Oncogene (2008) 27, 404–408

www.nature.com/onc

SHORT COMMUNICATION

Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice

G Howard[1], R Eiges[1], F Gaudet[2,3,4], R Jaenisch[2,3] and A Eden[1]

[1]*Department of Animal and Cell Biology, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel;* [2]*Whitehead Institute for Biomedical Research, Cambridge, MA, USA and* [3]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA*

Genomewide DNA hypomethylation is a consistent finding in human tumors, but the importance of this change for human tumorigenesis remains an open question. We have previously reported that mice carrying a hypomorphic allele for the maintenance DNA methyltransferase (*Dnmt1*$^{chip/-}$) are hypomethylated and develop thymic lymphomas, demonstrating that genomewide DNA hypomethylation can induce tumors. Hypomethylated cells exhibit inherent chromosomal instability, which is revealed in the lymphomas as a consistent trisomy of chromosome 15. We now report another aspect of the molecular basis for tumor development upon DNA hypomethylation. Seven out of 16 hypomethylation-induced lymphomas were found to contain an intracisternal A particle (IAP) somatic insertion in the middle of the *Notch1* genomic locus, leading to generation of an oncogenic form of Notch1 in the tumors. This finding suggests that the molecular basis for hypomethylation-induced tumors in this model involves chromosomal instability events accompanied by activation of endogenous retroviral elements. Our findings validate the proposed role of DNA methylation in suppression of transposable elements in mammalian cells and demonstrate the importance of DNA methylation for normal cell function as well as the potential consequences of spontaneously occurring or chemically induced DNA hypomethylation.
Oncogene (2008) **27,** 404–408; doi:10.1038/sj.onc.1210631; published online 9 July 2007

Keywords: DNA hypomethylation; transposable elements; Notch1; T-cell lymphoma

Proper methylation of genomic DNA is essential for normal development and for survival of somatic cells (Li *et al*., 1992; Jackson-Grusby *et al*., 2001). Human tumors display abnormal methylation patterns including genomewide hypomethylation and site-specific hypermethylation (Ehrlich, 2002; Jones and Baylin, 2002). To understand the effects of genomewide hypomethylation and the significance of this change in cancer, we developed a hypomorphic allele for the maintenance of DNA methyltransferase (*Dnmt1*^{Chip}) (Gaudet *et al*., 2003). Compound heterozygous mice carrying the hypomorphic allele over a null allele (*Dnmt1*$^{chip/-}$) are viable, but are hypomethylated and develop thymic lymphomas with a characteristic trisomy of chromosome 15 (Gaudet *et al*., 2003).

To understand further the molecular basis of hypomethylation-induced tumors, we compared the gene expression profile in thymic lymphomas to that of wild-type (wt) thymus. Interestingly, among the most significantly overexpressed genes were *c-myc* and *Notch1*. Upregulation of these genes was verified by northern blot analysis (Figure 1). Both genes have been shown to act as oncogenes and to cooperate in human and mouse T-cell lymphomas (Ellisen *et al*., 1991; Girard *et al*., 1996; Zweidler-McKay and Pear, 2004). Oncogenic activity of Myc is frequently the result of increase in gene copy number (amplification) and elevated expression level (reviewed in Nesbit *et al*., 1999). Using Southern blot analysis, we did not observe any drastic amplification of *c-myc* (data not shown). However, in mice, *c-myc* resides on chromosome 15. Thus, the consistent trisomy observed in hypomethylation-induced tumors may account for the oncogenic contribution of *c-myc*. Overexpression of *Notch1* was also confirmed by northern blot. Intriguingly, in addition to the normal full-length *Notch1* transcript, the hypomethylation-induced tumors displayed additional shorter transcripts of variable size (Figure 1). Oncogenic activation of *Notch1* often involves retroviral insertions or chromosomal translocations, which introduce an ectopic promoter to the region, and drive the expression of a truncated protein product, comprising the intracellular portion of the protein (Ellisen *et al*., 1991; Girard *et al*., 1996; Zweidler-McKay and Pear, 2004). We therefore searched the *Notch1* region for evidence of genomic rearrangements. Southern blot analysis revealed genetic changes in several tumors (Figure 2). Further Southern blot analysis mapped the changes to introns between exons D and F of *Notch1*. We applied inverse PCR (iPCR; Suzuki *et al*., 2002) to clone and sequence the mutated site. The sequence

