

IDENTIFICATION AND INITIAL CHARACTERIZATION OF GENES  
CONTROLLED BY DNA METHYLATION IN EMBRYONIC STEM CELLS

By

NEMANJA RODIC

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2005

Copyright 2005

by

Nemanja Rodic

This document is dedicated to my family in Yugoslavia.

## ACKNOWLEDGMENTS

I first want to thank my mentor, Dr. Naohiro Terada, for his positive attitude and exemplary conduct that he maintained throughout the years. I would like to thank to all members of the Terada Laboratory. I thank Dr. Masahiro Oka, for numerous discussions and invaluable insights into critical thinking. I am grateful to Dr. Takashi Hamazaki for technical advice he provided. I thank our laboratory manager, Amy Meacham, for frequent help she provided. I thank to my friends and fellow graduate students Amar Singh, Michael Rutenberg, and Sarah Kehoe, for providing me with many suggestions and help through the years. I would also like to acknowledge Dr. Bungert, Dr. Petersen, Dr. Resnick, Dr. Oh, and Dr. Yang along with their respective laboratory members for sharing their knowledge and technical advice. Finally, I am grateful to my wife, Dr. Keisha-Gay Hylton Rodić, for her love and care.

## TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS .....	iv
LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
ABSTRACT .....	x
CHAPTER	
1 INTRODUCTION .....	1
Epigenetics.....	1
DNA Methylation in Development .....	2
Epigenetic Studies in ES Cells .....	3
CpG Islands .....	4
DNA Methylation Patterns .....	6
DNA Methylation of Tissue-Specific Genes.....	6
DNA Methylation in Cancer .....	8
DNA Methylation Associated Factors.....	9
DNA Methyltransferases.....	10
DNA Methylation Associated Proteins .....	12
Chromatin Related Modification and Chromatin Imposed Boundaries .....	13
<i>De Novo</i> DNA Methylation.....	14
Drugs That Affect DNA Methylation.....	15
Nutrients That Affect DNA Methylation.....	16
Additional Comments.....	16
Study Design and Rationale.....	17
2 METHODS AND MATERIALS .....	20
<i>In Vitro</i> ES Cell Differentiation.....	20
Methylation Sensitive Restriction Fingerprinting (MSRF).....	20
MSRF Cloning and Sequencing .....	21
5'RACE.....	22
Construction and <i>in vitro</i> Methylation of Reporter Vectors.....	22
Chromatin Immunoprecipitation .....	23
FLAG Tagged Ant4 Protein Overexpression .....	25
Untagged Ant4 Protein Overexpression .....	25

Western Blotting.....	26
Immunohistochemistry .....	26
Bisulfite Sequencing and Combined Bisulfite Restriction Analysis (COBRA).....	27
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).....	28
Northern Blotting.....	29
<i>In situ</i> RNA Hybridization.....	29
Immunostaining for Ant4 .....	30
Primordial Germ Cell Preparation.....	31
Targeted Disruption of Mouse <i>Ant4</i> gene.....	31
3 RESULTS.....	33
Introduction.....	33
Identification of Differentially Methylated Genomic Regions.....	34
A Survey of DNA Methylation Screening Methods .....	34
Methylation Sensitive Restriction Fingerprinting (MSRF) Method .....	35
Identification of DNA Methylation Controlled Genes.....	37
Identification of a Novel ES and Germ Cell-Specific Gene Using the MSRF Method.....	40
<i>RIKENcDNA 1700034J06</i> Computational Analysis .....	41
5'RACE .....	42
<i>Ant4</i> Promoter Transcription is Inhibited by DNA Methylation.....	43
<i>Ant4</i> Genomic Locus Undergoes Epigenetic Restructuring During ES cell Differentiation.....	45
Semi-quantitative RT-PCR Analysis of the <i>Ant4</i> Transcript During ES cells Differentiation.....	46
5-aza-dC Treatment Reactivates <i>Ant4</i> Transcript Expression.....	47
Dnmt3 Activity Is Required for <i>Ant4</i> Repression.....	47
<i>Ant4</i> is a Germ Cell-specific Gene .....	48
<i>Ant4</i> Promoter DNA Methylation in Normal Adult Organs.....	50
4 DISCUSSION.....	76
On Newly Identified Differentially Methylated Genomic Regions .....	76
On Newly Identified DNA Methylation Controlled Genes.....	77
On the Link Between DNA Methylation and Germ-specific Genes .....	78
On the Newly Identified Adenine Nucleotide Translocase, <i>Ant4</i> .....	80
LIST OF REFERENCES.....	84
BIOGRAPHICAL SKETCH .....	91

