Slc38a4 acquires an imprinting mark[s] other than DNA methylation, which is required for the protection of the *Slc38a4* DMR from demethylation during preimplantation development but which is lost in somatic cells.

Gab1, *Sfmbt2* and *Slc38a4* may not require *de novo* DNA methylation for the establishment of imprinting (Table 1). *De novo* DNA methylation has already been shown to be dispensable for imprinting of the maternal X-chromosome (36), and *Xist* shows ectopic expression in nearly all cloned mouse embryos (16). It is interesting to speculate that the *de novo* DNA methylation-independent establishment of imprinting may be a common feature of imprinted genes showing consistent imprinting defects in cloned mice.

In conclusion, we found that *Gab1*, *Sfmbt2* and *Slc38a4* showed consistent loss of imprinting in cloned mice. It is likely that an imprinting mark[s] other than DNA methylation may be required for the establishment of imprinting of these genes. We also verified the correlation between loss of *Dlk1-Dio3* imprinting and embryonic lethality of cloned mice. These findings will have value both for elucidating the mechanisms involved in reprograming and also determining the potential risks of clinical applications of nuclear reprograming.

MATERIALS AND METHODS

Ethics statement

All animal experiments were performed at RIKEN Tsukuba Institute in accordance with the Animal Experimentation Committee's guiding principles.

SCNT

To generate [129xJF1]F1 mice, 129 females were mated with JF1 males. B6 females were mated with DBA males to generate [B6xDBA]F1 mice. Nuclear transfer was carried out with a Piezo-driven micromanipulator (PMM-150FU, Prime Tech, Ibaraki, Japan) as described in Wakayama *et al.* (37) and Ogura *et al.* (38). For the preparation of nuclear donor cells, cumulus cells were collected from female mice at 8–12 weeks of age, and Sertoli cells were collected from male mice at 1–9 days of age as described in Ogura *et al.* (38). Donor nuclei were injected into enucleated recipient oocytes collected from [B6xDBA]F1 female mice, and the reconstructed embryos were treated with 50 nM TSA (Sigma–Aldrich, St. Louis, MO, USA) for 8 h in total. To obtain the data shown in Supplementary Material, Figures S3B and S4G, the reconstructed embryos were not treated with TSA. For the analyses of term placentas, microinjection of *Xist*-siRNA was performed as described in Matoba *et al.* (18). After nuclear transfer, some embryos in the two-cell stage were transferred to pseudopregnant ICR mice, and others were collected at the blastocyst stage. [129xJF1]F1 embryos generated by conventional IVF (39) were used as controls for allelic expression and epigenetic analyses. Cloned embryos were delivered by cesarean section, and only placentas were collected and used for the analyses, shown in Figure 4E and Supplementary Material, Figure S4F. In the case that neonates at birth were alive, placentas were classified as 'placentas with viable fetuses'. In the case that fetuses were dead or absorbed, placentas were classified as 'placentas without viable fetuses'.

Whole transcriptome sequencing and data analysis

Total RNA was extracted from brains and placentas of E13.5 embryos. Four micrograms of total RNA was used for library construction using TruSeq RNA Sample Prep Kit v2 (Illumina, CA, USA) according to the manufacturer's protocol. Briefly, poly-A-containing mRNAs were purified using poly-T oligo-attached magnetic beads. The purified mRNAs were fragmented using divalent cations under elevated temperatures and then converted to dsDNA by two rounds of cDNA synthesis using reverse transcriptase and DNA polymerase I. After an end repair process, DNA fragments were ligated with adaptor oligos. The ligated products were amplified using 15 cycles of PCR to generate an RNA-seq library. Library integrity was verified by Bioanalyzer DNA1000 assay (Agilent Technologies, CA, USA). Sequencing was performed in 39-bp paired-end mode using a Genome Analyzer IIx (Illumina).

