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Expression of Various Genes is Controlled by DNA Methylation During Mammalian Development

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Abstract Despite thousands of articles about 5-methylcytosine (m^5C) residues in vertebrate DNA, there is still controversy concerning the role of genomic m^5C in normal vertebrate development. Inverse correlations between expression and methylation are seen for many gene regulatory regions [Heard et al., 1997; Attwood et al., 2002; Plass and Soloway, 2002] although much vertebrate DNA methylation is in repeated sequences [Ehrlich et al., 1982]. At the heart of this debate is whether vertebrate DNA methylation has mainly a protective role in limiting expression of foreign DNA elements and endogenous transposons [Walsh and Bestor, 1999] or also is important in the regulation of the expression of diverse vertebrate genes involved in differentiation [Attwood et al., 2002]. Enough thorough studies have now been reported to show that many tissue- or development-specific changes in methylation at vertebrate promoters, enhancers, or insulators regulate expression and are not simply inconsequential byproducts of expression differences. One line of evidence comes from mutants with inherited alterations in genes encoding DNA methyltransferases and from rodents or humans with somatically acquired changes in DNA methylation that illustrate the disease-producing effects of abnormal methylation. Another type of evidence derives from studies of in vivo correlations between tissue-specific changes in DNA methylation and gene expression coupled with experiments demonstrating cause-and-effect associations between DNA hyper- or hypomethylation and gene expression. In this review, I summarize some of the strong evidence from both types of studies. Taken together, these studies demonstrate that DNA methylation in mammals modulates expression of many genes during development, causing major changes in or important fine-tuning of expression. Also, I discuss previously established and newly hypothesized mechanisms for this epigenetic control. J. Cell. Biochem. 88: 899–910, 2003.
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Methylation of cytosine residues is the only genetically programmed modification of the bases of vertebrate genomes. This DNA alteration is ubiquitous in vertebrates. In human DNA, 5-methylcytosine (m^5C) accounts for 0.76–1.0% of all the bases, depending on the tissue type [Ehrlich et al., 1982]. While symmetrical methylation at CpG sites is predominant in vertebrates, CpA and CpT methylation occurs at some DNA sequences as well, especially early in development [White et al., 2002]. Thus far, three mammalian genes have been shown to encode DNA methyltransferases, *Dnmt1*/*DNMT1*, *Dnmt3a*/*DNMT3A*, and *Dnmt3b*/*DNMT3B* [Okano et al., 1999; Attwood et al., 2002]. Mouse knock-out mutants have demonstrated that these three genes are essential for normal development. In these mutants, death occurs from about the 10-somite stage to 4 weeks postnatally depending on which *Dnmt* gene is inactivated and how completely [Okano et al., 1999; Attwood et al., 2002].

Research on bacteria has proven that one of the functions of their genomic methylation, which can occur on adenine or cytosine residues, is the regulation of transcription of certain genes [Wallecha et al., 2002]. DNA methylation plays other critical roles in bacteria, including

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regulation of the initiation of DNA replication and direction of mismatch repair as well as protection against restriction [Palmer and Marinus, 1994]. There is increasing evidence for methylation of vascular plant genomes contributing to the control of expression of developmental genes [Li et al., 2002]. This review summarizes some of the evidence for the control of expression of diverse vertebrate genes during development by differential DNA methylation.

There are several caveats in studies of whether DNA methylation is a regulator of tissue-specific gene expression. Cultured mammalian cells can give misleading results due to abnormal DNA methylation and will be described only when their DNA methylation patterns were shown to mirror those in tissues. Another possible problem occurs when there is hypermethylation of a repressed promoter but a molecule-by-molecule analysis by genomic sequencing reveals that, nonetheless, only some of these promoter molecules are methylated in fully repressed tissues. This indicates that methylation of the studied sequence does not suffice for repression in the examined tissue, although it might in precursor cells. Also, DNA methylation changes may often not be an on/off switch but only a modulator of the efficiency of transcription that is primarily dependent on specific transcription factors. Alternatively, the region examined might not be the most important one for methylation control or the methylation might be only a consequence and not a cause of gene inactivity. For studies of in vitro-methylated reporter constructs, optimally, the imposed methylation pattern should resemble the in vivo pattern and be limited to the mammalian transcription control region or also present on control constructs to show that the effects on expression are not due to vector methylation. Lastly, while experiments involving inhibition of DNA methylation by 5-azadeoxycytidine (azaCdR) are an important part of the evidence for a causal effect of changes in methylation of a given promoter on the expression of the associated gene, they do not suffice because azaCdR causes widespread DNA demethylation, inhibition of replication, and activation of repair. Therefore, independent confirmation by other lines of evidence is necessary and has been reported for all the genes described in this review.

EFFECTS OF ABNORMAL MAMMALIAN DNA METHYLATION: INHERITED DISEASE-CAUSING MUTATIONS, EPIMUTATIONS, AND SPONTANEOUS ALTERATIONS IN DNA METHYLATION IN SOMATIC TISSUES

Dnmt1, Dnmt3a, and Dnmt3b activity are essential for normal murine development [Okano et al., 1999]. Because domains of these proteins other than the catalytic C-terminal domain interact with proteins involved in controlling DNA replication, the cell cycle, and gene expression [Attwood et al., 2002; Ordway and Curran, 2002], the non-viability of the *Dnmt* knockout mice alone does not prove that it is the loss of DNA methylation per se which leads to their phenotypes. However, point mutations in humans affecting only the C-terminal catalytic domain of DNMT3B can give rise to a rare recessive syndrome, ICF (immunodeficiency, centromeric region instability, facial anomalies) [Okano et al., 1999]. The catalytic domain of mammalian DNA methyltransferases has been implicated only in controlling catalysis. ICF results in the loss of $<10\%$ of genomic m^5C [Tuck-Muller et al., 2000]. This syndrome always involves immune problems affecting B-cell function. Various other congenital abnormalities are often seen in ICF patients, such as, facial anomalies, growth retardation, and neurological dysfunction. Although, the mechanism for how DNA hypomethylation causes the developmental deficiencies in ICF patients is still being examined (see below), the finding that ICF mutations usually specifically alter the catalytic domain of DNMT3B indicates that normal levels of DNA methylation in humans are important for regulating expression of certain genes involved in differentiation.

