMR1 probe illustrates relative levels of methylated (4.1-kb parent *Pvu*II band) and unmethylated (2.9-kb *Eag*I-cut band) DNA. DNA samples were from mouse kidney DNA (Som), ES cell lines or PGK12.1 XX ES cells differentiated (Diff) *in vitro*. Approximately 50% of somatic and XY ES samples remained undigested by *Eag*I (methylated, presumed maternal allele), whereas no methylation was detectable in XX ES cells before or after differentiation. (**c**) Analysis of maternally methylated *Igf2r-Air* DMR as in **b** except DNA was digested with *Pvu*II (P) and the methylation-sensitive enzyme *Mlu*I (M), and the Southern blot was probed with the *Igf2r-Air* probe. Hypomethylation occurred specifically in XX ES cells.

Late-passage PR13 cells also had a reduced X:autosome ratio, albeit to a lesser extent, which might reflect a mixed population of XX and XO cells where selection is still ongoing. These data support the hypothesis that retention of methylation provides a selective pressure for deletion of all or part of one X chromosome.

To study the mechanism of hypomethylation in XX ES cells, we analyzed levels of the enzymes involved in *de novo* (Dnmt3a and Dnmt3b; refs. 13,14) and maintenance methylation (Dnmt1; ref. 15) by western blotting (**Fig. 5a**). Levels of Dnmt3a were markedly lower in XX than in XY ES cell lines. Dnmt3b levels also seemed to be lower in XX cells, but this was more variable in the different cell lines. Levels of Dnmt1, on the other hand, were equivalent in XX and XY ES cells.

Levels of Dnmt3a2 and Dnmt3b1 (the major isoforms seen in ES cells) initially increased and then declined after *in vitro* differentiation of ES cell cultures (**Fig. 5b**). We observed similar patterns in both XY and XX ES cells, although overall levels of Dnmts were lower at all stages in XX cells. The transient increase during early differentiation stages could account for the reacquisition of methylation at major satellite repeats (**Fig. 1c**). Qualitative assessment of the distribution of Dnmt enzymes using immunofluorescence detected no differences between XY and XX ES cells (data not shown).

To test whether reduced levels of *de novo* Dnmts contributed to global hypomethylation, we transfected XX ES cells with Dnmt3a and Dnmt3b expression constructs. Western-blot analysis detected increased levels of the proteins in many of the transgenic cell lines, although levels were generally not as high as those observed in control

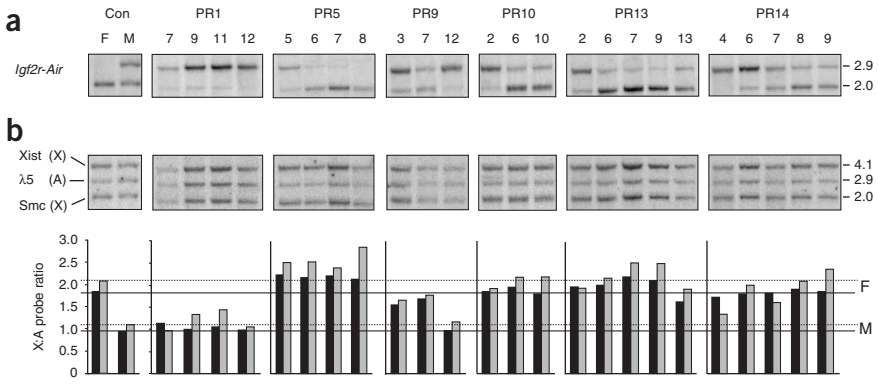


Figure 4 Progressive hypomethylation of the *Igf2r-Air* DMR correlates with X chromosome number in parthenogenetic ES cells. (**a**) Methylation analysis of *Igf2r-Air* DMR (as in **Fig. 3c**) in control (Con) XX female (F) and XY male (M) ES cell DNA and in DNA from different passages (indicated above lanes) of six independently derived parthenogenetic ES cell lines, PR1, PR5, PR9, PR10, PR13 and PR14. Molecular weights (kb) are shown to the right. (**b**) Upper panels show Southern-blot analysis used to estimate X chromosome number on the basis of the ratio of signal for two X-linked (X) probes, *Xist* and *Smc1l1* (Smc), and an autosomal (A) probe from the λ5preB locus (λ5). Molecular weights (kb) are shown to the right. Lower panels illustrate quantification of the ratio of X:autosome (A) signals for *Xist* (filled bars) and *Smc1l1* (shaded bars). Solid and dashed lines highlight levels observed in control XY (M) and XX (F) cells. Equivalent results were obtained in an independent repeat of this experiment.