A total of 2 012365 798 reads were obtained for 25 samples. Sequenced reads were all filtered by the sequence quality score. If the average quality score of at least one of a paired read was <20, the paired read was discarded. Filtered reads were mapped to the reference mouse genome (UCSC mm9) by using Novoalign (V.2.07.13) (http://novocraft.com/) with the parameter '-r Random'. Mapping results were further processed using the Picard MarkDuplicate program (version 1.67) (http://picard.sourceforge.net/) to remove duplicate reads. Samples obtained from 129 and JF1 inbred mice were used for the identification of SNPs. SNP candidates were searched for with the The Genome Analysis Toolkit (GATK) (version 1.6–5) (40) according to its recommended procedure, and a total of 1 756 512 SNPs were identified. SNPs meeting the following criteria were used for further analysis: $\geq 90\%$ reads of 129-derived samples and $<10\%$ reads of JF1-derived samples identical to the reference sequence, or the opposite case.

Known imprinted genes were selected based on imprinted gene databases (http://igc.otago.ac.nz/ and http://www.har.mrc.ac.uk/). Imprinted genes annotated by RefSeq, except miRNAs and snoRNAs, were analyzed. Placenta-specific imprinted genes *Tfpi2*, *Tspan32*, *Cd81* and *Ano1* were excluded because these genes were highly expressed by maternal cells in the placenta. Only genes that had at least one SNP site with ≥ 10 reads in all brain or placental samples were used for the allelic expression analysis. For the analyses of allelic expression of imprinted genes, we used all mapped reads for all SNPs identified (including SNPs with <10 reads), and the numbers of paternal and maternal reads were summed for each gene. The maternal read number divided by the paternal read number (M/P ratio) was calculated. The maternal expression and paternal expression are defined as [M/P ratio] >2 and [M/P ratio] <0.5, respectively.

Preparation of embryos from *Dnmt3L*-deficient and oocyte-specific *Dnmt3a/3b*-deficient females

Production of mice with the conditional alleles, referred to as $Dnmt3a^{2lox}$ and $Dnmt3b^{2lox}$, was described previously (5,41). To disrupt the conditional alleles in growing oocytes, the mice were crossed with those carrying a *Zp3*-Cre gene (42). The precise timing of conditional deletion of *Dnmt3a* and *Dnmt3b* by *Zp3*-Cre is described elsewhere (43). By crossing [$Dnmt3a^{2lox/2lox}$, $Dnmt3b^{2lox/2lox}$, *Zp3*-Cre] females with wild-type

JF1 male mice, we obtained [*Dnmt3a*$^{-/+}$, *Dnmt3b*$^{-/+}$] E9.5 embryos. *Dnmt3L*-knockout female mice (44) were also crossed with wild-type JF1 male mice to obtain *Dnmt3L*$^{-/+}$ E9.5 embryos.

Real-time RT–PCR

Total RNA was prepared using an RNeasy mini Kit and RNase-free DNase (Qiagen, CA, USA). First-strand cDNA was synthesized from total RNA using PrimeScript II (Takara Bio, Shiga, Japan). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of target mRNA was determined from the appropriate standard curve and normalized to the amount of *β-actin* mRNA. The primer sets are shown in Supplementary Material, Table S3.

Analysis of allelic expression

PCR amplification was performed using KOD FX (TOYOBO, Osaka, Japan). PCR products were Sanger-sequenced, and the sequence chromatograms were analyzed with Sequencing Analysis Software v5.4 (Applied Biosystems, CA, USA). Multiple sequence alignments were done using GENETYX version 10.0.3 (GENETYX, Tokyo, Japan). For the quantification of expression alleles in Figure 4E, the peak heights of sequence chromatograms were used as expression levels. The allelic expression of *Rtl1* was analyzed using 3′ rapid amplification of cDNA ends (RACE) as previously reported (45). The primer sets and SNP positions are shown in Supplementary Material, Table S3.

Bisulfite sequencing

DNA samples were treated with sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, Orange, CA) and PCR-amplified using TaKaRa EpiTaq^{TM} HS (Takara Bio). The PCR products were cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA), and individual clones were sequenced. Primers used are listed in Supplementary Material, Table S3.