The fragile X syndrome is usually caused by expansion of a CGG repeat at the 5′ end of the X-linked *FMR1* gene followed by de novo methylation of the CpG-rich *FMR1* promoter leading to silencing of the gene [Pietrobono et al., 2002]. This silencing is reversed in fragile X cell lines by treatment with azaCdR. There are rare phenotypically normal males with expansion of the *FMR1* CGG repeat, who do not display the hypermethylation of the repeat and adjacent promoter almost invariably associated with this trinucleotide expansion. Moreover, chorionic villi of one male fetus with this CGG expansion in the X-linked *FMR1* did not have

promoter methylation while the fetus did; only the fetus displayed *FMR1* silencing [Sutcliffe et al., 1992]. This indicates that the expansion-induced hypermethylation, rather than the expansion per se, was responsible for the disease-causing inhibition of *FMR1* expression. Even a gene with a trinucleotide repeat expansion that does not contain a CpG within this triplet repeat can be dysregulated as a result of ensuing aberrant DNA methylation. Analysis of the *DMPK* gene in congenital myotonic dystrophy suggests that its disease-associated over-expression is due to abnormal hypermethylation of CpGs near a CTG expansion within the gene [Filippova et al., 2001]. This over expression might occur by abrogating the negative function of an insulator, a transcription regulatory element, that was shown to be present in this region of *DMPK*.

The changes in DNA methylation very early in embryogenesis are dramatic. There is active demethylation of the paternal genome within hours of fertilization followed by slower, passive demethylation of the maternal genome [Santos et al., 2002]. Subsequent remethylation of DNA of the inner cell mass, but not the trophectoderm, begins at the blastocyst stage. Aberrant DNA methylation during embryo formation in mammals cloned by nuclear transfer may be partly responsible for the very high rate of prenatal lethality and growth abnormalities in surviving embryos [Kang et al., 2002]. Furthermore, there is evidence in mammals, bacteria, and vascular plants for epimutations due to inherited variations in methylation patterns, which can result in only partially stable alleles [Li et al., 2002; Rakyan et al., 2002; Wallecha et al., 2002].

Instability of DNA methylation postnatally is a major factor contributing to oncogenesis. This may be related to age-associated increases and decreases in methylation at certain DNA sequences in specific tissues [Attwood et al., 2002]. Both local increases in DNA methylation and global decreases in genomic methylation are extremely common in human cancer [Ehrlich, 2002]. Hypermethylation of CpG-rich promoter regions of tumor suppressor genes (TSG) can promote oncogenesis or tumor progression. Sometimes promoter hypermethylation is the sole detectable explanation for the complete loss of expression of both TSG alleles [Ordway and Curran, 2002]. Silencing of gene expression in cancer cell lines can often be reversed by azaCdR treatment. Additional evidence for a causal role of cancer-associated promoter hypermethylation in TSG silencing comes from the findings that this hypermethylation can occur very early in tumorigenesis and serve as the second hit to inactivate a functional TSG allele while the mutated allele remains unmethylated in the tumor [Ordway and Curran, 2002]. The loss of gene products by such epigenetic silencing can occur at higher frequencies than by mutation, deletion, or loss of heterozygosity. Furthermore, for some tumor suppressor genes, like *E-cadherin*, the possible reversibility of the silencing by the subsequent loss of de novo hypermethylation can be advantageous to tumor progression [Strathdee, 2002]. In contrast to cancer-linked DNA hypermethylation, cancer-associated hypomethylation of DNA is seen most frequently in highly repetitive DNA sequences [Ehrlich, 2002]. However, such hypomethylation might also result in gene dysregulation indirectly, as will be described below.

Aberrant postnatal DNA methylation changes may contribute to other diseases, like lupus, in this case, by hypomethylation increasing levels of lymphocyte function-associated antigen-1 (LFA-1) [Attwood et al., 2002]. The proximal 380 bp of the promoter of the *ITGAL* gene, which encodes one of the two subunits of LFA-1, is hypomethylated in T cells of active lupus patients vs. those of controls as determined by bisulfite genomic sequencing. Patch methylation of CpGs in this 380-bp promoter region in a reporter plasmid with the −1818 to +79 region of *ITGAL* driving transcription led to consistent 4-fold decreases in reporter gene expression upon transient transfection while patch methylation of 1.4 kb distal to this region gave much lower decreases [Lu et al., 2002]. Treatment of T cells with azaCdR increases levels of *ITGAL* RNA but not of actin RNA and decreases methylation in the proximal 380-bp region of the *ITGAL* promoter. The treated T cells are capable of causing a lupus-like disease in syngeneic mice.

PROGRAMMING OR MAINTENANCE OF DEVELOPMENTALLY LINKED TRANSCRIPTION CONTROL BY DNA METHYLATION

One of the sets of genes for which studies of animals, humans, and cultured cells clearly demonstrate a role of DNA methylation in the

regulation of expression are those on the X chromosome. Although DNA methylation is not necessary for establishing silencing of the inactive X chromosome (X_i), it seems to be important in efficiently protecting the one X chromosome that needs to stay active from inactivation (see below). Also, it is needed to prevent reactivation of many silenced genes on X_i once this inactivation is established [Heard et al., 1997]. For example, normally the rate of biallelic expression of the inactivation-susceptible X-linked genes in female mammalian cells is extremely low. The 5′ CpG-rich regions of these genes are highly methylated just on X_i. In fibroblasts or lymphoblastoid cell lines from DNMT3B-deficient ICF syndrome patients, there was a much higher rate of biallelic expression than normal in two out of five analyzed X_i genes displaying abnormal hypomethylation of the 5′ region [Hansen et al., 2000]. Moreover, in a study involving experimental induction of hypomethylation on X_i in murine fibroblasts by treatment with azaCdR or *Dnmt1* conditional excision, there was increased reactivation of the two assayed X_i genes [Csankovszki et al., 2001]. The rate of activation for the X_i *Hprt* gene was 6×10^{-9} in the absence of treatment and 4×10^{-7} when the cells were treated with azaCdR. Conditional excision of *Dnmt1* gave yet more DNA demethylation than the azaCdR treatment and was almost 100-fold more effective than azaCdR at increasing expression of an ectopic X_i-linked reporter gene. DNA methylation was shown to act synergistically with the *Xist* gene, described below, and also in concert with other factors to keep X_i genes turned off subsequent to conversion of one of the female X chromosomes to X_i.