Correspondence: Dr A Eden, Department of Animal and Cell Biology, Institute of Life Sciences, The Hebrew University, Givat-Ram, Jerusalem 91904, Israel.
E-mail: eden@vms.huji.ac.il
[4]Current address: Novartis Institutes for Biomedical Research, Cambridge, MA, USA.
Received 3 January 2007; revised 30 April 2007; accepted 23 May 2007; published online 9 July 2007

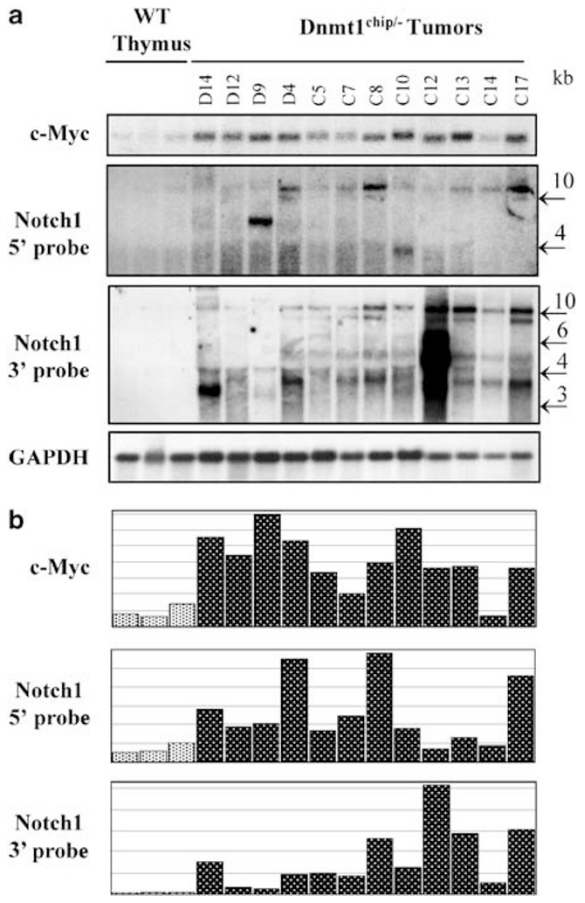

Figure 1 Upregulation of *c-Myc* and *Notch1* in the *Dnmt1*$^{chip/-}$ tumors. (**a**) Northern blot analysis showing c-myc and Notch1 overexpression in hypomethylation-induced T-cell lymphomas (dnmt1$^{chip/-}$ tumors) compared to WT thymus. Twenty micrograms total RNA were loaded. The full-length *Notch1* transcripts (two bands in the 10 kb range) are found at low levels in wt thymus and elevated levels in the tumors. In addition, short transcripts are detected in the tumors, primarily with the 3′ probe corresponding to the intracellular portion of Notch1. Probe A (from 5′ of transcript) and probe K (covering most of the intracellular (3′) portion of the transcript) are described in (Hoemann *et al*., 2000). (**b**) Quantification by densitometry of blots from panel (**a**). Signals were quantified using Image Gauge V3.45 and normalized to GAPdH levels. White columns, WT samples; Black columns, dnmt1/$^{chip-}$ tumors. GAPdH, glyceraldehyde-3-phosphate dehydrogenase

