Dnmt3L and the Establishment of Maternal Genomic Imprints

Déborah Bourc'his,[1] Guo-Liang Xu,[1]* Chyuan-Sheng Lin,[2] Brooke Bollman,[1]† Timothy H. Bestor[1]‡

Complementary sets of genes are epigenetically silenced in male and female gametes in a process termed genomic imprinting. The *Dnmt3L* gene is expressed during gametogenesis at stages where genomic imprints are established. Targeted disruption of *Dnmt3L* caused azoospermia in homozygous males, and heterozygous progeny of homozygous females died before midgestation. Bisulfite genomic sequencing of DNA from oocytes and embryos showed that removal of Dnmt3L prevented methylation of sequences that are normally maternally methylated. The defect was specific to imprinted regions, and global genome methylation levels were not affected. Lack of maternal methylation imprints in heterozygous embryos derived from homozygous mutant oocytes caused biallelic expression of genes that are normally expressed only from the allele of paternal origin. The key catalytic motifs characteristic of DNA cytosine methyltransferases have been lost from Dnmt3L, and the protein is more likely to act as a regulator of imprint establishment than as a DNA methyltransferase.

Genomic imprinting imposes a requirement for biparental reproduction. Uniparental mammalian conceptuses are inviable (*1*–*3*), and lack of imprinting of specific chromosomes or chromosome segments as a result of uniparental isodisomy or deletions of imprinting centers causes distinct developmental defects according to the chromosomal region involved (*4*, *5*). The poor success rate and unpredictable phenotypic variation seen in mammals produced by cloning procedures are also likely to involve disturbances of genomic imprints (*6*). However, very little is known about the mechanisms that establish and maintain genomic imprints.

Maternal imprints are established in growing diplotene oocytes and paternal imprints in perinatal prospermatogonia (*7*–*10*). In many cases imprinting involves the de novo methylation of regulatory regions of affected genes (*4*); these methylation marks are maintained throughout development and are only erased and reestablished in the germ line. The DNA methyltransferases and regulatory factors involved in the establishment of imprints in germ cells have not been identified. Dnmt3L (*11*) became a candidate for such an activity on the basis of sequence similarity to Dnmt3A and Dnmt3B, which have been shown to catalyze de novo methylation (*12*). However, Dnmt3L lacks the sequence motifs shown to be involved in activation of the target cytosine, binding of the methyl donor *S*-adenosyl L-methionine, and sequence recognition (*13*). Similarity of Dnmt3L to Dnmt3A and Dnmt3B is largely restricted to a cysteine-rich region of unknown function and regions between catalytic motifs (Fig. 1A).

Dnmt3L was disrupted by homologous recombination in mouse embryonic stem (ES) cells by means of a deletion-replacement mutation that removed four exons and inserted a β-galactosidase–neomycin phosphotransferase (β-geo) fusion gene (*14*) under the control of the endogenous *Dnmt3L* promoter (Fig. 1, B and C). The disrupted allele was termed *Dnmt3L*^{G}. Heterozygous *Dnmt3L*^{G} mice were of normal phenotype and showed high-level expression of the β-geo marker exclusively in the cell types in which genomic imprints are established (*7*–*10*): growing oocytes in adult females and prospermatogonia in perinatal males (Fig. 2, A and B). Homozygous animals of both sexes were viable and of normal visible phenotype, but both sexes were sterile. Testes of homozygous *Dnmt3L*^{G} animals contained normal complements of germ cells at birth, but adult testes had severe hypogonadism and Sertoli cell–only phenotype (Fig. 2, C to E).

Oogenesis was normal in homozygous *Dnmt3L*^{G} females, but the mutation behaved as a maternal-effect lethal in that heterozygous progeny of homozygous females failed to develop past 9.5 days postcoitum (dpc). The most notable anatomical abnormalities within the embryo proper were pericardial edema with exencephaly and other neural tube defects (Fig. 3A). These defects, together with death at midgestation, are common consequences of abnormalities of extraembryonic tissues (*15*). In the case of heterozygous progeny of homozygous *Dnmt3L*^{G} females these included a failure of chorio-allantoic fusion (Fig. 3, C and D), hyperproliferation of secondary trophoblastic giant cells and overgrowth of the chorion, hyperproliferation of yolk sac endoderm, and excess maternal blood in the vicinity of the ectoplacental cone (Fig. 3D). The defects were not due to uterine environment effects, because a similar phenotype was seen when heterozygous progeny of homozygous females were trans-