The better known role of *Xist* is in establishing X inactivation. Around the time of implantation, *Xist* transcription is upregulated in one randomly chosen X chromosome in each cell of the inner cell mass of female mammalian embryos. This results in the production of a nontranslated, but functional RNA, Xist RNA, which coats the *Xist*-expressing chromosome (X_i) and inactivates most of its genes [Heard et al., 1997]. X_i has little or no methylation in the 5′ *Xist* region while the active X chromosome (X_a) has extensive methylation throughout the 5′ region of *Xist* and lacks *Xist* expression. In males, the same kind of extensive methylation of the promoter and 5′ end of *Xist* is found as on X_a of females and, again, this is associated with the lack of X inactivation. In normal males, there is no *Xist* expression in somatic tissues after implantation but in *Dnmt1* double-knockout mice embryos displaying very low levels of genomic m^5C, about 15% of the cells from E8.5 or 9.5 male embryos had an active *Xist* on the single X chromosome and about one-third of these had silencing of the two assayed protein-encoding X-linked genes. Similar leakiness in protection from inactivation of X_a occurs in female *Dnmt1*$^{-/-}$ embryos. Also, *Xist* activation was found in 4–8% of male E15.5 and E19.5 cortical fetal brain cells during short in vitro culture when the cells came from embryos with partial genomic hypomethylation due to the conditional deletion of *Dnmt1* in neural precursors at E9–E10 [Fan et al., 2001]. Analogous control male cells showed no *Xist* activation. In summary, methylation of the *Xist* 5′ region on X_a seems to be necessary to insure that 100% of the cells keep one X chromosome active at the time of X inactivation. Promoter methylation is also needed subsequent to X inactivation to prevent substantial leakiness in maintaining repression of *Xist* on X_a and repression of many X_i genes.

Differential DNA methylation is a critical signal for mammalian gene imprinting, which gives monoallelic expression of these genes [Plass and Soloway, 2002]. For most of the studied clusters of imprinted genes, one allele is very highly methylated and the other unmethylated or methylated at only a small percentage of CpGs in a 1–5-kb CpG-rich region (differentially methylated region, DMR). The gamete-specific differences in DMR methylation patterns, which are usually at least partially retained during embryogenesis, appear to generally be the primary imprinting mark. Among the imprinted genes improperly expressed in *Dnmt1*$^{-/-}$ mouse embryos are *H19*, whose paternal allele is normally silent, and the nearby *Igf-2*, whose maternal allele is normally silent. In these mutant embryos, the paternal *H19* allele is abnormally activated, and the reciprocally imprinted, paternal *Igf-2* allele is abnormally silenced [Li et al., 1993; Plass and Soloway, 2002]. Consistent with the *Dnmt1* mutation acting through its effect on DNA methylation, this mutation decreases methylation of the paternally imprinted DMR (an insulator, see below) between *H19* and *Igf-2* in mutant embryos. Conversely, hypermethylation of this DMR on the paternal chromosome

as a result of engineered strong overexpression of *DNMT1* in murine embryonic stem cells is concomitant with biallelic expression of *Igf-2* [Biniszkiewicz et al., 2002]. In humans, inappropriate methylation of this DMR in the paternal *IGF2*- and *H19*-containing imprinted gene cluster due to *cis*-acting imprinting mutations is found in certain patients with the Beckwith-Wiedemann syndrome as well as in various cancers [Ehrlich, 2002]. Accompanying this hypermethylation is biallelic *IGF2* expression, resulting in abnormally high levels of its encoded mitogen and fetal growth promoting protein. Both losses and gains of methylation in DMRs may contribute to carcinogenesis via the resulting abnormal expression of imprinted genes, which requires alteration of only one allele for phenotypic changes [Ehrlich, 2002; Plass and Soloway, 2002].

Given the strong multifocal evidence that DNA methylation modulates the activity of imprinted genes and X chromosome genes, it is reasonable to expect that it also controls the expression of some other developmentally regulated genes. There is such evidence although, to date, it is less extensive for individual genes than for the imprinted and X-linked genes. Some low-copy-number CpG-rich regions show tissue-specific differences in DNA methylation [Zhang et al., 1987; Shiota et al., 2002]. For certain of these CpG islands that overlap promoters, there is evidence that changes in DNA methylation help regulate expression. Tables I and II present various examples of development-specific differences in methylation of 5′ CpG island and 5′ non-island promoter or enhancer regions functionally implicated in normal differentiation by experiments providing cause-and-effect evidence. Because of space limitations, many genes for which there is persuasive evidence for roles of DNA methylation in differentiation-controlled gene expression are not listed. Included in Table I are genes that have testis-specific expression and testis-specific promoter hypomethylation, namely, the many genes encoding testis cancer-antigens in the *MAGE-LAGE-GAGE-BAGE* superfamily, the *Alf* gene which specifies a germ cell-specific TFIIA subunit, and a testis-specific lactate dehydrogenase gene. The cancer-testis antigen genes resemble satellite DNAs of centromeric and juxtacentromeric heterochromatin in that they display hypomethylation invariably in sperm and often in cancers [Ehrlich, 2002].