## LIST OF TABLES

<u>Table</u>	<u>page</u>
1-1 Molecular mechanisms of <i>de novo</i> DNA methylation .....	19
3-1 Characteristics of MSRF derived differentially methylated fragments in ES differentiation model. ....	53

## LIST OF FIGURES

<u>Figure</u>	<u>page</u>
3-1 Morphology of ES cells and methylation sensitive restriction fingerprinting (MSRF) method outline. ....	52
3-2 RT-PCR analyses of levels of <i>RIKEN</i> cDNA 1700034J06, <i>Viaat</i> , <i>Nr4A1</i> , <i>AK005608</i> , and $\beta$ - <i>actin</i> transcripts during ES cell differentiation. ....	54
3-3 Germ cell-specific genes are repressed by DNA methylation during ES cells differentiation. ....	55
3-4 Germ cell-specific genes are repressed by DNA in NIH3T3 somatic cell line. ....	56
3-5 Germ cell-specific promoters are repressed by DNA methylation during ES cell differentiation. ....	57
3-6 Identification of differentially methylated CpG-rich fragment in ES cells and EBs. ....	58
3-7 MF1 genomic localization. MF1 nucleotide sequence is identical to exon 1/intron 1 boundry region of a previously unknown sequence, RIKEN cDNA 1700034J06. ....	59
3-8 RIKEN cDNA 1700034J06 encodes Ant4, a novel isoform of adenine nucleotide translocase. ....	60
3-9 Transcription initiation site of the Ant4 gene determination using 5'RACE sequencing. ....	61
3-10 Analysis of <i>Ant4</i> promoter activity by a luciferase reporter assay in R1 ES and NIH3T3 cells. ....	62
3-11 Promoter hypermethylation silences expression of <i>Ant4</i> . Analysis of <i>Ant4</i> promoter activity by a luciferase reporter assays in R1 ES cells. ....	63
3-12 Ant4 promoter region is hypomethylated in undifferentiated ES cells. ....	64
3-13 Ant4 promoter region undergoes DNA methylation in differentiated EB cells. ....	65
3-14 Ant4 promoter region undergoes covalent histone 3 changes during ES cell differentiation. ....	66

3-15	Ant4 is repressed by DNA methylation during ES cell differentiation.....	67
3-16	Dnmt3 is required for Ant4 repression during ES cell differentiation.....	68
3-17	Dnmt3 is required for Ant4 repression during ES cell differentiation.....	69
3-18	Ant4 is expressed selectively in testis.....	70
3-19	Localization of <i>Ant4</i> demonstrated by <i>in situ</i> hybridization. ....	71
3-20	Ant4 is expressed in male and female germ cells. ....	72
3-21	<i>Ant4</i> is expressed in primordial germ cells. ....	73
3-22	Bisulfite analysis of the <i>Ant4</i> promoter in various organs from adult mice.....	74
3-23	COBRA analysis of the <i>Ant4</i> promoter for DNA methylation levels in various adult mice organs. ....	75
4-1	Disruption of <i>Ant4</i> .....	83

Abstract of Dissertation Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Doctor of Philosophy

IDENTIFICATION AND INITIAL CHARACTERIZATION OF GENES  
CONTROLLED BY DNA METHYLATION IN EMBRYONIC STEM CELLS

By

Nemanja Rodić

August 2005

Chair: Naohiro Terada

Major Department: Molecular Cell Biology (IDP)

Capacity for cellular differentiation is governed by the repertoire of available transcription factors and by the accessibility of cis-regulatory elements. Studying changes in epigenetic modifications during stem cell differentiation will help us to understand how cells maintain or lose differentiation potentials. I have investigated changes in DNA methylation during pluripotent embryonic stem (ES) cell transitions into differentiated cell types. Using a methylation-sensitive restriction fingerprinting method, I identified a novel adenine nucleotide translocase gene (*Ant4*) that was selectively hypomethylated and expressed in undifferentiated ES cells. In contrast to other pluripotent stem cell-specific genes, *Oct4* and *Nanog*, repression of the *Ant4* gene during ES cell differentiation was dependent on functional Dnmt3 expression. Additionally, 5-aza-2'-deoxycytidine readily up-regulates the *Ant4* gene but not the *Oct4* or *Nanog* gene in differentiated cells. These results indicate that DNA methylation plays a primary role in the transcriptional silencing of *Ant4* during stem cell differentiation. While the deduced

amino acid sequence of Ant4 is highly homologous to the previously identified Ant isoforms, Ant1 and Ant2, the expression of Ant4 and its promoter hypomethylation were uniquely restricted to germ cells in adult mice.