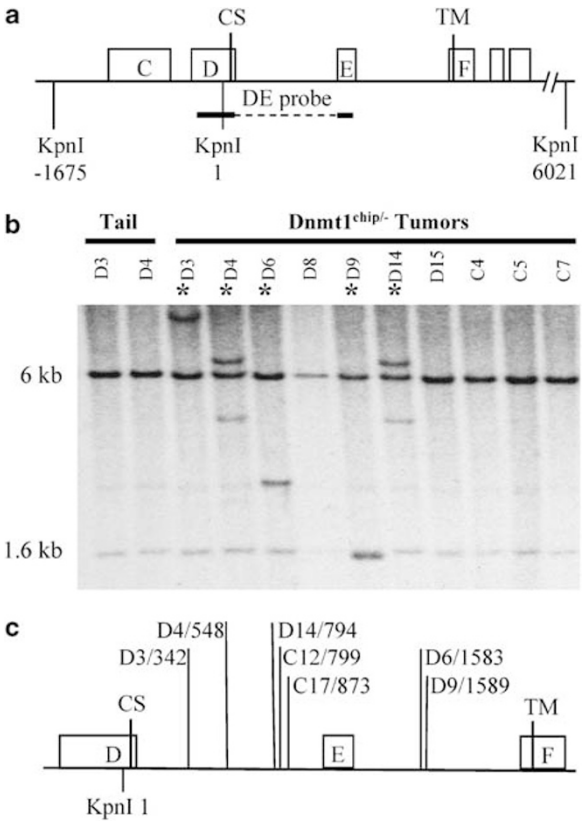

Figure 2 IAP integration into *Notch1* in hypomethylation-induced tumors. (**a**) Schematic showing exons C–F of the *Notch1* locus (nomenclature according to Hoemann *et al*., 2000). CS-region in exon D coding for the extracellular protease cleavage site on Notch1. TM-region in exon F coding the transmembranal portion of Notch1. (**b**) Southern blot analysis of genomic DNA from tail and from hypomethylation-induced T-cell lymphomas showing genomic rearrangement in the tumors (asterisks). Fifteen micrograms DNA were digested with *Kpn*I. DE probe was generated by PCR on Notch1 cDNA using primers: NotchDf: 5′-CCTAG ACTGTGCTGAGCATGTACCC-3′ and NotchEr: 5′-ACATCG GTGGCACTCTGGAA-3′. (**c**) Schematic summarizing IAP integration events. Integration sites are labeled with tumor ID and position (defined as distance from *Kpn*I site in exon D). Integration sites were determined by sequencing inverse PCR (iPCR) products (Suzuki *et al*., 2002) generated from genomic DNA re-ligated after *Kpn*I digestion and using primers K3: 5′-AACAGCAGGAGCTG GAGCACT-3′ and K4: 5′-CAGTGTGTCCCTGGGTTCTCT AAC-3′. Additional integration sites were determined by sequencing PCR products generated using U-IAP-F primer: 5′-ATGAC TACTTGTGCTCTGCCTTCC-3′ and NotchEr or NotchFr: 5′-GCCACGTACATGAGGTGCAG-3′ primers. IAP, intracisternal A particle.

revealed that the genetic change was the result of transposition of an endogenous retroviral-like element known as intracisternal A particle (IAP) into the *Notch1* locus. Further analysis confirmed this observation in 7/16 tumors (Figure 2c). All insertions were in the forward direction and included a 4 bp target site duplication characteristic for retroviral integration mechanism. Tail DNA from the same mice carrying the tumors did not contain the transposition, indicating that this was a somatic event. Importantly, all integrations occurred between exon D (which includes the extracellular protease cleavage site of the Notch receptor) and exon F, which codes the transmembranal region of the protein (Figure 2c).