Among the developmentally regulated genes in Table II is one for which methylation appears to contribute to a kind of cellular memory, the liver-specific *Tat* gene, which is associated with methylation-linked memorization of glucocorticoid (GC) stimulation [Grange et al., 2001]. *Tat* is induced by activated glucocorticoid receptor (GR), which causes demethylation of the glucocortocoid response unit (GRU) 2.5 kb upstream of the gene. Following GC induction in a rat hepatoma cell line, demethylation of the *Tat* GRU occurs and is stable even upon subsequent hormone withdrawal, unlike the GC-induced chromatin remodeling in the promoter. Transcriptional activation resulting from a second GC stimulation is stronger, which can be ascribed to GRU demethylation induced by the first GC treatment. The kinetics of protein binding and demethylation suggests that binding of the activated GR to GRU causes GRU demethylation which, in turn, facilitates binding of two other transcription factors to this site. These methylation and expression changes in the hepatoma cell line are mirrored in the rat, late in embryogenesis and at birth (Table II) and illustrate that DNA methylation's effects on transcription can be subtle, but important. Furthermore, they show that binding of one transcription factor can cause DNA demethylation affecting the binding of others and imparting a type of cell memory.

There is much evidence that correct patterns of DNA methylation are necessary for proper development and functioning of the lymphoid system. The importance of DNA methylation to T-cell development is seen in analysis of the *IFN-*γ gene in T-cell fractions derived in vivo at different stages of development, in cell culture studies involving inhibition of DNMT1 production using an antisense construct, and in studies of hypermethylation of the *IFN-*γ promoter upon HIV-1 infection (Table II). A more general role of genomic methylation in T-cell lymphogenesis is apparent from analysis of conditional $Dnmt1^{-/-}$ mice displaying T-cell precursor-specific deletion of *Dnmt1* [Lee et al., 2001]. In mutant mice that lose *Dnmt1* expression early in T-cell development, there is greatly enhanced cell death of TCR$\alpha\beta$-lineage T cells and an increase in TCR$\alpha\delta^+$ thymocytes and T cells even under conditions in which TCR$\alpha\beta^+$ cells do not decrease. Also, these mutant mice display atypical activation of expression of CD8α in TCR$\alpha\delta^+$ cells with a corresponding decrease in

TABLE I. Some Mammalian Genes for Which There is Evidence That Differentiation-Associated DNA Methylation Controls Their Expression[a]

Gene	Tissue-specific differences in methylation	Tissue-specific expression	Demethylation-induced expression	De novo methylation-induced repression	References
X-linked & imprinted genes	See text for examples of regulation of X-linked protein-encoding genes, *Xist*, and imprinted genes by in vivo DNA meth[b]				
Genes encoding cancer-testis antigens, including, members of the *MAGE*, *LAGE*, *GAGE*, & *BAGE* families	Promoter almost unmeth in human testes & sperm but very highly meth in somatic tissues by clonal genomic sequencing; part of a large group of related genes with testis & sperm-specific hypometh	Expressed specifically in the male germ line	Promoter hypometh seen in a variety of tumors is associated with expression; azaCdR activates the genes' expression in tumor cell lines that do not originally have this hypometh & gives *MAGE*-1 promoter hypometh	In vitro CpG meth decreases expression of a *MAGE*-1 promoter-containing reporter plasmid; meth of Ets motifs in the *MAGE*-1 promoter interferes with binding of Ets family transcription factor in DNA-binding assays	[De Smet et al., 1999; Ehrlich, 2002]
TFIIAα/β-like factor (*Alf*)	No promoter meth in mouse testes & sperm; high overall meth in postnatal somatic tissues but extent varies much by clonal genomic sequencing; hypometh precedes expression in prepubertal testes	Expressed almost exclusively in testis	azaCdR activates expression in somatic cell lines	In vitro CpG meth decreases expression of *Alf* promoter-containing reporter plasmid much more than an analogous SV40 promoter-containing reporter plasmid	[Xie et al., 2002]
Lactate dehydrogenase C (*mLdhC*)	Proximal promoter essentially unmethylated in murine testis but mostly meth in murine somatic tissues by genomic bisulfite sequencing of PCR product	Expressed exclusively in cells of germinal epithelium	Not reported	In vitro CpG meth decreases expression of a *mLdhC* promoter-containing reporter plasmid	[Kroft et al., 2001]
Oxytocin receptor (*OTR*)	Hypermeth of 5′ end of gene in human liver and mononuclear blood cells relative to term myometrium; meth analysis by semi-quantitative PCR of *Hpa*II-treated DNA	Blood and liver are non-expressing tissues; term myometrium has high expression	azaCdR activates expression in a hepatoblastoma cell line	*OTR* promoter/5′ region-containing reporter construct shows suppression of expression by CpG meth; this was largely reversed by deleting part of the *OTR* 5′ region (part of CpG island)	[Kusui et al., 2001]

[a]Tables I and II present lists of just some of the genes for which there is strong evidence for differential DNA methylation controlling their expression during normal mammalian development and a summary of the experimental evidence. For descriptions of some of the evidence for DNA methylation-controlled expression of X-linked genes and imprinted genes, see the text.

[b]Meth, methylation; hypometh, hypomethylation; demeth, demethylation; hypermeth, hypermethylation; clonal genomic sequencing, bisulfite-based sequencing by analysis of molecular clones to give a molecule-by-molecule analysis of all C methylation in a given DNA region; genomic sequencing with hydrazine and piperidine or bisulfite sequencing of PCR product gives the average methylation at every C; Southern blotting, methylation analysis by Southern blotting of DNA digested by a CpG methylation-sensitive restriction endonuclease.