## CHAPTER 1 INTRODUCTION

### **Epigenetics**

During the early period of molecular biology studies spanning the 1930s to the 1960s, epigenetic phenomena were thought of as a form of heredity independent of the nucleus. The term epigenetics, originally coined by Waddington in 1942 (1,2), referred to a process whereby the genome gives rise to the phenotype. Such a broad definition of epigenetics would include many processes that by today's standards are not considered epigenetic. These include T-cell rearrangement, bacterial conjugation, prion-like phenomenon, and monocellular morphological heredity. With the proposition of the operon model by Monod and Jacob, the concept of a genetic program of development became available. It is with such notion of restricted cellular potency that the modern definition of epigenetics came to existence as a study of mitotically heritable gene function that cannot be explained by DNA sequence alone.

Today we consider eukaryotic gene transcription as a process that requires a competent genomic DNA sequence, availability of transcription factors, and a permissive state of the chromatin. If the surrounding chromatin state, such as CpG dinucleotide DNA methylation or covalent histone modifications (histone code), is faithfully propagated through cell division, then such regulation is called an epigenetic gene regulation. DNA methylation is the most widely studied epigenetic program. DNA methylation contributes to numerous processes, both physiological and pathological in nature. The wave of genome wide *de novo* methylation during early embryonic

development, imprinting of differentially methylated regions during gametogenesis, and DNA methylation associated with aging and cancer are all examples of how widely the epigenetic process influences cell fate.

DNA methylation was first studied as a prototypic prokaryotic immune program, restriction-modification system, targeted against bacteriophage infection (2,3). Shortly after, the involvement of DNA methylation in eukaryotic gene expression became evident during both gene silencing and X-chromosome inactivation (4). The DNA methylation within mammalian somatic nuclei occurs mostly within the context of CpG dinucleotide sequences. However, low levels of CpA dinucleotide methylation have also been reported (5-7). A 5-methyl cytosine group constitutes anywhere from 1 to 9% of all cytosine residues within a normal eukaryotic nuclei (2). In mammals, DNA methylation patterns of both single copy and repetitive regions are propagated with high fidelity in a tissue specific manner and are highly evolutionary conserved (2).

### **DNA Methylation in Development**

Gene expression changes are part of concerted cellular programs, restricted in a temporal and spatial manner. We know of several well-characterized instances in which epigenetic phenomena, mostly DNA methylation, play a decisive role during cellular differentiation. On an organism level, the Jaenisch group found that normal mouse development requires the activity of DNA methyltransferase (*Dnmt*) *Dnmt1*, *Dnmt3a*, and *Dnmt3b* methyltransferase genes (8-10). In the absence of these genes, embryonic lethality ensues. On a tissue-restricted level, it is known that mesenchymal cell line 10T1/2 commitment toward myogenic differentiation is controlled by demethylation of the myogenic determination (*MyoD*) promoter region (11). Differentiation of other cell types, such as primordial germ cells, has also been found to be controlled by DNA

methylation (12). An early embryo undergoes genome wide demethylation prior to implantation. However, after implantation most of the non-imprinted genes in the embryo proper undergo a high degree of a genome wide *de novo* methylation around embryonic day 6 (E 6). Interestingly, extraembryonic lineages (yolk sac, placenta) undergo genome wide demethylation following differentiation (13). Some genes, such as octamer binding protein (*POU5F1/Oct4*), a well studied transcription factor essential for early embryonic development, undergo delayed *de novo* methylation, around embryonic day E 6.5 (14). Primordial germ cells undergo genome wide demethylation by embryonic day E 9.5, which erases DNA methylation imprints (15,16). A subset of genes undergo parental specific methylation in migrating primordial germ cells, which ensures there will be a regulated pattern of expression of these genes in the developing embryo.

### **Epigenetic Studies in ES Cells**

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a E 3.5 embryo blastocyst. ES cells indefinitely expand *in vitro* under appropriate culturing conditions. When grown in the presence of leukemia inhibitory factor (LIF), mouse ES cells undergo cell proliferation and retain their ability to contribute to all chimeric mouse cell lineages. In the absence of LIF, ES cells undergo molecular, phenotypic, and functional differentiation dependent changes, which are considered to recapitulate the early processes of mammalian development.