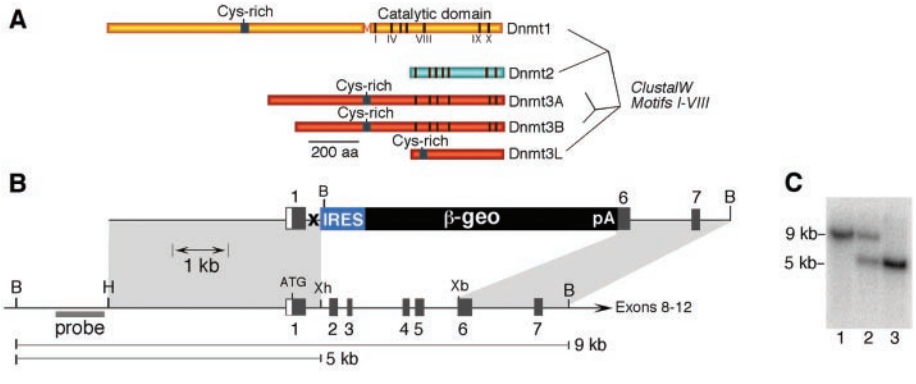


Fig. 1. Relationship of Dnmt3L to other mammalian DNA methyltransferases and disruption of *Dnmt3L* gene. (**A**) Sequence relationships among mammalian DNA methyltransferases. Catalytic motifs are designated with roman numerals. Motifs are absent from Dnmt3L, whereas the cysteine-rich regions and other sequences show strong similarities with Dnmt3A and Dnmt3B. At right is a ClustalW representation of sequence similarities within the region spanning catalytic motifs I to VIII. The corresponding region of Dnmt3L was identified by alignment with Dnmt3A and Dnmt3B. (**B**) Disruption of the Dnmt3L gene by homologous recombination in ES cells. Methods were as described (*25*, *27*), except that CSL3 ES cells derived from blastocysts of strain 129SvEv/Tac were used. The disruption brings the β-geo reporter/resistance gene under the control of the endogenous *Dnmt3L* promoter. The X 3′ of exon 1 indicates the site of three in-frame stop codons; the polyadenylation signal prevents expression of downstream exons. Restriction endonuclease sites: B, Bam HI; H, Hind III; Xh, Xho I; Xb, Xba I. DNA blot hybridization (**C**) after cleavage of ES cell DNA with Bam HI confirmed that the expected homologous recombination event had taken place. The mutant allele was designated *Dnmt3L*^{G}.

[1]Department of Genetics and Development, [2]Transgenic Animal Facility, Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA.

*Present address: Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China.

†Present address: Department of Biological Sciences, Dartmouth College, Hanover, NH, USA.

‡To whom correspondence should be addressed. E-mail: thb12@columbia.edu

ferred to oviducts of wild-type foster females (Fig. 3B).

A role for Dnmt3L in the establishment of genomic imprints was suggested by the specific expression in germ cells at stages where imprints are established, the sequence affinities with known DNA methyltransferases, and the maternal-effect phenotype. Bisulfite genomic sequencing (*16*) of the differentially methylated region (DMR) of the imprinted and maternally repressed *Snrpn* gene (*17*) revealed that the DMR was heavily methylated at all tested sites in DNA of control oocytes but was markedly undermethylated in oocytes of homozygous *Dnmt3L*^{G} females (Fig. 4A). DNA blot hybridization after cleavage with the methylation-sensitive restriction endonuclease Hha I was used to determine whether the methylation deficiency present in the oocyte persisted on the maternal allele in progeny derived from crosses to wild-type males. Neither allele of *Snrpn* was detectably methylated in such heterozygous embryos (Fig. 4B). Bisulfite genomic sequencing showed that one-half of the alleles of the imprinted genes *H19*, *Snrpn*, and *Peg1* were methylated in DNA of control embryos. *H19*, one of the rare genes whose maternal expression is enforced by methylation of the paternal allele (*18*, *19*), showed normal allele-specific methylation in heterozygous progeny of homozygous females, whereas the maternally imprinted *Snrpn* and *Peg1* genes were unmethylated on both alleles (Fig. 4C). These results showed that Dnmt3L is required for the establishment of maternal methylation imprints during oogenesis, and that a maternal store of Dnmt3L is not required for the maintenance of paternal methylation imprints. Global genome methylation (the large majority of which resides in repeated sequences) was not notably reduced in DNA of heterozygous progeny of homozygous *Dnmt3L*^{G} mutant females (Fig. 4B), and demethylation imposed by Dnmt3L deficiency during oogenesis was largely restricted to the DMRs of maternally imprinted genes.