To determine whether the IAP insertion contributes an ectopic promoter that directs expression of new transcripts coding for a truncated Notch1 product, we first searched for hybrid transcripts containing IAP sequences followed by Notch coding exons. Using reverse transcription–PCR analysis with a forward primer from the IAP LTR region and a reverse primer from *Notch1* exon F, we observed PCR products of variable size in the tumors that contained the IAP transposition (Figure 3a). Sequencing the PCR products confirmed the nature of the hybrid transcripts, with

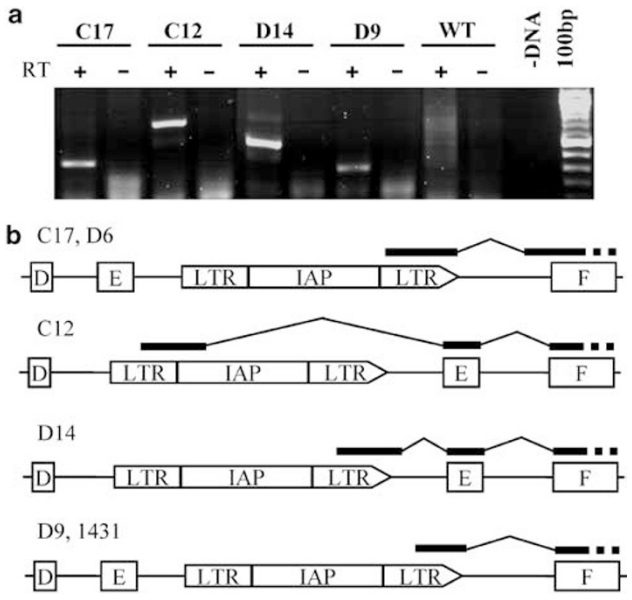

Figure 3 IAP-notch fusion transcripts were detected in tumors. (**a**) RT–PCR using U-IAP-F forward primer (corresponding IAP LTR) and NotchFr reverse primer yields products with variable size, demonstrating the presence of IAP-Notch1 fusion transcripts in the tumors, but not in wt thymus. (**b**) Schematic showing representative types of fusion transcripts as determined by sequencing PCR products from panel (**a**). Labels indicate tumor ID. IAP, intracisternal A particle; RT–PCR, reverse transcription–PCR; wt, wild type.