This *in vitro* ES cell differentiation system has served as an excellent model to study transcription factors required for stem cell self-renewal and pluripotency (17-19). Recent studies have begun revealing that pluripotent ES cells have a unique expression pattern of Dnmts and methyl-binding proteins (MBPs) (10) and have a differential loci specific DNA methylation pattern when compared to their descendant differentiated cells

(20,21). Additionally, trophoblast stem cells also have differential DNA methylation patterns relative to trophoblast derived differentiated cells (20,22). However, due to technical limitations, the exact genomic positions of such differentially methylated regions are yet to be determined.

Judging by a nearest-neighbor analysis of ES and ES-derived differentiated cells, there are no genome wide changes in the overall levels of DNA methylation during ES cell differentiation (23). Epigenetic state of ES cells is unusually unstable, as evidenced by variation in imprinted gene expression and DNA methylation of imprinting centers (ICs) loci (24). An *in vitro* growth of ES cells is not affected by loss of genome wide DNA methylation; however the ability of ES cells to form teratocarcinomas *in vivo* is severely diminished in the absence of DNA methylation activity. Finally, the ability of ES cells to undergo differentiation into multiple lineages *in vitro* is nearly abolished in the absence of DNA methylation. In conclusion, while DNA methylation seems dispensable for ES cell maintenance, DNA methylation is required for both *in vitro* and *in vivo* ES cell differentiation.

### **CpG Islands**

The term “CpG island” was coined in the late 1970s after a characteristic appearance of DNA restriction fragment on an agarose gel. The Bird group first detected such “islands” of DNA with an average size of 1kb when genomic DNA from a variety of vertebrates was digested with HpaII and HhaI restriction enzymes (2). Such regions were later found to represent non-repetitive DNA segments. Today, we know that more than half of all genes in the mouse genome contain CpG islands in their respective 5'end region. A CpG island is a genomic sequence that satisfies three factors: (i) at least 200bp in length, (ii) >50% sequence containing cytosine and guanine nucleotides, and (iii)

observed/expected CpG content of  $>0.6$ . However, it is imperative to remember that on a genome wide scale, the majority of available CpG dinucleotides are present within the context of highly repetitive sequences. In fact, more than half of all methylcytosines are present in satellite DNA (2). There is an appreciable amount of tissue variation in satellite DNA methylation. For instance, in premeiotic spermatogonial cells, both major and minor satellites are undermethylated relative to somatic cells. Such undermethylation is thought to play a role in pairing and recombination (2).

Changes in gene specific and genome wide DNA methylation are referred to as *de novo* DNA methylation events. In vertebrate organisms, DNA methylation is observed in CpG island regions as well as on a genome wide basis. Genome wide methylation is a vertebrate characteristic, and it is not observed in invertebrate organisms. However, gene specific methylation precedes the invertebrate to vertebrate boundary, suggesting that it might be an evolutionary more conserved mechanism of gene control (25).

A CpG island is an important factor that participates in the regulation of *de novo* methylation. CpG islands have been proposed to contain functionally important CpG sites within a DNA sequence, such that they might present unique molecular targets for trans-acting factors (26). Supporting this model is the work from the Grummt group concerning the DNA methylation dependent repression of ribosomal genes in mammalian cells. Santoro and Grummt provided clear evidence supporting the existence of critical CpG residues in ribosomal promoter regions (27). Furthermore, specific methylation of such critical CpG sites is required and necessary for transcriptional repression of ribosomal genes. Additionally, methylation of CpA sequences has been proposed to potentially serve as a target of *de novo* methyltransferase activity and proceeds

methylation of entire CpG islands (7). Methylation density also plays a decisive role in the maintenance of transcriptional repression in a given genomic locus. Partially methylated viral constructs do not repress reporter gene transcription as well as fully methylated constructs (28). Finally, certain CpG islands from highly expressed genes, such as the adenine phosphoribosyltransferase (*APRT*) gene, can protect neighboring flanking DNA sequences from differentiation induced methylation (29,30).

### **DNA Methylation Patterns**

Historically, DNA methylation has been studied as a developmental program that controls the expression of endogenous genes, and has been shown to be an important factor in transcriptional repression of numerous retroviral sequences. DNA methylation has been implicated as a dominant factor in chromatin condensation during development (31). An *in vitro* methylated DNA fragments is maintained through subsequent mitosis, with a repressed chromatin state and a repressive histone code faithfully propagated (32). Methylation induced blockage of transcription is most likely mediated by blocking the RNA polymerase initiation step (32).