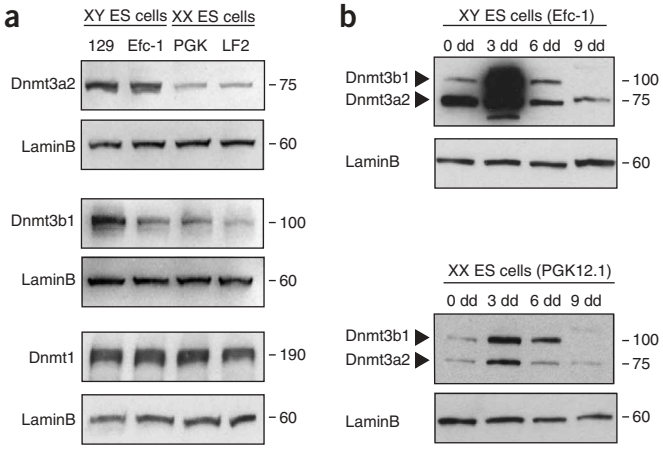


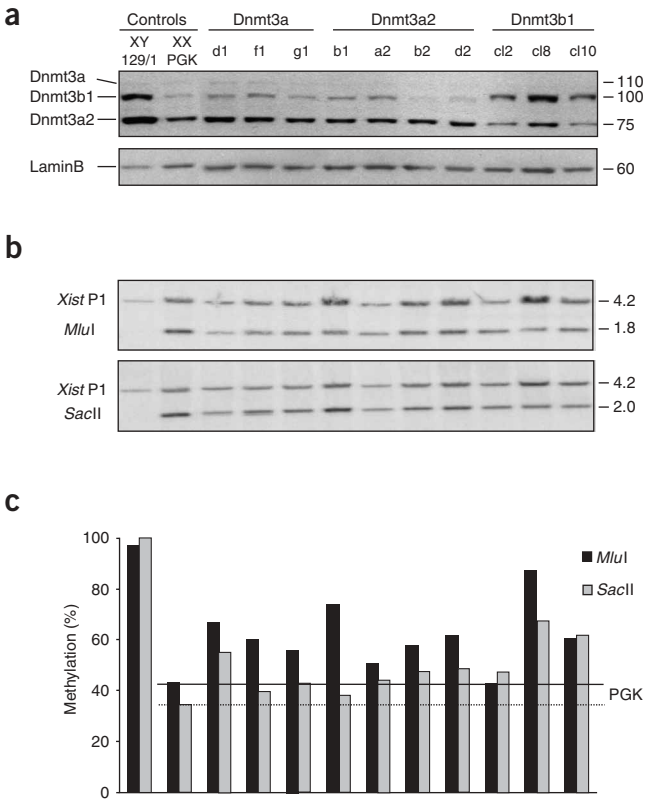
Figure 5 Reduced levels of *de novo* methyltransferases in XX ES cells. (**a**) Western-blot analysis of Dnmt3a, Dnmt3b and Dnmt1 in cell extracts from XY and XX ES cells. PGK, PGK12.1 ES cells. Equal loading of samples is illustrated by probing blots with antibody for LaminB. Molecular weights (kDa) are shown on the right. Selected regions of western blots show the major isoforms seen in ES cells, Dnmt3a2 and Dnmt3b1. (**b**) Western-blot analysis of Dnmt3a2 and Dnmt3b1 in XY and XX ES cells differentiated *in vitro* for 0, 3, 6 or 9 days (dd). Equal loading of samples is indicated by LaminB controls.

XY ES cells (**Fig. 6a**). Ectopic expression of Dnmt3a and Dnmt3b increased methylation levels in the *Xist* promoter region, generally ~10–30% higher than in the parental PGK12.1 XX ES cells (**Fig. 6b**,**c**). In cell line Dnmt3b cl8, methylation levels approached those observed in normal XY ES cells.