ChIP and SNuPE

ChIP analysis was performed using a Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA) according to the manufacturer's protocol. The following antibodies were used: dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The precipitated DNA was PCR-amplified, and the allelic histone modifications were analyzed using SNuPE. SNuPE analysis was performed using a SNaPshot Multiplex Kit (Applied Biosystems) according to the manufacturer's protocol. The peak height was determined using GeneMapper v4.1 (Applied Biosystems). Primers used are listed in Supplementary Material, Table S3.

Imprinted gene expression in bi-maternal placentas

Microarray data for three types of bi-maternal placentas (28) were used for the expression analysis presented in Table 1. For *Dlk1*, we used the data for embryos in which the *Igf2-H19* domain in the distal region on chromosome 7 was switched from the maternal to the paternal epigenotype. For *Igf2*, we used the data for embryos in which the *Dlk1-Dio3* domain in the distal region on chromosome 12 was switched from the maternal to the paternal epigenotype. For other imprinted genes, we used the data for embryos in which the both domains in the distal regions on chromosomes 7 and 12 were switched from the maternal to the paternal epigenotype.

5′ RACE

5′ RACE analysis of *Gab1* was performed using a CapFishing Full-length cDNA Isolation Kit (Seegene, Maryland, USA). The gene specific-primer sequence was 5′-GACTGGAGGCTGGTGCT GTACTTA-3′.

Accession numbers

All sequencing data are deposited in DDBJ Sequence Read Archive (DRA) under the accession number DRA000627.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank Ms N. Miyauchi, Ms F. Sato, Ms M. Tsuda, Ms N. Koshita, Ms M. Kikuchi and Mr K. Kuroda for technical assistance and Dr Rosalind M. John for support and valuable suggestions. The microarray data of bi-maternal placentas were kindly provided by Dr T. Kono and Mr F. Cao. We also thank the Biomedical Research Core of Tohoku University Graduate School of Medicine for technical support.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research (KAKENHI) from Japan Society for the Promotion of Science (22800084, 23220011, 23390385, 24613001 and 25670691), KAKENHI from the Ministry of Education, Culture, Sports, Science and Technology (20062010, 23013003 and 25112009) and the Takeda Science Foundation.

REFERENCES

1. Dean, W., Santos, F. and Reik, W. (2003) Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. *Semin. Cell Dev. Biol.*, **14**, 93–100.
2. Jouneau, A. and Renard, J.P. (2003) Reprogramming in nuclear transfer. *Curr. Opin. Genet. Dev.*, **13**, 486–491.
3. Yang, X., Smith, S.L., Tian, X.C., Lewin, H.A., Renard, J.P. and Wakayama, T. (2007) Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat. Genet.*, **39**, 295–302.
4. Surani, M.A. (1998) Imprinting and the initiation of gene silencing in the germ line. *Cell*, **93**, 309–312.
5. Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E. and Sasaki, H. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, **429**, 900–903.