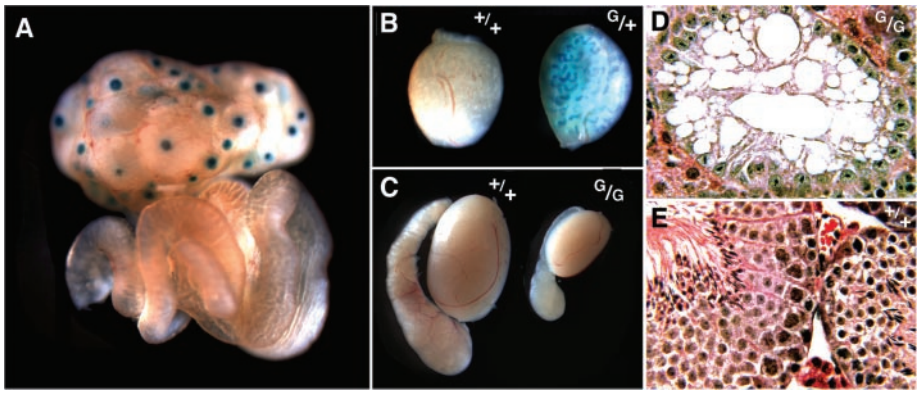


Fig. 2. Expression of *Dnmt3L* gene in male and female germ cells and sterility of homozygous *Dnmt3L*^{G} males. (**A**) Accumulation of β-geo reporter under control of the *Dnmt3L* promoter specifically in growing oocytes as assessed by staining adult ovaries with X-Gal. Growing oocytes at all stages are stained, but primary oocytes and somatic cells are unstained. The oviduct is at bottom. (**B**) Transcription from *Dnmt3L* promoter in seminiferous tubules of fetal testis. Staining for β-geo with X-Gal was as in (A). (Left) Wild-type testis from 17.5 dpc mouse fetus; (right) testis from heterozygous littermate. Staining within seminiferous tubules was present in prospermatogonia (*29*). Postpartum and adult testes showed much less β-Gal activity (*29*). (**C**) Hypogonadism in homozygous *Dnmt3L*^{G} testes. Testis of wild-type littermate is at left. (**D**) Sertoli cell–only phenotype in seminiferous tubules of homozygous *Dnmt3L*^{G} adult males. The tubule lumen is occupied only by cytoplasmic processes of Sertoli cells. (**E**) Section of seminiferous tubule from wild-type littermate.