TABLE II. More Mammalian Genes for Which There is Evidence That Differentiation-Associated DNA Methylation Controls Their Expression[a]

Gene	Tissue-specific differences in methylation and expression	Demethylation-induced expression	De novo methylation-induced repression	References
Tyrosine aminotransferase (*Tat*), a glucocorticoid (GC)-responsive gene	Upstream glucocorticoid response unit (GRU) containing 4 CpGs: in fetal rat liver parenchymal cells, largely meth at E15 & becomes mostly demeth, specifically in liver, during last days before birth; further demeth in proximal promoter begins at birth and is also liver-specific; expression begins specifically in liver at birth upon stimulation by hypoglycemia; meth analysis by genomic sequencing with hydrazine & piperidine	Prolonged GC treatment of a hepatoma cell line causes slow but stable demeth and recruitment of additional transcription factors to GRU CpG sites	Not reported	[Thomassin et al., 2001]
Glial fibrillary acidic protein (*GFAP*)	Murine astrocyte-specific GFAP synthesis requires STAT3 (has a binding site at −1176) & also a state of responsiveness that coincides with CpG demeth of *GFAP* STAT3 site in fetal brain neuroepithelial cells from E11.5 to E14.5 (84 & 38% meth, respectively); this hypometh occurs during astrogenesis; also see this site-specific hypometh in GFAP-expressing adult brain astrocytes (8–20% meth) vs. adult brain neurons or various other unexpressing tissues (80–100% meth); meth analysis by clonal genomic sequencing & Southern blotting	Primary cultures of cytokine-treated E11.5 neuroepithelial cells, which do not normally express GFAP, do so after azaCdR treatment	Inhibition of expression by CpG meth of a reporter plasmid with a minimal promoter containing eight STAT3 sites necessary for expression; CpG meth of STAT3 site inhibits STAT3 binding in DNA-binding assays & associated with lack of binding in vivo (by ChIP assay) even in the presence of activated STAT3	[Condorelli et al., 1994; Takizawa et al., 2001]
Leukosialin	*CD43* is expressed and its promoter almost completely unmeth at assayed sites in leukocytes, hypometh in leukocyte-rich tissues, & highly meth in leukocyte-poor tissues; tight correlations between expression and promoter hypometh in cell lines too; meth analysis by Southern blotting	azaCdR converts non-expressing to expressing cell lines & decreases *CD43* promoter meth	CpG meth decreases expression of *CD43* promoter/reporter plasmids but not an SV40-promoter plasmid in human cells; in *Drosophila* cells, this meth-caused repression is dependent on co-transfecting an MeCP2 expression plasmid	[Kudo, 1998]
Interferon-γ (*IFNγ*)	Proximal promoter hypometh in expressing murine Th1 T cell clones & almost completely meth in nonexpressing Th2 clones; hypometh near or in the gene in a small percentage of T cells vs. T-cell depleted lymphocytes & non-lymphoid cells; CpG & CpT/CpA promoter meth levels in human T cells were inversely related to expression in adult vs. cord blood, in cord $CD8^+$ vs. $CD4^+$ cells; promoter hypermeth in cord blood $CD4^+$ T cells may contribute to poor T-cell memory against viral infection in neonates; promoter meth may control differentiation of $CD4^+$ cells; meth analysis by Southern blotting & clonal genomic sequencing	Stable transfection of a lymphoid cell line with a *DNMT1*-AS expression plasmid increased IFNγ expression; Cre/loxP *Dnmt1* deletion specifically in embryonic mouse thymocytes increased the percentage of $IFN\gamma^+$ naive T cells & hypometh of their IFNγ promoter	Hypermeth of promoter in lymphoid cell lines infected with HIV-1 downregulates IFNγ synthesis but not if the cells had been previously stably transfected with *DNMT1*-AS expression plasmid; *in vitro* meth of a sequence from the proximal promoter inhibited protein-complex formation in DNA-binding assays	[Young et al., 1994; Katamura et al., 1998; Mikovits et al., 1998; Lee et al., 2002; White et al., 2002]

[a]See Table I for explanation of abbreviations. ChIP, chromatin immunoprecipitation; MeCP2, a vertebrate transcription repressor that preferentially binds to m^5CpG-containing DNA sequences; AS, antisense.

methylation of this locus in those cells. In the mutant mice with loss of *Dnmt1* expression programmed later in T-cell development, T cells develop normally but the fraction of $CD44^{hi}$ memory T cells is reduced, which suggests impairment of replication-dependent maturation of naive into memory T cells. Also, there is an increase in IFN-γ, IL-2, IL-3, and IL-4 mRNAs in naive T cells from these mutant mice. It has been proposed that normally cytokine expression by naive T cells, which is facilitated by passage through S phase, may rely partially on replication-associated demethylation and chromatin remodeling at cytokine loci. The mutant mice were shown to have hypomethylation at IFN-γ and *IL-3* sites that are fully methylated in nonexpressing cell types but hypomethylated in expressing T cells. This study implicates IFN-γ hypomethylation in regulation of this gene's expression, as do those mentioned above.

Moreover, direct or indirect effects of DNA hypomethylation probably lead to misregulation of gene expression in the B-cell lineage and defects in later stages of B-cell development. This conclusion is derived from analyses of microarray expression and surface immunoglobulin proteins in ICF vs. control B-cell lines and the symptoms of ICF patients [Ehrlich et al., 2001]. As mentioned above, ICF patients, who have only a minor decrease in genomic m^5C levels [Tuck-Muller et al., 2000], often display neurological dysfunction. This might be related to the finding that DNA hypomethylation perturbs the function and survival of neurons in postnatal mice with conditional deletion of *Dnmt1* in neural precursor cells [Fan et al., 2001].

Additional evidence for the involvement of DNA methylation in expression of many diverse genes comes from a study of murine embryonic fibroblasts made *Dnmt1*$^{-/-}$ by Cre-activated *Dnmt1* deletion [Jackson-Grusby et al., 2001]. The fibroblasts were *Trp53*$^{-/-}$ to counteract DNA demethylation-induced apotosis. Microarray expression analysis showed that 4–10% of studied genes were upregulated when these cells first become highly m^5C-deficient. Also, it has been demonstrated that *Dnmt1* overexpression results in murine embryonic lethality just like *Dnmt1* underexpression [Biniszkiewicz et al., 2002], consistent with the importance of maintaining correct DNA methylation levels for normal mammalian development.