6. Bourc'his, D., Xu, G.L., Lin, C.S., Bollman, B. and Bestor, T.H. (2001) Dnmt3l and the establishment of maternal genomic imprints. *Science*, **294**, 2536–2539.
7. Ferguson-Smith, A.C. (2011) Genomic imprinting: the emergence of an epigenetic paradigm. *Nat. Rev. Genet.*, **12**, 565–575.
8. Smith, F.M., Garfield, A.S. and Ward, A. (2006) Regulation of growth and metabolism by imprinted genes. *Cytogenet. Genome Res.*, **113**, 279–291.
9. Chavatte-Palmer, P., Camous, S., Jammes, H., Le Cleac'h, N., Guillomot, M. and Lee, R.S. (2012) Review: placental perturbations induce the developmental abnormalities often observed in bovine somatic cell nuclear transfer. *Placenta.*, **33**(Suppl), S99–S104.
10. Dinnyes, A., Tian, X.C. and Yang, X. (2008) Epigenetic regulation of foetal development in nuclear transfer animal models. *Reprod. Domest. Anim.*, **43**(Suppl 2), 302–309.
11. Whitworth, K.M. and Prather, R.S. (2010) Somatic cell nuclear transfer efficiency: how can it be improved through nuclear remodeling and reprogramming? *Mol. Reprod. Dev.*, **77**, 1001–1015.
12. de Waal, E., Yamazaki, Y., Ingale, P., Bartolomei, M.S., Yanagimachi, R. and McCarrey, J.R. (2012) Gonadotropin stimulation contributes to an increased incidence of epimutations in ICSI-derived mice. *Hum. Mol. Genet.*, **21**, 4460–4472.
13. Mann, M.R., Chung, Y.G., Nolen, L.D., Verona, R.I., Latham, K.E. and Bartolomei, M.S. (2003) Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol. Reprod.*, **69**, 902–914.
14. Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W.M. 3rd, Biniszkiewicz, D., Yanagimachi, R. and Jaenisch, R. (2001) Epigenetic instability in ES cells and cloned mice. *Science*, **293**, 95–97.
15. Ogawa, H., Ono, Y., Shimozawa, N., Sotomaru, Y., Katsuzawa, Y., Hiura, H., Ito, M. and Kono, T. (2003) Disruption of imprinting in cloned mouse fetuses from embryonic stem cells. *Reproduction*, **126**, 549–557.
16. Inoue, K., Kohda, T., Sugimoto, M., Sado, T., Ogonuki, N., Matoba, S., Shiura, H., Ikeda, R., Mochida, K., Fujii, T. *et al.* (2010) Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. *Science*, **330**, 496–499.
17. Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F. and Ogura, A. (2002) Faithful expression of imprinted genes in cloned mice. *Science*, **295**, 297.
18. Matoba, S., Inoue, K., Kohda, T., Sugimoto, M., Mizutani, E., Ogonuki, N., Nakamura, T., Abe, K., Nakano, T., Ishino, F. *et al.* (2011) RNAi-mediated knockdown of Xist can rescue the impaired postimplantation development of cloned mouse embryos. *Proc. Natl. Acad. Sci. USA*, **108**, 20621–20626.
19. Okae, H., Hiura, H., Nishida, Y., Funayama, R., Tanaka, S., Chiba, H., Yaegashi, N., Nakayama, K., Sasaki, H. and Arima, T. (2012) Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression. *Hum. Mol. Genet.*, **21**, 548–558.
20. Wilkinson, L.S., Davies, W. and Isles, A.R. (2007) Genomic imprinting effects on brain development and function. *Nat. Rev. Neurosci.*, **8**, 832–843.
21. Mizuno, Y., Sotomaru, Y., Katsuzawa, Y., Kono, T., Meguro, M., Oshimura, M., Kawai, J., Tomaru, Y., Kiyosawa, H., Nikaido, I. *et al.* (2002) Asb4, Ata3, and Dcn are novel imprinted genes identified by high-throughput screening using RIKEN cDNA microarray. *Biochem. Biophys. Res. Commun.*, **290**, 1499–1505.
22. Smith, R.J., Dean, W., Konfortova, G. and Kelsey, G. (2003) Identification of novel imprinted genes in a genome-wide screen for maternal methylation. *Genome Res.*, **13**, 558–569.
23. Kobayashi, H., Sakurai, T., Imai, M., Takahashi, N., Fukuda, A., Yayoi, O., Sato, S., Nakabayashi, K., Hata, K., Sotomaru, Y. *et al.* (2012) Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. *PLoS Genet.*, **8**, e1002440.
24. da Rocha, S.T., Edwards, C.A., Ito, M., Ogata, T. and Ferguson-Smith, A.C. (2008) Genomic imprinting at the mammalian Dlk1-Dio3 domain. *Trends Genet.*, **24**, 306–316.
25. Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R. and Shiota, K. (2001) Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol. Reprod.*, **65**, 1813–1821.