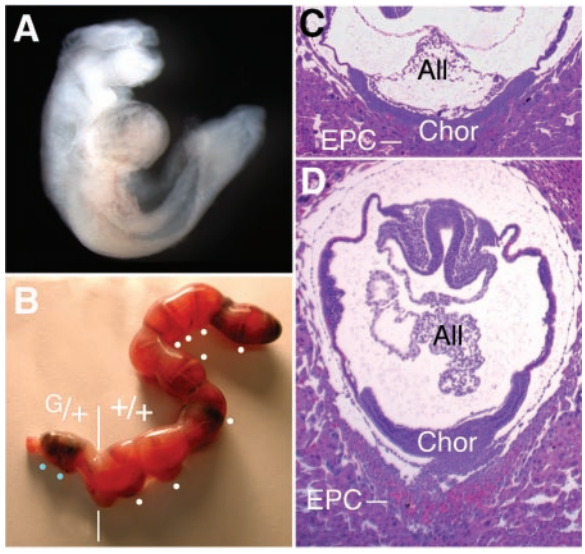


Fig. 3. Maternal-effect lethal phenotype in heterozygous embryos derived from homozygous *Dnmt3L*^{G} females. (**A**) Exencephalic embryo at 9.5 dpc. (**B**) Developmental failure of heterozygous progeny of homozygous *Dnmt3L*^{G} females when transferred to uteri of wild-type females. The left uterine horn received 15 mutant embryos; the right horn received 15 wild-type control embryos. At 13.5 dpc mutant conceptuses are represented only by necrotic implantation sites (blue spots at left), whereas development of wild-type conceptuses (white spots) in the right horn is normal. (**C**) Normal chorio-allantoic fusion in a wild-type conceptus at 8.5 dpc. (**D**) Abnormalities of extraembryonic structures and failure of chorio-allantoic fusion in a heterozygous *Dnmt3L*^{G} 9.5 dpc embryo derived from a homozygous oocyte. Note the abnormal separation of allantois (All) and chorion (Chor) by comparison with (C), thickened chorion, and hyperproliferation of secondary trophoblastic giant cells in ectoplacental cone (EPC) and of yolk sac endoderm (lateral to allantois). Excess maternal blood apposed to ectoplacental cone is also apparent.

The effect of abnormal methylation imprints on imprinted gene expression was tested in embryos derived from crosses of *Mus musculus* females homozygous for *Dnmt3L*^{G} to wild-type *M. m. castaneus* (CAST) males or to a strain in which CAST chromosome 7 had been introgressed into a C57BL/6J strain background to improve breeding efficiency (*20*). Expressed polymorphisms allowed assignment of the parental origin of transcripts (*20*). As shown in Fig. 5, *Snrpn*, *Necdin*, *Zfp127*, *Kcnq1ot1*, and *Peg3* were transcribed from both alleles in heterozygous *Dnmt3L*^{G} progeny of homozygous females but only from the paternal allele in control *M. musculus* × CAST embryos. The paternally methylated and imprinted *H19* gene remained maternally expressed, in agreement with the retention of paternal *H19* methylation seen in Fig. 4C. *Igf2*, which is normally maternally repressed (*3*), retained paternal-specific expression as predicted by unperturbed *H19* imprinting (*4*, *21*). *Cdkn1* and *Ipl* were not expressed from either allele, as assessed by reverse transcription–polymerase chain reaction (PCR) with specific primers and an internal Necdin control (Fig. 5B). This is likely due to repression of the maternal allele of *Cdkn1* and *Ipl* as a result of reactivation of the maternal allele of the nearby *Kcnq1ot1* gene, which is predicted to repress in cis the active allele of other imprinted genes in the cluster (*22*–*24*). Development of heterozygous *Dnmt3L*^{G} embryos is most similar to that of embryos reconstituted from a normal sperm nucleus and a haploid nucleus derived from a nongrowing oocyte, which lacks both maternal and paternal imprints (*7*).

Mutations in *Dnmt3L* and each of the known DNA methyltransferases produce distinct phenotypes. Deletion of the somatic form of *Dnmt1* causes global genome demethylation with dysregulation of imprinted genes and ectopic X-chromosome inactivation (*25*, *26*), whereas deletion of the oocyte-specific form of *Dnmt1* causes a pure maternal-effect phenotype in which

one-half of the normally silent alleles of certain imprinted genes are demethylated and reactivated in heterozygous progeny of homozygous females (*27*). Mutations in *Dnmt3B* prevent the methylation of specific types of pericentric satellite DNA and cause the human immunodeficiency and chromosome instability disease known as ICF syndrome (*28*). Although demethylation has not been reported to occur in Dnmt3A-deficient cells, *Dnmt3A- Dnmt3B* double-mutant ES cells have been reported to undergo