We analyzed global methylation levels in selected transgenic cell lines. In Dnmt3a d1 and Dnmt3b cl10 cells, CpG methylation was elevated to a level intermediate between the levels observed in XX and XY ES cells (**Fig. 6d**). In Dnmt3b cl8 cells, which showed the highest levels of *Xist* promoter methylation, methylation levels were essentially indistinguishable from those observed in XY ES cells. Taken together, these results indicate that the methylation deficit in XX ES cells could be complemented by ectopic expression of either Dnmt3a or Dnmt3b. Methylation at the KvDMR1 and *Igf2r-Air* DMR was not restored in transgenic XX cell lines (**Fig. 6e**). This is probably because methylation at imprinted DMRs (unlike repeat sequences such as the major satellite; **Fig. 1**) is completely lost. This could also explain why methylation at imprinted DMRs does not recover after differentiation of XX ES cells (**Fig. 3b**,**c**).

Hypomethylation of the genome in XX ES cells may account for previously unexplained findings, including *Xist* promoter hypomethylation[4–6], elevated levels of chromatin modifications associated with gene activity on the X chromosome in XX ES cells[16], and hypomethylation of the *H19* DMR in XX but not XY embryonic germ cells[17,18]. Our findings also provide a possible explanation for instability of the XX karyotype in ES cells, although we cannot rule out alternative explanations at this time. The fact that some XX ES cell lines, notably PGK12.1 and LF2 used in this study, tolerate hypomethylation and retain both X chromosomes may indicate that a secondary mutation compensates for the proposed selective disadvantage.

Hypomethylation in XX ES cells seems to be attributable to reduced levels of *de novo* methyltransferases, most notably Dnmt3a. This is consistent with the demonstrated role of these enzymes in maintenance of methylation in ES cells[7,19,20]. We speculate that the



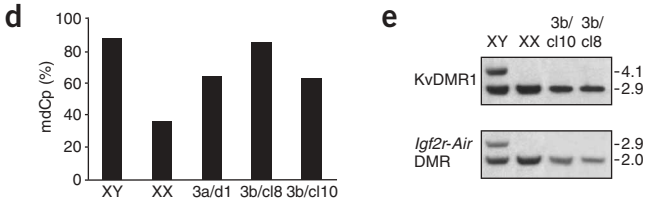


Figure 6 Ectopic expression of *de novo* methyltransferases restores methylation in XX ES cells. (**a**) Western-blot analysis illustrating levels of *de novo* methyltransferases in control 129/1 XY and PGK12.1 (PGK) XX ES cells and in a series of PGK12.1 derivative cell lines obtained by stable transfection with constructs expressing one of the two major isoforms of Dnmt3a (Dnmt3a and Dnmt3a2) or the major isoform of Dnmt3b (Dnmt3b1). LaminB was used as a loading control. In the case of Dnmt3a, only the short 100-kDa isoform (normally the predominant isoform in ES cells[21]) was overexpressed, regardless of whether the Dnmt3a or Dnmt3a2 construct was used. This suggests that the translation initiation codon for the short form is preferentially selected in ES cells. Molecular weights (kDa) are shown to the right. (**b**) Southern-blot analysis to estimate methylation levels at *Mlu*I and *Sac*II sites in the promoter (P1) of the gene *Xist* (as in **Fig. 2d**). Molecular weights (kb) are shown to the right. (**c**) Quantitative analysis of Southern blots in **b** showing methylated fraction as percentage of total. Solid and dashed lines highlight methylation levels in control PGK12.1 (PGK) cells. (**d**) Quantification of nearest-neighbor analysis showing percentage of total dCp that is methylated (mdCp) in control XY (129/1) and XX (PGK12.1) ES cells and in transgenic cell lines Dnmt3a (3a) d1 and Dnmt3b (3b) cl8 and cl10. (**e**) Southern-blot analysis of methylation levels in KvDMR1 and *Igf2r-Air* DMR (as in **Fig. 3b**,**c**) in control XY (129/1) and XX (PGK12.1) ES cells and in transgenic cell lines Dnmt3b (3b) cl8 and cl10.

X chromosome encodes a modifier locus whose product represses *de novo* methyltransferases. Cells with two active X chromosomes will overexpress the modifier and therefore have reduced levels of the enzymes. Additional factors may also be involved in genome-wide hypomethylation, particularly because the rate of loss of methylation at imprinted DMRs in parthenogenetic ES cells is much more rapid than the reported loss of methylation in ES cells lacking both Dnmt3a and Dnmt3b (refs. 7,14,19,20).