In summary, the evidence presented in Tables I and II for tissue-specific gene expression controlled by DNA methylation comes from studies of tissue-specific expression and corresponding promoter/enhancer hypomethylation in liver, brain, leukocytes, testes, or myometrium. Developmentally programmed alterations in DNA methylation in these gene regions may cause major changes in their transcription, fine-tune their upregulation, or help maintain repression. For these genes, demethylation induced by azaCdR, hormone treatment, *DNMT1* antisense RNA, or *Dnmt1* conditional deletion increases expression. Methylation changes were shown to affect binding of a transcription factor to the regulatory region or to alter test promoter/reporter gene expression in transfection assays (Tables I and II). For the *CD43* promoter, it was demonstrated that the repressive effects of DNA methylation on reporter plasmid expression were dependent on the presence of a methylation-specific DNA-binding protein [Kudo, 1998]. Further evidence for DNA methylation regulating gene expression is provided by examination of immune gene dysregulation and other developmental anomalies in ICF syndrome patients with missense mutations in the catalytic domain of *DNMT3B* as well as by studies of the various developmental problems of *Dnmt1* homozygous knockout, conditional deletion, or overexpression mice and *Dnmt3a* or *Dnmt3b* knockout mice.

MECHANISMS FOR DNA METHYLATION REGULATING GENE EXPRESSION

It has been demonstrated in many studies that DNA methylation can affect histone modifications and chromatin structure, which, in turn, can alter gene expression [Nguyen et al., 2001; Attwood et al., 2002; Ordway and Curran, 2002]. Usually, more methylation of a transcription control gene leads to less expression. Decreases or increases in DNA methylation can affect chromatin structure by altering binding of sequence-nonspecific methylated DNA binding proteins, which, in turn, recruit histone deacetylases or other proteins to regulate transcription (Fig. 1). Alternatively, changing methylation of DNA sequences can alter their interactions with sequence-specific DNA-binding proteins that bind either less or more avidly to their CpG-containing recognition sites when those sites are methylated and can act as

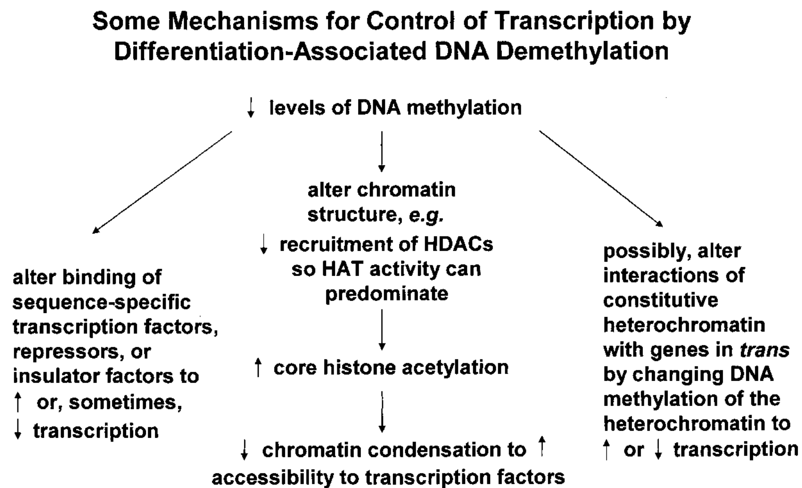

Fig. 1. Some of the mechanisms by which DNA demethylation may control transcription in mammals are illustrated. HDAC, histone deacetylase; HAT, histone acetyltransferase.

transcription activators or repressors [De Smet et al., 1999; Sengupta et al., 1999; Takizawa et al., 2001] (Tables I and II). During methylation of hemimethylated DNA sequences in newly replicated DNA, a recruited DNA methyltransferase may help regulate transcription by interacting itself with histone deacetylases or other transcriptional repressors [Ordway and Curran, 2002]. These interactions involve methyltransferase domains other than the catalytic domain. Also, gene expression can be regulated by differential methylation at insulators, DNA-protein complexes that repress long-distance interactions in *cis* between an enhancer on one side of the insulator and a promoter on the other side [Filippova et al., 2001; Kanduri et al., 2002; Plass and Soloway, 2002]. Methylation of the insulator can positively control expression from that promoter by interfering with binding of a required insulator-specific, CpG methylation-sensitive DNA-binding protein, e.g., CTCF [Filippova et al., 2001].

Another level of control of eucaryotic gene expression can be exerted by nuclear compartmentalization [Gasser, 2001]. Centromeric heterochromatin, which tends to cluster in interphase mammalian nuclei, has been implicated in the heritable downregulation of expression in mice of certain early lymphogenesis genes that have to be turned off as lymphogenesis proceeds [Brown et al., 1999]. Ikaros acts as a positive or negative transcription regulatory factor and can multimerize [Trinh et al., 2001]. It is involved in euchromatin–heterochromatin interactions in *trans* and can bind specifically to target gene promoters and to γ-satellite DNA, a component of centromeric heterochromatin [Cobb et al., 2000]. For example, Ikaros negatively controls the $\lambda 5$ gene through cognate promoter sites. Both Ikaros and the $\lambda 5$ gene are sequestered at centromeric heterochromatin 3–4 days after stimulation of splenic B cells [Brown et al., 1999]. The γ-satellite heterochromatin may serve as a reservoir facilitating delivery of Ikaros to the $\lambda 5$ promoter for repression of the gene [Sabbattini et al., 2001]. A related phenomenon is the association of centromeric heterochromatin with the β-globin locus and with NF-E2p18 when the locus is repressed [Francastel et al., 2001]. NF-E2p18 is one subunit of a transcription factor for this locus but, by itself, acts as a repressor. The β-globin locus' potential to be expressed, rather than its transcription per se, correlates with its movement and that of NF-E2p18 away from centromeric clusters. Concomitant with this movement, NF-E2p18 can heterodimerize with its erythroid-specific partner NF-E2p45 to form an activator of the β-globin locus. There is a repressor-activator duality also in the case of the above-mentioned Ikaros transcription control protein. While it represses a number of genes, it activates others. Recently, it has been shown that when Ikaros acts as a potentiator of transcription, its binding to γ-satellite-containing heterochromatin can be likewise involved [Koipally et al., 2002].