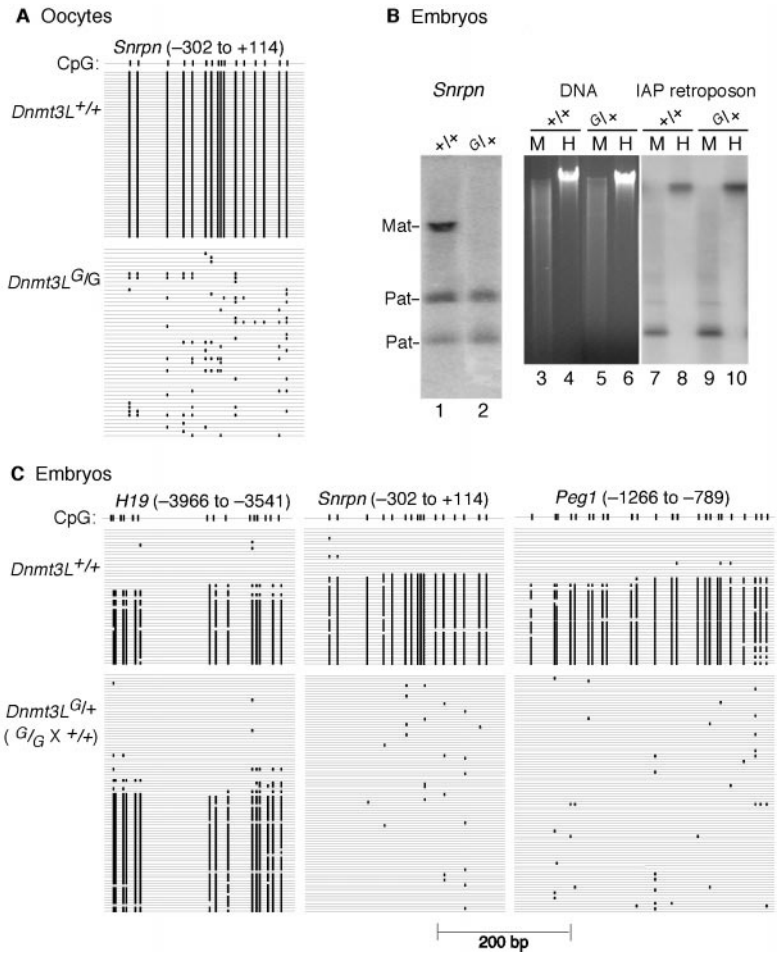
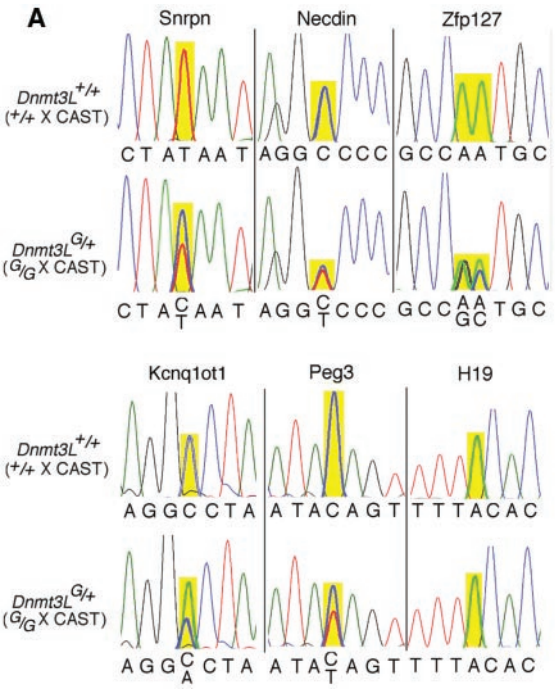


Fig. 4. Lack of maternal methylation imprints in homozygous *Dnmt3L*^{G} oocytes and in heterozygous embryos derived from them. (**A**) Methylation of all CpG dinucleotides in the DMR of *Snrpn* in control embryos and light and variable methylation of the same sequence in homozygous *Dnmt3L*^{G} oocytes. (**B**) Lanes 1 and 2: lack of methylation at Hha I sites in the DMR of the maternal allele of *Snrpn* in heterozygous embryos derived from homozygous *Dnmt3L*^{G} oocytes. DNA was digested with Hha I and Nde I. Lanes 3 to 6: global genome methylation was not decreased in heterozygous embryos derived from homozygous *Dnmt3L*^{G} oocytes, as assessed by sensitivity to Hpa II (lanes 4 and 6). Lanes 3 and 5 contained Msp I digests. Lanes 7 to 10, IAP retroposons (*30*) and pericentric satellite DNA (*29*) are not demethylated in heterozygous embryos derived from homozygous *Dnmt3L*^{G} oocytes. In all cases DNA was purified from embryos at 8.5 dpc. Samples marked G/+ were from heterozygous progeny of homozygous *Dnmt3L*^{G} females. (**C**) Loss of allele-specific methylation at the maternally imprinted genes *Snrpn* and *Peg1* with normal monoallelic methylation at the paternally imprinted *H19* gene. Controls show monoallelic methylation for all tested sequences [upper portion of (C)].