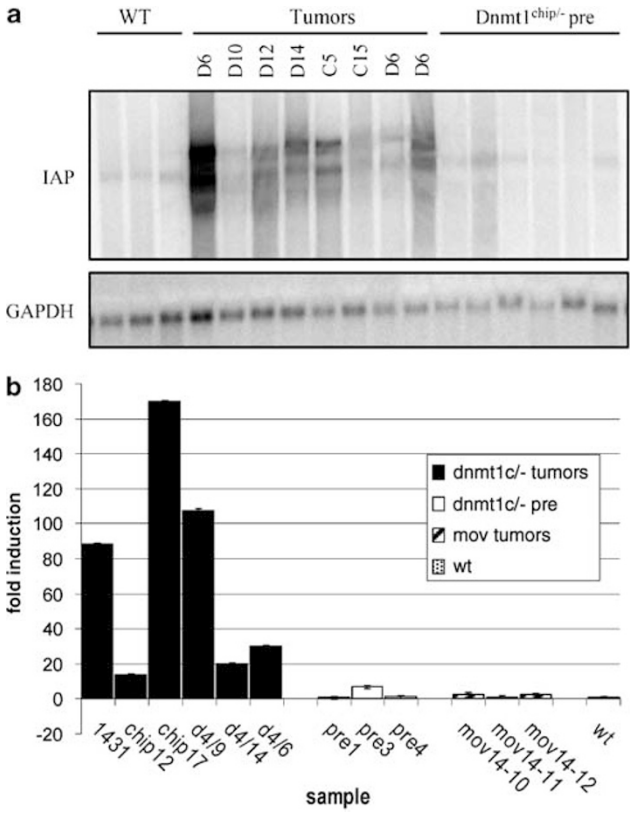

Figure 4 (**a**) Northern blot analysis showing IAP expression in hypomethylation induced T-cell lymphomas (tumors) but not in hypomethylated thymus (Dnmt1$^{chip/-}$ pre) or wild-type (WT) thymus. (**b**) Quantification by real-time RT–PCR of IAP levels normalized to β-actin in *Dnmt1*$^{chip/-}$ tumors, pre-leukemic *Dnmt1*$^{chip/-}$ thymus, wild-type (wt) thymus and MOV-induced tumors – T-cell lymphomas induced by spontaneous activation of an endogenous Moloney virus transgene (Mov14 mice (Jahner and Jaenisch, 1980). IAP primers IAPEzF: 5′-AGCAAGAAAAGAAGCCCGTGA-3′; IAPEzR: 5′-ATGCCAGAACATGTGTCAAGTG-3′, β-actin control primers: BActinF: 5′-CACAGCTTCTTTGCAGCTCCT-3′; BActinR: 5′-GTCATCCATGGCGAACTGG-3′. MOV, Moloney virus; RT–PCR, reverse transcription–PCR.

some transcripts originating from the 5′-LTR and others driven by 3′-LTR into the intronic region downstream of the insertion point and are then spliced into exons E or F of *Notch1* (Figure 3b). This situation highly resembles lymphomas in which Moloney virus (MOV) integrations into the same introns of *Notch1* were shown to produce oncogenic, constitutively active, truncated Notch1 products (Ellisen *et al*., 1991; Girard *et al*., 1996; Zweidler-McKay and Pear, 2004). We next used 5′-RACE (Ansari-Lari *et al*., 1996) to define the transcription start site of the hybrid products in a representative number of tumors. We identified several start sites with variation between the tumors, and possibly multiple start sites within a single tumor (Supplementary Figure 1). Ectopic expression driven by IAP promoters has been reported in the past. Different transcription start sites were reported in these cases and transcripts were found to originate from multiple sites, sometime in both sense and antisense directions (Christy and Huang, 1988; Michaud *et al*., 1994; Rakyan *et al*., 2003; Druker *et al*., 2004). Thus, the ectopic transcription start site may vary depending on the insertion site, the type of IAP, and possibly, the epigenetic status (for example, methylation pattern) of the inserted element. To support further the presence of an ectopic promoter, we exclude the possibility that the IAP-Notch1 hybrid transcripts are driven by the canonical *Notch1* promoter, since we could find hybrid transcripts between IAP and downstream *Notch1* exons, but not upstream exons (Supplementary Figure 2).

Large-scale studies using retroviral insertional mutagenesis to induce T-cell lymphoma in mice identified 152 tumor-associated retroviral integration sites including *Notch1* and other Notch pathway members (Suzuki *et al*., 2002). To search for additional transposition events in hypomethylation-induced tumors, we expanded the Southern blot analysis to cover 35 kb of the *Notch1* locus and to additional proto-oncogenes that are known to play a role in T-cell lymphomas including *c-myc*, *Pim1* and *Bmi1*, but could not identify evidence of genomic rearrangement in these loci (summarized in Supplementary Table). Considering the large number of possible integration sites, it is not possible to determine if tumors without IAP integration in *Notch1* develop by a different mechanism or if transposon-mediated insertional mutagenesis occurred elsewhere in these tumors. However, elevated IAP expression was observed in all Dnmt1$^{Chip/-}$ tumors (Figure 4), supporting the later possibility.

IAP elements are found in about 1000 copies per haploid genome and are normally epigenetically silenced (Boeke and Stoye, 1997). IAP transposition is documented in cultured cells (Kuff, 1990) or as germ line mutations *in vivo* (Michaud *et al*., 1994). To the best of our knowledge, this is the first example of a cancer-causing IAP transposition that occurred *in vivo* in somatic tissue. To determine whether the same IAP element was activated in all tumors or different elements were activated, we evaluated the size of the inserted element in different tumors using Southern blot analysis (data not shown). On the basis of their size, the IAP elements inserted into *Notch1* in different tumors were not identical and could be divided into three size groups, suggesting that random activation of different IAP copies rather than activation of a single, specific IAP copy was responsible for the different events. This was further supported by sequence data obtained from the IAP-Notch1 hybrid transcripts: all cloned hybrids varied in their IAP portion suggesting that they originate from different IAP elements (Supplementary Figure 3).