Because centromeric DNA is normally highly methylated in postnatal somatic cells in mammals (but not in sperm or extraembryonic

tissues), we proposed that its methylation may influence the above-described chromatin interactions [Ehrlich et al., 2001]. In microarray analysis of six ICF B-cell lines vs. five control B-cell lines, we found that 32 genes had consistent and significant changes in RNA levels [Ehrlich et al., 2001]. The observed dysregulation of twelve of these genes can explain the major immune defects seen in ICF patients. However, examination of methylation in the promoter regions of several of these genes revealed no ICF-specific differences. Given the catalytic domain-specific missense mutations in *DNMT3B* in most of these patients, hypomethylation is probably responsible for their symptoms, e.g., by dysregulation of unidentified transcription factor genes via promoter hypomethylation or by effects related to those described above for centromeric heterochromatin. With respect to the latter postulated pathway, the satellite DNA in the large juxtacentromeric (centromere-adjacent) heterochromatin regions of chromosomes 1, 9, and 16 is always hypomethylated in cells from ICF patients [Jeanpierre et al., 1993]. In contrast, in normal postnatal somatic cells of vertebrates, constitutive heterochromatin has highly methylated DNA. We hypothesize that the DNA hypomethylation in juxtacentromeric heterochromatin of ICF lymphoid cells interferes with chromatin interactions in *trans* and, thereby, changes expression of certain genes necessary later in lymphogenesis, such as the cytokine-responsive regulator of G-protein signaling-1 gene, which is specific for B cells [Ehrlich et al., 2001]. If DNA methylation influences transcription-regulatory interactions of constitutive heterochromatin with various euchromatic gene regions, then the frequent hypomethylation of centromeric and juxtacentromeric satellite DNA sequences in cancer [Ehrlich, 2002] could lead to a novel type of dysregulation of gene expression by alteration of *trans* interactions of chromatin.

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REFERENCES

Attwood JT, Yung RL, Richardson BC. 2002. DNA methylation and the regulation of gene transcription. Cell Mol Life Sci 59:241–257.

Biniszkiewicz D, Gribnau J, Ramsahoye B, Gaudet F, Eggan K, Humpherys D, Mastrangelo MA, Jun Z, Walter J, Jaenisch R. 2002. Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. Mol Cell Biol 22:2124–2135.

Brown KE, Baxter J, Graf D, Merkenschlager M, Fisher AG. 1999. Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol Cell 3: 207–217.

Cobb BS, Morales-Alcelay S, Kleiger G, Brown KE, Fisher AG, Smale ST. 2000. Targeting of Ikaros to pericentromeric heterochromatin by direct DNA binding. Genes Dev 14:2146–2160.

Condorelli DF, Nicoletti VG, Barresi V, Caruso A, Conticello S, de Vellis J, Giuffrida Stella AM. 1994. Tissue-specific DNA methylation patterns of the rat glial fibrillary acidic protein gene. J Neurosci Res 39:694–707.

Csankovszki G, Nagy A, Jaenisch R. 2001. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol 153:773–784.

De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. 1999. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 19:7327–7335.

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

Ehrlich M, Gama-Sosa M, Huang L-H, Midgett RM, Kuo KC, McCune RA, Gehrke C. 1982. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. Nucleic Acids Res 10:2709–2721.

Ehrlich M, Buchanan K, Tsien F, Jiang G, Sun B, Uicker W, Weemaes C, Smeets D, Sperling K, Belohradsky B, Tommerup N, Misek D, Rouillard J-M, Kuick R, Hanash S. 2001. DNA methyltransferase 3B mutations linked to the ICF syndrome cause dysregulation of lymphocyte migration, activation, and survival genes. Hum Mol Genet 10:2917–2931.

Fan G, Beard C, Chen RZ, Csankovszki G, Sun Y, Siniaia M, Biniszkiewicz D, Bates B, Lee PP, Kuhn R, Trumpp A, Poon C, Wilson CB, Jaenisch R. 2001. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. J Neurosci 21:788–797.

Filippova GN, Thienes CP, Penn BH, Cho DH, Hu YJ, Moore JM, Klesert TR, Lobanenkov VV, Tapscott SJ. 2001. CTCF-binding sites flank CTG/CAG repeats and form a methylation- sensitive insulator at the DM1 locus. Nat Genet 28:335–343.

Francastel C, Magis W, Groudine M. 2001. Nuclear relocation of a transactivator subunit precedes target gene activation. Proc Natl Acad Sci USA 98:12120–12125.

Gasser SM. 2001. Positions of potential: Nuclear organization and gene expression. Cell 104:639–642.

Grange T, Cappabianca L, Flavin M, Sassi H, Thomassin H. 2001. In vivo analysis of the model tyrosine aminotransferase gene reveals multiple sequential steps in glucocorticoid receptor action. Oncogene 20:3028–3038.

Hansen RS, Stoger R, Wijmenga C, Stanek AM, Canfield TK, Luo P, Matarazzo MR, D'Esposito M, Feil R, Gimelli G, Weemaes CM, Laird CD, Gartler SM. 2000. Escape from gene silencing in ICF syndrome: Evidence for advanced replication time as a major determinant. Hum Mol Genet 9:2575–2587.

Heard E, Clerc P, Avner P. 1997. X-chromosome inactivation in mammals. Annu Rev Genet 31:571–610.

Jackson-Grusby L, Beard C, Possemato R, Tudor M, Fambrough D, Csankovszki G, Dausman J, Lee P, Wilson C, Lander E, Jaenisch R. 2001. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet 27:31–39.

Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, Fischer A, Viegas-Pequignot E. 1993. An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 2:731–735.

Kanduri C, Fitzpatrick G, Mukhopadhyay R, Kanduri M, Lobanenkov V, Higgins M, Ohlsson R. 2002. A differentially methylated imprinting control region within the Kcnq1 locus harbors a methylation-sensitive chromatin insulator. J Biol Chem 277:18106–18110.