### **DNA Methylation of Tissue-Specific Genes**

DNA methylation is a part of repressive transcription complexes that control multiple genomic loci. One group of such loci are tissue-specific genes. For instance, neuroepithelial cell differentiation into astrocytes is precluded by DNA methylation of an important cell differentiation gene marker, glial fibrillary acidic protein (*GFAP*) (33). *GFAP* promoter methylation increases during differentiation, which prohibits the transcription factor, signal transducer and activator of transcription 3 (*STAT3*), from binding. This results in transcriptional repression of *GFAP*. Another tissue-specific model devised by the Rosenfeld group provides insight into how neuronal-specific genes

are repressed in non-neuronal cell types. DNA methylation, as well as histone deacetylation, represses specific target genes by recruiting additional repressor factors. One such repressor factor, neuron-restrictive silencer factor (NSRF), binds target promoter regions and represses neuronal gene expression in non neuronal tissues *in vivo* (34). Repressor molecules can provide molecular platforms to allow binding of multiple heterochromatic factors, such as heterochromatin protein 1 (HP1) and Dnmts. However, repressor molecules can associate with a wide range of functionally distinct molecular complexes depending on the state of differentiation. Harter and Mal show that the MyoD transcription factor complexes with histone acetyl transferase (HAT) and histone deacetylase (HDAC), in myoblasts and myotubes, respectively (35).

DNA methylation also regulates *Oct4* in somatic cells in a methylation-dependent manner (36). This methylation is a trans-factor dependent process in which repression is mediated at the transcription level, and absence of methylation is correlated with high expression of the *Oct4* transcript. A region in 5'proximity of the *Oct4* transcription start site, the so called proximal enhancer region, was implicated as a cis-acting factor necessary for *Oct4* locus demethylation in *Oct4* expressing cells (14). Additionally, the homeobox (*Hox*) genes, a set of genes important in axial patterning, are transcriptionally inactivated in certain somatic organs. The *Hox* genes exhibit tissue specific expression, deregulation in malignant cells, and transcriptional downregulation in embryogenesis . The *HoxA5* gene is transcriptionally inactivated in somatic cells, as is the whole neighboring locus spanning ~25kb (37).

DNA methylation in germ cells represents another important topic in differentiation-dependent methylation. DNA methylation is a critical factor involved in

the propagation of imprints (38), and has been used widely as an imprinting status marker in numerous studies. General chromatin factors such as DNA methyltransferase proteins Dnmt3a and Dnmt3L were found to be essential in the establishment of maternally derived imprints (39). Other locus specific factors, such as the zinc finger containing protein (CTCF), were found to be essential for the activity of certain ICs, such as the IC for Igf2 and the H19 imprinting locus (40). Imprinting might not be the only target of DNA methylation in germ cells. It also appears that DNA methylation can dominantly control the cell-intrinsic program of differentiation from primordial germ cell derivatives into post migratory germ cells (12). As such, DNA methylation is implicated as being part of an intrinsic cellular memory mechanism, controlling cellular differentiation without extracellular instructions.

### **DNA Methylation in Cancer**

It is widely accepted that the alteration of chromatin structure is one of the hallmarks of cancerous cells. The Dnmt1 is known to increase in activity in the latter stages of cancer progression, and is thought to be responsible in part for the genome wide *de novo* methylation that is commonly found in cancer (41). It is also known that Dnmt1 is transcriptionally upregulated by c-fos, and that this up-regulation might be responsible for at least a part of the methylation changes seen in early transformation. Dnmt1 up-regulation is followed by a genome wide *de novo* wave of methylation, and it is required for the transforming ability of the proto oncogene (c-fos) gene (41). However, the opposite effect of DNA methylation, that is, genome wide hypomethylation, is another hallmark of cancer. A recent study by the Jaenisch group points out that the Dnmt1 hypomorphic expression can cause a genome wide hypomethylation that in turns leads to an increase in chromosomal breaks (42). This crucial finding points out that DNA

methylation is not only associated with cancer, but that DNA methylation alterations are actually involved in cancer initiation and progression.

Histone modifying factors, such as histone deacetylase and methylase activities, are commonly found in promoter regions of aberrantly regulated genes involved in carcinogenesis (43). Numerous target genes undergo *de novo* methylation/demethylation events during tumorigenesis. Numerous genes involved in diverse cellular processes such as: cell cycle regulation (*p16*, *p21*), metabolism (asparagine synthetase), differentiation (*AML-RAR*, *pinin*), and adhesion (*E-catherin*) are commonly affected by methylation-dependent changes in human cancer. This suggests that methylation plays an important role in cancer (44). Cancer-related *de novo* DNA methylation is thought to require cell division. For instance, methylation of the tumor suppressor p16 can only take place when cells are progressing through the cell cycle (45). Nevertheless, the basic question of how *de novo* methylation targets a specific gene locus is still poorly understood. An important mechanistic study by the Pelicci group proposed a mechanism for locus specific DNA methylation, whereby transforming fusion transcription factor PML-RAR can bring about *de novo* methylation to a targeting locus by interacting with Dnmt1 and Dnmt3a proteins (46).