IAP expression is suppressed by DNA methylation and was found to be significantly elevated in demethylated ES cells and embryos (Walsh *et al*., 1998). Mice carrying the hypomorphic allele $Dnmt1^{Chip/-}$ exhibit genomewide DNA hypomethylation in all tissues and throughout embryonic development (Gaudet *et al*., 2003), yet we could not find any significant increase in IAP expression in a variety of tissues from the pre-leukemic $Dnmt1^{Chip/-}$ mice or in MOV-induced lymphomas (Figure 4). Elevated IAP expression was detected only in the $Dnmt1^{Chip/-}$ tumors (Figure 4). Although IAP transcripts of several sizes are observed in the tumors, it remains to be determined whether IAP expression in the tumors is originating primarily from the newly inserted copy/ies or whether upregulation of IAPs precedes transposition and is triggered by further demethylation or by additional events.

DNA methylation imposes a significant burden on cells, but is essential for mammalian development and for cell survival (Li *et al*., 1992; Jaenisch, 1997; Jaenisch and Bird, 2003). It has been proposed that like in plants, DNA methylation in mammals plays a role in suppression of transposable elements (Yoder *et al*., 1997). IAP activation and transposition *in vivo* observed in hypomethylated mice provide clear evidence for the indispensable role of DNA methylation in maintenance of genome ecology in mammals.

Genomewide DNA hypomethylation is a consistent finding in human tumors, but the importance of this change for human tumorigenesis remains an open question. By analysing a mouse model, we have demonstrated that hypomethylation can result in tumorigenesis and identified two mechanisms that mediate this effect. Hypomethylated cells exhibit inherent chromosomal instability, which can lead to loss of heterozygosity in soft tissue tumors (Eden *et al*., 2003) and in intestinal adenomas (Yamada *et al*., 2005), and is revealed in hypomethylation-induced lymphomas as a consistent trisomy of chromosome 15 (Gaudet *et al*., 2003). Although the mechanism is still not clear, accumulating evidence now link hypomethylation of certain repetitive elements to chromosomal instability in human cancer (Wong *et al*., 2001; Schulz *et al*., 2002; Tsuda *et al*., 2002; Ehrlich *et al*., 2003). We now find that in addition to reducing chromosomal stability, hypomethylation can promote tumor development in mice by promoting activation and transposition of endogenous retroviral elements. Endogenous transposable elements are not considered important players in human disease, and the relevance of this mechanism to hypomethylation in human tumors remains to be determined.

Acknowledgements

We thank Dr M Van-Lohuizen and Dr P Jolicoeur for probes and reagents. This study was supported by the Chief Scientist, Israeli Ministry of Health and by DKFZ-MOS grant #CA-121.

References

Ansari-Lari MA, Jones SN, Timms KM, Gibbs RA. (1996). Improved ligation-anchored PCR strategy for identification of 5′ ends of transcripts. *Biotechniques* **21**: 34–36, 38.

Boeke JD, Stoye JP. (1997). Retroviruses. In: Coffin JM, Hughes SH, Varmus HE (eds), Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp 343–436.

Christy RJ, Huang RC. (1988). Functional analysis of the long terminal repeats of intracisternal. A-particle genes:sequences within the U3 region determine both the efficiency and the direction of promoter activity. *Mol Cell Biol* **8**: 1093–1102.

Druker R, Bruxner TJ, Lehrbach NJ, Whitelaw E. (2004). Complex patterns of transcription at the insertion site of a retrotransposon in the mouse. *Nuc Ac Res* **32**: 5800–5808.

Eden A, Gaudet F, Waghmare A, Jaenisch R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* **300**: 455.