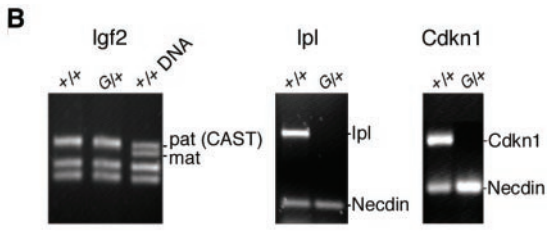


Fig. 5. Biallelic expression of maternally imprinted genes in heterozygous progeny of homozygous *Dnmt3L*^{G} females. Expressed polymorphisms were introduced by crossing homozygous *Dnmt3L*^{G} females to *M. m. castaneus* males or to males containing *M. m. castaneus* chromosome 7 on a C57BL/6J strain background (*20*). Regions containing the polymorphisms were recovered by RT-PCR and the allele-specific expression determined by direct sequencing of the products in (A) or by agarose gel electrophoresis in (B). (**A**) *Snrpn*, *Necdin*, *Zfp127*, *Kcnq1ot1*, and *Peg3* were expressed only from the paternal allele in control embryos (top row) but were expressed from both alleles in heterozygous progeny of homozygous *Dnmt3L*^{G} females (bottom row). *H19* remained imprinted and was expressed only from the maternal allele, as predicted from the methylation data of Fig. 4. (**B**) Expression of *Igf2* remained monoallelic and paternal, in agreement with the retention of imprinted expression of *H19* and the reciprocal imprinting of *Igf2* and *H19* (*4*). The *Cdkn1* and *Ipl* genes were silenced biallelically as a result of reactivation of maternal *Kcnq1ot1* transcription (*22–24*). Polymorphisms are described in (*31*). Samples marked G/+ were from heterozygous progeny of homozygous *Dnmt3L*^{G} females.

global genome demethylation and a loss of the ability to methylate newly integrated retroviral DNA (*12*). Dnmt3L is required specifically for the establishment of genomic imprints but is dispensable for their propagation, and *Dnmt3L* is the only gene known to be essential for the de novo methylation of single-copy DNA sequences. The results of this and prior studies (*27*) confirm that the methylation of single-copy sequences and repeated sequences are independently regulated. The sequence of Dnmt3L suggests that the protein is likely to function not directly as a DNA methyltransferase but as a regulator of methylation at imprinted loci, and identification and characterization of germ cell factors that interact with Dnmt3L should lead to a better understanding of the mechanisms that establish genomic imprints.

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31. GenBank accession codes and expressed polymorphisms are as follows, with the *M. musculus* allele preceding and the *M. m. castaneus* allele following nucleotide position: Snrpn: MMSMM, C915T; Kcnq1ot1: AF119385, T3976G; Zfp127: MMU19106, G1544AATGCCT; Ndn: MUSNECDIN, T117C; Peg3: AF038939, A3451G; Igf2: MMU71085, $(CA)_{18}25435(CA)_{21}$. Allele-specific expression of Igf2 was assessed by RT-PCR followed by digestion of the product with Nde I and Hae III and then separation of the fragments by agarose gel electrophoresis. H19 polymorphism was as described in (*21*). Sequences of PCR primers are available on request.
32. We are grateful to M. Mann and M. Bartolomei for providing unpublished data on expressed polymorphisms in imprinted genes and to J. R. Chaillet and M. Bartolomei for providing mice bearing CAST chromosome 7 on the C57BL/6J strain background. We also thank K. Anderson and M. Goll for comments on the manuscript, K. Hadjantonakis and V. E. Papaioannou for advice and discussions, U. Beauchamp for DNA sequencing, and J. R. Chaillet for probes. Supported by NIH grants GM59377 and HD37687 (T.H.B.). D.B. was supported by a fellowship from the Bourse Lavoisier du Ministère des Affaires Etrangères.