### **DNA Methylation Associated Factors**

Mammalian DNA methylation is achieved by a family of conserved eukaryotic Dnmts 1-3, which contains a C-terminal methyl-cytosine transferase active domain. It has been difficult to determine the exact DNA methyltransferases responsible for *de novo* DNA methylation. This is most likely due to the synergistic nature and functional overlap of known Dnmts. For instance, the Li group showed that *de novo* DNA

methylation of exogenous MMLV viral sequences requires both Dnmt3a and Dnmt3b enzyme activity (10).

### **DNA Methyltransferases**

The Dnmt1 gene product is believed to be responsible for the majority of methylation maintenance, which is the activity that propagates preexisting methylation patterns following cell division. The Dnmt1 null murine embryonic stem (ES) cells retain the ability to *de novo* methylate integrated virus sequences, indicating that *de novo* machinery can operate even in the absence of Dnmt1 protein (10). The Dnmt1 transcript increases in the G1/S boundary and during S phase of the cell cycle (47). In fact, this may partially delineate mechanisms leading to hypermethylation during neoplasia. The Dnmt1 might be regulated by polyadenosine diphosphate-ribose polymerase (PARP), which regulates Dnmt1's association with proliferating cell nuclear antigen (PCNA) (47). Dnmt1 also binds, through its N-terminal domain, to HDAC2 and DNA methyltransferase associated protein 1 (DMAP1). This association implicates Dnmt1 activity in not only the maintenance of pre-existing methylation patterns, but also in the formation of new transcriptionally inactive chromatin (48). The Dnmt3b gene appears to be an important co-operator that modulates DNA methylation alongside Dnmt1. In Dnmt1 and 3b double null cells centromeric satellite sequences, imprinted genes, and tumor suppressor genes all suffer nearly a complete loss of methylation (49). This loss of methylation is associated with a severe loss in cell growth. Again, functional overlap with other chromatin factors is illustrated by the fact that human colon cancer Dnmt1 null cells largely retain normal methylation patterns. In Dnmt1 null cells centromeric satellite repeats undergo demethylation; however, gene specific methylation remains unaffected (50).

Other Dnmts include Dnmt3a, Dnmt3b, Dnmt3L, and Dnmt2. Both Dnmt3a and Dnmt3b are expressed in ES cells; they both undergo a decrease in expression after differentiation. Both Dnmts are expressed at low levels in adult tissues. Dnmt3a and Dnmt3b are thought to be responsible for *de novo* methylation, as genomic disruption of these genes causes a loss of *de novo* methylation at proviral sequences in ES cells (51). More specifically, a loss of Dnmt3a and Dnmt3b in double null ES cells leads to a loss of methylation in the *Igf2* and *Xist* genes (51). There also exist two additional Dnmt3a isoforms, Dnmt3a- $\beta$  and Dnmt3a2, in murine ES cells. Both of these proteins lack the N-terminal domain of the Dnmt3a isoform. The Dnmt3a2 isoform is produced by an internal intronic promoter (52). Since Dnmt3a2 is localized in euchromatin, and is expressed in both physiological settings, such as mouse ES cells, and in pathological settings, such as breast and ovarian cancer cell lines, it is possible that Dnmt3a2 may be responsible for *de novo* methylation (52). The Dnmt3a- $\beta$  isoform is highly expressed in ES cells and is expressed at lower levels in adult tissues (53). Also, Dnmt3a was shown to methylate DNA in a distributive manner, which is a characteristic of *de novo* methylase activity (54). A cooperation between Dnmt3a, Dnmt3b and Dnmt1 is illustrated by the Jones group, where Dnmt3a and Dnmt3b double null ES cells and Dnmt1 null ES cells were used to study novel hypomethylated genomic targets. Mouse LINE-1 promoters were found to be extensively hypomethylated in all null ES cells (55). Also, these investigators showed that Dnmt1 could not methylate certain target genes in the absence of Dnmt3a and Dnmt3b proteins. An additional Dnmt enzyme, Dnmt3L, is known to be a catalytically inactive Dnmt-like protein. Furthermore, Dnmt3L binds to other Dnmts to modulate maternal imprinting (39) and is exclusively expressed in ES

cells and testis (56). Also, Dnmt3L contains a zinc finger region, which is thought to modify protein DNA interactions (56). Genomic deletion of Dnmt3L exhibit maternal infertility, with a loss of imprinting in maternally imprinted genes (57). The significance of this finding lies in the fact that Dnmt3L knockout studies led, for the first time, to sequence-specific DNA methylation changes. To date, Dnmt3L is the only known gene to selectively influence locus-specific methylation *in vivo* (57). Cooperation between Dnmt3L and Dnmt3a has been implicated in genome wide methylation, which includes ICs and endogenous repetitive sequence methylation (58). Lastly, Dnmt3L did not synergize with the Dnmt3b enzyme in *de novo* methylation of small nucleolar RNAs (SNRPN) IC region (58).