Ehrlich M, Hopkins NE, Jiang G, Dome JS, Yu MC, Woods CB *et al*. (2003). Satellite DNA hypomethylation in karyotyped Wilms tumors. *Cancer Genet Cytogenet* **141**: 97–105.

Ehrlich M. (2002). DNA methylation in cancer: too much, but also too little. *Oncogene* **21**: 5400–5413.

Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD *et al*. (1991). TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**: 649–661.

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW *et al*. (2003). Induction of tumors in mice by genomic hypomethylation. *Science* **300**: 489–492.

Girard L, Hanna Z, Beaulieu N, Hoemann CD, Simard C, Kozak CA *et al*. (1996). Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. *Genes Dev* **10**: 1930–1944.

Hoemann CD, Beaulieu N, Girard L, Rebai N, Jolicoeur P. (2000). Two distinct Notch1 mutant alleles are involved in

the induction of T-cell leukemia in c-myc transgenic mice. *Mol Cell Biol* **20**: 3831–3842.

Jackson-Grusby L, Beard C, Possemato R, Tudor M, Fambrough D, Csankovszki G *et al*. (2001). Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat Genet* **27**: 31–39.

Jaenisch R, Bird A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33**(Suppl): 245–254.

Jaenisch R. (1997). DNA methylation and imprinting: why bother? *Trends Genet* **13**: 323–329.

Jahner D, Jaenisch R. (1980). Integration of Moloney leukaemia virus into the germ line of mice: correlation between site of integration and virus activation. *Nature* **287**: 456–458.

Jones PA, Baylin SB. (2002). The fundamental role of epigenetic events in cancer. *Nat Rev Genet* **3**: 415–428.

Kuff EL. (1990). Intracisternal A particles in mouse neoplasia. *Cancer Cells* **2**: 398–400.

Li E, Bestor TH, Jaenisch R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**: 915–926.

Michaud EJ, van Vugt MJ, Bultman SJ, Sweet HO, Davisson MT, Woychik RP. (1994). Differential expression of a new dominant agouti allele (Aiapy) is correlated with methylation state and is influenced by parental lineage. *Genes Dev* **8**: 1463–1472.

Nesbit CE, Tersak JM, Prochownik EV. (1999). MYC oncogenes and human neoplastic disease. *Oncogene* **18**: 3004–3016.

Rakyan VK, Chong S, Champ ME, Cuthbert PC, Morgan HD, Luu VK *et al*. (2003). Transgenerational inheritance of epigenetic states at the murine Axin^{Fu} allele occurs after maternal and paternal transmission. *Proc Natl Acad Sci USA* **100**: 2538–2543.

Schulz WA, Elo JP, Florl AR, Pennanen S, Santourlidis S, Engers R *et al*. (2002). Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. *Genes Chromosomes Cancer* **35**: 58–65.

Suzuki T, Shen H, Akagi K, Morse HC, Malley JD, Naiman DQ *et al*. (2002). New genes involved in cancer identified by retroviral tagging. *Nat Genet* **32**: 166–174.

Tsuda H, Takarabe T, Kanai Y, Fukutomi T, Hirohashi S. (2002). Correlation of DNA hypomethylation at pericentromeric heterochromatin regions of chromosomes 16 and 1 with histological features and chromosomal abnormalities of human breast carcinomas. *Am J Pathol* **161**: 859–866.

Walsh CP, Chaillet JR, Bestor TH. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat Genet* **20**: 116–117.

Wong N, Lam WC, Lai PB, Pang E, Lau WY, Johnson PJ. (2001). Hypomethylation of chromosome 1 heterochromatin DNA correlates with q-arm copy gain in human hepatocellular carcinoma. *Am J Pathol* **159**: 465–471.

Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H *et al*. (2005). Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc Natl Acad Sci USA* **102**: 13580–13585.

Yoder JA, Walsh CP, Bestor TH. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* **13**: 335–340.

Zweidler-McKay PA, Pear WS. (2004). Notch and T cell malignancy. *Semin Cancer Biol* **14**: 329–340.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).