26. Yevtodiyenko, A. and Schmidt, J.V. (2006) Dlk1 expression marks developing endothelium and sites of branching morphogenesis in the mouse embryo and placenta. *Dev. Dyn.*, **235**, 1115–1123.
27. Ogura, A., Inoue, K., Ogonuki, N., Lee, J., Kohda, T. and Ishino, F. (2002) Phenotypic effects of somatic cell cloning in the mouse. *Cloning Stem Cells*, **4**, 397–405.
28. Kawahara, M., Morita, S., Takahashi, N. and Kono, T. (2009) Defining contributions of paternally methylated imprinted genes at the Igf2-H19 and Dlk1-Gtl2 domains to mouse placentation by transcriptomic analysis. *J. Biol. Chem.*, **284**, 17751–17765.
29. Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T. and Hochedlinger, K. (2010) Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature*, **465**, 175–181.
30. Carey, B.W., Markoulaki, S., Hanna, J.H., Faddah, D.A., Buganim, Y., Kim, J., Ganz, K., Steine, E.J., Cassady, J.P., Creyghton, M.P. *et al.* (2011) Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells. *Cell Stem Cell*, **9**, 588–598.
31. Boiani, M., Eckardt, S., Scholer, H.R. and McLaughlin, K.J. (2002) Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.*, **16**, 1209–1219.
32. Cattanach, B.M., Beechey, C.V. and Peters, J. (2004) Interactions between imprinting effects in the mouse. *Genetics*, **168**, 397–413.
33. Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M. and Hirano, T. (2000) Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell. Biol.*, **20**, 3695–3704.
34. Constancia, M., Angiolini, E., Sandovici, I., Smith, P., Smith, R., Kelsey, G., Dean, W., Ferguson-Smith, A., Sibley, C.P., Reik, W. *et al.* (2005) Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proc. Natl. Acad. Sci. USA*, **102**, 19219–19224.
35. Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E.S., Golub, T.R. and Jaenisch, R. (2002) Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc. Natl. Acad. Sci. USA*, **99**, 12889–12894.
36. Chiba, H., Hirasawa, R., Kaneda, M., Amakawa, Y., Li, E., Sado, T. and Sasaki, H. (2008) De novo DNA methylation independent establishment of maternal imprint on X chromosome in mouse oocytes. *Genesis*, **46**, 768–774.
37. Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, **394**, 369–374.
38. Ogura, A., Inoue, K., Ogonuki, N., Noguchi, A., Takano, K., Nagano, R., Suzuki, O., Lee, J., Ishino, F. and Matsuda, J. (2000) Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. *Biol. Reprod.*, **62**, 1579–1584.
39. Mochida, K., Ohkawa, M., Inoue, K., Valdez, D.M. Jr, Kasai, M. and Ogura, A. (2005) Birth of mice after in vitro fertilization using C57BL/6 sperm transported within epididymides at refrigerated temperatures. *Theriogenology*, **64**, 135–143.
40. DePristo, M.A., Banks, E., Poplin, R., Garimella, K.V., Maguire, J.R., Hartl, C., Philippakis, A.A., del Angel, G., Rivas, M.A., Hanna, M. *et al.* (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.*, **43**, 491–498.
41. Dodge, J.E., Okano, M., Dick, F., Tsujimoto, N., Chen, T., Wang, S., Ueda, Y., Dyson, N. and Li, E. (2005) Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. *J. Biol. Chem.*, **280**, 17986–17991.
42. de Vries, W.N., Binns, L.T., Fancher, K.S., Dean, J., Moore, R., Kemler, R. and Knowles, B.B. (2000) Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis*, **26**, 110–112.
43. Kaneda, M., Hirasawa, R., Chiba, H., Okano, M., Li, E. and Sasaki, H. (2010) Genetic evidence for Dnmt3a-dependent imprinting during oocyte growth obtained by conditional knockout with Zp3-Cre and complete exclusion of Dnmt3b by chimera formation. *Genes Cells*, **15**, 169–179.
44. Hata, K., Okano, M., Lei, H. and Li, E. (2002) Dnmt3l cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development*, **129**, 1983–1993.
45. Sekita, Y., Wagatsuma, H., Irie, M., Kobayashi, S., Kohda, T., Matsuda, J., Yokoyama, M., Ogura, A., Schuster-Gossler, K., Gossler, A. *et al.* (2006) Aberrant regulation of imprinted gene expression in Gtl2lacZ mice. *Cytogenet. Genome Res.*, **113**, 223–229.