### **DNA Methylation Associated Proteins**

Heterochromatin-related proteins are thought to be important in the maintenance of a repressive epigenetic code, that is in translating DNA methylation as an actual repressive message. Methyl-binding proteins (MBPs) complexed with multiple protein factors can recognize methylated DNA as a molecular binding platform. The methyl-CpG-binding protein 2 (MeCP2) and the HDAC complex were first precipitated together in extracts from frog embryos; this finding paved the way for an understanding of molecular synergy between DNA methylation and histone modifications (59). However, MBPs have also been implicated in binding transcriptional activators. For instance, MBP interacting protein (MBPin) was found to relieve MeCP2 transcriptional repression (60). Interestingly, this relief from transcriptional repression comes without any loss of methylated CpG bases. Another major repressor molecule is heterochromatin protein 1 (HP1), initially identified as a protein responsible for position effect variegation (PEV) in mice (61). The PEV refers to a chromosomal context dependent expression of a

transgene. The HP1 family of genes consists of three isoforms, each has distinct nuclear localization and numerous genomic targets. Isoforms are known to interact and also antagonize between distinct target genes. For instance, asparagine synthetase is known to be repressed by HP1- $\alpha$  and HP1- $\beta$  overexpression, but is enhanced when HP1- $\gamma$  is selectively overexpressed (62).

### **Chromatin Related Modification and Chromatin Imposed Boundaries**

Differentiation-dependant methylation could conceivably be signal dependent as many cell-to-cell signaling occurs during differentiation. Current thinking is that reversible covalent modification largely affects the histone code, which in turn creates alternative chromatin states. One recent finding points to a novel histone kinase protein, which dominantly up-regulates inositol responsive gene (*INO*) transcription in response to a lack of inositol in the feeding media. These investigators show that the kinase acts upstream and in conjunction with histone acetylation (63).

Since DNA methylation is assumed to be a genome wide epigenetic regulatory mechanism, scientists have focused on the identification of cis-acting boundary element factors that might separate differentially regulated chromosomal regions. Studies in budding yeast provided some of the most complete data with respect to this question. The Grewal group described the existence of a heterochromatic epigenetic code across a mating type locus region spanning around 20kb of DNA. Heterochromatin is separated by inverted repeat DNA elements, which serve as boundary elements between euchromatin and heterochromatin. Heterochromatin contains highly methylated histones across the entire region. Also, the same investigators showed that the histone modification was carried out by the heterochromatin protein homolog (Swi6) protein (64). Heterochromatin initiation was also found to depend on RNAi machinery (65).

Studies in yeast thus defined basic concepts of heterochromatin initiation and spreading. Other loci are implicated in organizing chromatin into functional segments. For instance, tissue-specific heterochromatic proteins are also involved in the silencing of targeted genes in their given cell populations. The nuclear matrix association region-binding protein (SATB1) is a thymocyte-specific nuclear protein, which is distributed through thymocytes' heterochromatin where it is required for both silencing and activation of target genes (66).

### ***De Novo DNA Methylation***

The molecular mechanism of *de novo* DNA methylation is not entirely clear. Practically, this means that although we know of proteins responsible for *de novo* DNA methylation, namely Dnmt3a and Dnmt3b, we have little knowledge as to how these molecules are selectively recruited to distinct genomic sites *in vivo* (Table 1-1).

During the past five years, two proposed models have gained grounds. The first model was originally proposed by the Pelicci group (Table 1-1). This so called “recruitment” model proposes that DNA methyltransferases are recruited to selective genomic loci by the action of sequence-specific transcription factors (46). Supporting this model is the study by the Kohn group, where reporter gene DNA methylation-dependent repression can be relieved by point mutations that disrupt repressor transcription factor binding or by the creation of binding sites for transcription activators (67). The Pelicci group showed that a fusion generated an aberrant PML-RAR transcription factor that is responsible for the recruitment of DNA methyltransferase proteins to targeted promoters (46). Recently, this same group reported that the well-known transcription factor (c-myc), also recruits DNA methyltransferase to target gene promoters. The interaction with DNA methyltransferases is critical in c-myc mediated

repression of the p21 promoter, and is also necessary for c-myc mediated cellular transformation (68). In support of this model, recent data points out that small interfering RNAs (siRNAs), mediated transcriptional repression in human cells operates by recruiting *de novo* DNA methylation to target promoters (69,70).