29 August 2001; accepted 7 November 2001
Published online 22 November 2001;
10.1126/science.1065848
Include this information when citing this paper.

Requirement of Heterochromatin for Cohesion at Centromeres

Pascal Bernard,[1]* Jean-François Maure,[2]* Janet F. Partridge,[1] Sylvie Genier,[2] Jean-Paul Javerzat,[2] Robin C. Allshire[1]†

Centromeres are heterochromatic in many organisms, but the mitotic function of this silent chromatin remains unknown. During cell division, newly replicated sister chromatids must cohere until anaphase when Scc1/Rad21–mediated cohesion is destroyed. In metazoans, chromosome arm cohesins dissociate during prophase, leaving centromeres as the only linkage before anaphase. It is not known what distinguishes centromere cohesion from arm cohesion. Fission yeast Swi6 (a Heterochromatin protein 1 counterpart) is a component of silent heterochromatin. Here we show that this heterochromatin is specifically required for cohesion between sister centromeres. Swi6 is required for association of Rad21-cohesin with centromeres but not along chromosome arms and, thus, acts to distinguish centromere from arm cohesion. Therefore, one function of centromeric heterochromatin is to attract cohesin, thereby ensuring sister centromere cohesion and proper chromosome segregation.

Before anaphase onset, each duplicated chromosome must be bilaterally attached to the mitotic spindle. This is achieved by sister centromeres and their associated kinetochores attaching to microtubules that emanate from opposite spindle poles. Accurate chromosome segregation requires that sister chromatids remain associated until all chromosomes have bilaterally attached to the spindle; only then can anaphase ensue. Sister chromatid cohesion is mediated by a conserved protein complex, known as cohesin (*1*). Anaphase is triggered by the cleavage of the Scc1/Rad21 subunit of cohesin allowing sister chromatid separation (*2*). In most organisms, cohesin is concentrated at centromeric regions (*3*–*7*). Metaphase chromosome spreads suggest that centromeres have a specialized role in holding sister chromatids together. Indeed, mammalian and fly cohesin is retained only at centromeric regions until anaphase (*6*, *7*). What distinguishes cohesion at centromeres from cohesion along chromosome arms? The integrity of centromeric heterochromatin is known to be important for normal chromosome segregation, although its role has not been elucidated (*8*). One possibility is that heterochromatin maintains cohesion between sister centromeres. Several observations suggest that heterochromatin may play a role in sister chromatid cohesion (*9*, *10*). Here we demonstrate that the high concentration of cohesin and, thus, cohesion at centromeres is an intrinsic property of the underlying heterochromatin.

Schizosaccharomyces pombe (fission yeast) centromeres contain two distinct silenced chromatin domains composed of different proteins (*11*). Swi6 coats the outer repeat regions, whereas Mis6 and Cnp1 (the homolog of CENP-A) are restricted to the central domain (Fig. 1A). Mutations affecting these proteins alleviate transcriptional repression of a marker gene inserted only within their respective domains (*11*–*13*). Mutations or conditions that disrupt silencing over the outer repeats lead to a high incidence of lagging chromosomes on late anaphase spindles (*14*–*16*).

Recently, it has been shown that Rad21 strongly associates with the outer repeat regions (*5*, *17*). To test for a link between outer repeat chromatin and cohesion, a strain was used in which Swi6 synthesis was driven by the repressible *nmt1* promoter (*18*). Growth in the presence of thiamine represses *swi6*$^+$ expression. Although Swi6 alone is not essential for cell viability, its withdrawal from *rad21-K1* cells (conditionally defective in cohesin function) (*5*) results in loss of viability (Fig. 1, B and C). Synthetic lethality often indicates functional

[1]MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK. [2]Institut de Biochimie et Génétique Cellulaires, CNRS, Unité Mixte de Recherche 5095, 1 Rue Camille Saint Saëns, 33077 Bordeaux Cedex, France.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: robin.allshire@hgu.mrc.ac.uk