Kang YK, Park JS, Koo DB, Choi YH, Kim SU, Lee KK, Han YM. 2002. Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. EMBO J 21:1092–1100.

Katamura K, Fukui T, Kiyomasu T, Iio J, Tai G, Ueno H, Heike T, Mayumi M, Furusho K. 1998. IL-4 and prostaglandin E2 inhibit hypomethylation of the 5′ regulatory region of IFN-gamma gene during differentiation of naive CD4+ T cells. Mol Immunol 35:39–45.

Koipally J, Heller EJ, Seavitt JR, Georgopoulos K. 2002. Unconventional potentiation of gene expression by Ikaros. J Biol Chem 277:13007–13015.

Kroft TL, Jethanandani P, McLean DJ, Goldberg E. 2001. Methylation of CpG dinucleotides alters binding and silences testis-specific transcription directed by the mouse lactate dehydrogenase C promoter. Biol Reprod 65:1522–1527.

Kudo S. 1998. Methyl-CpG-binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated. Mol Cell Biol 18:5492–5499.

Kusui C, Kimura T, Ogita K, Nakamura H, Matsumura Y, Koyama M, Azuma C, Murata Y. 2001. DNA methylation of the human oxytocin receptor gene promoter regulates tissue-specific gene suppression. Biochem Biophys Res Commun 289:681–686.

Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, Cherry SR, Tsai JH, Tucker SM, Weaver WM, Kelso A, Jaenisch R, Wilson CB. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity 15:763–774.

Lee DU, Agarwal S, Rao A. 2002. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. Immunity 16:649–660.

Li E, Beard C, Jaenisch R. 1993. Role for DNA methylation in genomic imprinting. Nature 366:362–365.

Li G, Hall TC, Holmes-Davis R. 2002. Plant chromatin: Development and gene control. Bioessays 24:234–243.

Lu Q, Kaplan M, Ray D, Gutsch D, Richardson B. 2002. Effect of DNA methylation and chromatin structure on *ITGAL* expression. Blood 99:4503–4508.

Mikovits JA, Young HA, Vertino P, Issa JP, Pitha PM, Turcoski-Corrales S, Taub DD, Petrow CL, Baylin SB, Ruscetti FW. 1998. Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in de novo methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. Mol Cell Biol 18: 5166–5177.

Nguyen CT, Gonzales FA, Jones PA. 2001. Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: Correlation of accessibility, methylation, MeCP2 binding, and acetylation. Nucleic Acids Res 29:4598–4606.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 98: 247–257.

Ordway JM, Curran T. 2002. Methylation matters: Modeling a manageable genome. Cell Growth Differ 13:149–162.

Palmer BR, Marinus MG. 1994. The dam and dcm strains of *Escherichia coli*–a review. Gene 143:1–12.

Pietrobono R, Pomponi MG, Tabolacci E, Oostra B, Chiurazzi P, Neri G. 2002. Quantitative analysis of DNA demethylation and transcriptional reactivation of the FMR1 gene in fragile X cells treated with 5-azadeoxycytidine. Nucleic Acids Res 30:3278–3285.

Plass C, Soloway PD. 2002. DNA methylation, imprinting, and cancer. Eur J Hum Genet 10:6–16.

Rakyan VK, Blewitt ME, Druker R, Preis JI, Whitelaw E. 2002. Metastable epialleles in mammals. Trends Genet 18:348–351.

Sabbattini P, Lundgren M, Georgiou A, Chow C, Warnes G, Dillon N. 2001. Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. EMBO J 20:2812–2822.

Santos F, Hendrich B, Reik W, Dean W. 2002. Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172–182.

Sengupta PK, Ehrlich M, Smith BD. 1999. A methylation-responsive MDBP/RFX site is in the first exon of the collagen alpha2(I) promoter. J Biol Chem 274:36649–36655.

Shiota K, Kogo Y, Ohgane J, Imamura T, Urano A, Nishino K, Tanaka S, Hattori N. 2002. Epigenetic marks by DNA methylation specific to stem, germ, and somatic cells in mice. Genes Cells 7:961–969.

Strathdee G. 2002. Epigenetic versus genetic alterations in the inactivation of E-cadherin. Semin Cancer Biol 12: 373–379.

Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, Warren ST. 1992. DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1:397–400.

Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T. 2001. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1:749–758.

Trinh LA, Ferrini R, Cobb BS, Weinmann AS, Hahm K, Ernst P, Garraway IP, Merkenschlager M, Smale ST. 2001. Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. Genes Dev 15:1817–1832.

Tuck-Muller CM, Narayan A, Tsien F, Smeets D, Sawyer J, Fiala ES, Sohn O, Ehrlich M. 2000. DNA hypomethylation and unusual chromosome instability in cell lines

from ICF syndrome patients. Cytogenet Cell Genet 89: 121–128.

Wallecha A, Munster V, Correnti J, Chan T, van der Woude M. 2002. Dam- and OxyR-dependent phase variation of agn43: Essential elements and evidence for a new role of DNA methylation. J Bacteriol 184:3338–3347.

Walsh CP, Bestor TH. 1999. Cytosine methylation and mammalian development. Genes Dev 13:26–34.

White GP, Watt PM, Holt BJ, Holt PG. 2002. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. J Immunol 168:2820–2827.

Xie W, Han S, Khan M, DeJong J. 2002. Regulation of ALF gene expression in somatic and male germ line tissues involves partial and site-specific patterns of methylation. J Biol Chem 277:17765–17774.

Young HA, Ghosh P, Ye J, Lederer J, Lichtman A, Gerard JR, Penix L, Wilson CB, Melvin AJ, McGurn ME, Lewis DB, Taub DD. 1994. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. J Immunol 153:3603–3610.

Zhang X-Y, Loflin PT, Gehrke CW, Andrews PA, Ehrlich M. 1987. Hypermethylation of human DNA sequences in embryonal carcinoma cells and somatic tissues but not sperm. Nucleic Acids Res 15:9429–9449.