What are the possible implications of our findings? A first consideration is whether similar hypomethylation occurs in human XX ES cells. Such epigenetic instability would have important consequences for the use of ES cells in cell therapy. A second consideration is that both X chromosomes are active during certain stages in normal development. In these cases there are probably insufficient cell divisions to cause a marked reduction in CpG methylation, but more subtle effects could occur. Possibly related to this, Beckwith-Wiedeman syndrome associated with hypomethylation of the KvDMR1 occurs at a relatively high frequency in monozygotic twins, and in almost all cases, the affected twins are female[21,22]. It will be important to examine this connection further and also to determine whether female bias occurs in association with other diseases with an epigenetic component.

METHODS

Cell lines and mouse strains. ES cell lines PGK12.1, LF2, 129/1 and Efc-1, and standard methods for maintaining and differentiating ES cells, have been described in detail elsewhere[4,5]. Parthenogenetic ES cell lines were described previously[12]. B3 cells, a subline of PGK12.1 XX ES cells, and A11 cells, a line that arose in the course of deriving new ES cells, were both verified to be 39 X0 by karyotypic analysis and DNA FISH using X- and Y-linked probes. XX and XY somatic cell DNA was prepared from kidneys of (C57Bl6 × CBA) F_1 mice using standard methods.

We generated the Dnmt3a and Dnmt3a2 expression constructs by cloning their respective cDNAs into the pEF6-V5-His-TOPO vector (Invitrogen). The vector contains the human EF1α promoter for constitutive expression, and the cDNAs were expressed as C-terminal fusions with the V5 and polyhistidine tags. To make the Dnmt3b1 expression construct, we initially cloned the cDNA into pIND-V5-HISA plasmid for fusion with the V5 and polyhistidine tags and then cloned the fusion construct into the pCAG1-Puro expression vector (a gift from S. Sheardown; Glaxo Smith-Kline, Harlow, UK), which contains the chicken β-actin promoter for constitutive expression in mammalian cells. We prepared stable PGK12.1 derivative cell lines by transfection of the constructs using Lipofectamine 2000 (Invitrogen). We expanded blasticidin-resistant (Dnmt3a and Dnmt3a2) or puromycin-resistant (Dnmt3b) colonies to produce stable cell lines for further analysis.

Methylation assays. For Southern-blot assays, DNA was digested with appropriate restriction enzymes in accordance with the manufacturers' recommendations (New England Biolabs) and then separated by electrophoresis on 1% agarose gels and Southern blotted using standard procedures. We probed blots with radioactively labeled pGammasat (mouse major satellite repeats, gift from N. Dillon; MRC-CSC, London, UK), pMo (C-type retrovirus, gift from L. Lefebvre; Life Science Center, Vancouver, Canada), MG1 (ref. 4; *Xist* promoter), sc24 probe[23] (KvDMR1), a 330-bp PCR probe described previously[24] (*Igf2r-Air* DMR), a 385-bp probe from exons 1–3 of *Smc1l1* generated by PCR from genomic DNA (*Smc1l1*) or λ5 probe D (genomic DNA probe from the mouse λ5vpreB locus; gift from N. Dillon). Blots were exposed on a phosphorimager, and the results quantified using ImageQuant 5.1.

For bisulphite assays, we processed DNA samples using a bisulphite assay kit (Chemicon International) in accordance with the manufacturer's protocols. After nested PCR amplification, we cloned samples into the vector pCR-4-TOPO (Invitrogen) and sequenced them. For the *H19* DMR, we used PCR primers in regions a and b as described[25]. Other primer sequences are given in **Supplementary Table 1** online.

We carried out quantitative nearest-neighbor analysis as described previously[26].

Western-blot analysis. We prepared whole-cell extracts as described[27]. We separated proteins by electropheresis on SDS-PAGE (8% or 4–15% gradient) gels and wet-blotted them in 10 mM Tris, 8.3 mM NaAc and 0.5 mM EDTA (pH 7.8). We carried out western blotting using TBS (0.9% NaCl, 100 mM Tris). We obtained antibodies to Dnmt3a and Dnmt3b from Alexis Biochemicals. The Dnmt3a antibody, which recognizes Dnmt3a and Dnmt3a2 isoforms and also cross-reacts with Dnmt3b (ref. 28), was diluted 1:250. The Dnmt3b antibody recognizes all isoforms of Dnmt3b and was used at a 1:250 dilution. The Dnmt1 antibody was from Abcam (ab5208) and was diluted 1:10,500. LaminB antibody was from Santa Cruz and was diluted 1:2,000. We used secondary antibodies linked to horseradish peroxidase and carried out ECL detection (Amersham) in accordance with the manufacturer's recommendations.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Smith, A.G. Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.* **17**, 435–462 (2001).
2. Bird, A. DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6–21 (2002).
3. Sanford, J., Forrester, L., Chapman, V., Chandley, A. & Hastie, N. Methylation patterns of repetitive DNA sequences in germ cells of *Mus musculus*. *Nucleic Acids Res.* **12**, 2823–2836 (1984).
4. Norris, D.P. *et al.* Evidence that random and imprinted Xist expression is controlled by preemptive methylation. *Cell* **77**, 41–51 (1994).
5. Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S. & Brockdorff, N. Requirement for Xist in X chromosome inactivation. *Nature* **379**, 131–137 (1996).
6. Sado, T., Tada, T. & Takagi, N. Mosaic methylation of Xist gene before chromosome inactivation in undifferentiated female mouse embryonic stem and embryonic germ cells. *Dev. Dyn.* **205**, 421–434 (1996).
7. Jackson, M. *et al.* Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. *Mol. Cell. Biol.* **24**, 8862–8871 (2004).
8. Wutz, A. *et al.* Imprinted expression of the Igf2r gene depends on an intronic CpG island. *Nature* **389**, 745–749 (1997).
9. Fitzpatrick, G.V., Soloway, P.D. & Higgins, M.J. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat. Genet.* **32**, 426–431 (2002).
10. Robertson, E.J., Evans, M.J. & Kaufman, M.H. X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J. Embryol. Exp. Morphol.* **74**, 297–309 (1983).
11. Surani, M.A. *et al.* The inheritance of germline-specific epigenetic modifications during development. *Phil. Trans. R. Soc. Lond. B* **339**, 165–172 (1993).
12. Dean, W. *et al.* Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125**, 2273–2282 (1998).
13. Okano, M., Xie, S. & Li, E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.* **19**, 219–220 (1998).
14. Okano, M., Bell, D.W., Haber, D.A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99**, 247–257 (1999).
15. Bestor, T.H. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.* **11**, 2611–2617 (1992).
16. O'Neill, L.P. *et al.* X-linked genes in female embryonic stem cells carry an epigenetic mark prior to the onset of X inactivation. *Hum. Mol. Genet.* **12**, 1783–1790 (2003).
17. Tada, T. *et al.* Epigenotype switching of imprintable loci in embryonic germ cells. *Dev. Genes Evol.* **207**, 551–561 (1998).
18. Durcova-Hills, G., Burgoyne, P. & McLaren, A. Analysis of sex differences in EGC imprinting. *Dev. Biol.* **268**, 105–110 (2004).

19. Liang, G. *et al.* Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol. Cell. Biol.* **22**, 480–491 (2002).
20. Chen, T., Ueda, Y., Dodge, J.E., Wang, Z. & Li, E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol. Cell. Biol.* **23**, 5594–5605 (2003).
21. Lubinsky, M.S. & Hall, J.G. Genomic imprinting, monozygous twinning, and X inactivation. *Lancet* **337**, 1288 (1991).
22. Weksberg, R. *et al.* Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. *Hum. Mol. Genet.* **11**, 1317–1325 (2002).
23. Smilinich, N.J. *et al.* A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *Proc. Natl. Acad. Sci. USA* **96**, 8064–8069 (1999).
24. Durcova-Hills, G., Ainscough, J. & McLaren, A. Pluripotential stem cells derived from migrating primordial germ cells. *Differentiation* **68**, 220–226 (2001).
25. Warnecke, P.M., Mann, J.R., Frommer, M. & Clark, S.J. Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. *Genomics* **51**, 182–190 (1998).
26. Ramsahoye, B.H. Nearest-neighbor analysis. *Methods Mol. Biol.* **200**, 9–15 (2002).
27. Mermoud, J.E., Costanzi, C., Pehrson, J.R. & Brockdorff, N. Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. *J. Cell Biol.* **147**, 1399–1408 (1999).
28. Chen, T., Ueda, Y., Xie, S. & Li, E. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active *de novo* methylation. *J. Biol. Chem.* **277**, 38746–38754 (2002).