The “protection” model, postulates that genomic region undergoes *de novo* DNA methylation in the absence of protection from transcription factors (Table 1-1). Such a model does not require the existence of proper *de novo* activity, since DNA methylation maintenance activity could be responsible for all DNA methylation events. In support of this model, the Hsieh group provided evidence that DNA methylation of the Lac operon occurs in the absence of transcription. Independent study into molecular mechanisms of imprinting within H19/Igf2 IC also support “protection” model (71). The Bartolomei group provided evidence that CCCTC-binding factor (CTCF) protects the maternal allele from constitutive action of Dnmts, thereby acting as a protection molecule (72). Consistent with this idea, the Karlsson group showed that lentivirus proviral sequences are more resistant to differentiation-dependent repression when transcriptional activator, stimulatory protein 1 (Sp1), binding site is introduced into the promoter region (73). Finally, it is entirely possible that there could be several *de novo* DNA methylation mechanisms, each operating independently and requiring distinct molecules.

### **Drugs That Affect DNA Methylation**

Historically, one of the most frequently used drugs for disrupting DNA methylation has been 5-aza-2'-deoxycytidine (5-aza-dC). This 5-aza-dC drug is a nucleotide analog and has multiple effects on both DNA and RNA metabolism; however, its effect on disruption of genomic DNA methylation patterns is mediated through covalent trapping of DNA methyltransferase proteins (74). It is important to note that human Dnmt1 null

and Dnmt 3 double null cells exhibit sensitivity to 5-aza-dC, even though their genomes are virtually devoid of any detectable methylated cytidines (49). A molecule with opposing effects, S-adenosyl methionine (SAM) has been used to increase genome wide DNA methylation by providing ample methyl groups in cells. The SAM molecule can also be used to delay genome wide demethylation processes (75).

### **Nutrients That Affect DNA Methylation**

A lack of numerous molecules, mainly SAM and folate, has been causally related to genomic wide hypomethylation. A low availability of these nutrients can serve as an initiator for localized disruption of DNA methylation (76). Also, deficiencies of zinc and selenium can lead to genome wide hypomethylation and a greater incidence of cancer (77). Gene-to-nutrient interactions have been documented for methylenetetrahydrofolate reductase (MRHF), an enzyme important in methyl group metabolism. This gene has been associated with a loss-of-activity point mutation that leads to inadequate levels of methyl groups and an elevation of blood homocysteine levels. Furthermore, MTHF mutations are implicated in numerous pathological processes such as carcinogenesis, neural tube defects, cardiovascular diseases, and colorectal cancer (76).

### **Additional Comments**

Changes in DNA methylation during cellular differentiation and even cellular transformation towards cancer has been recognized for nearly two decades (78). DNA methylation is a heritable and stable form of epigenetic modification that is used in the reprogramming of germ cells and in a developing embryo. In fact, one of the major issues in reprogramming somatic cells towards pluripotency is a genome wide DNA methylation instability (79).

Still, little is known as to how such genome wide changes occur. Currently it is suggested that epigenetic inheritance is maintained by the histone code, an array of post-translation modifications of histone molecules (80). DNA methylation is a dominant factor involved in the formation and propagation of heterochromatin, a condensed form of chromatin. Characteristics of heterochromatin include histone hypoacetylation and methylation (81). The causality between two forms of epigenetic memory, DNA methylation and histone modification, remains to be determined. Experiments indicate the presence of either independent or combined pathways of heterochromatin formation (34,81). A more complete understanding of the genomic targets of such epigenetic machineries will help to elucidate the pathways by which DNA methylation exerts its role on cellular differentiation.

### **Study Design and Rationale**

An *in vitro* ES cell differentiation model has served as an invaluable tool in studies of early development. During differentiation, ES cells can give rise to multiple cell lineages, which can be purified and used for transplantation studies or functional analysis. Such developmental plasticity is thought to depend, at least in part, on the unique epigenetic state of ES cells.

Thus far, little is known about the molecular targets of such ES-derived epigenetic machinery. To that end, we tried to identify genomic regions that are the targets of ES associated DNA methylation activities. Using a methylation sensitive restriction fingerprinting (MSRF) method, we identified 10 unique genomic loci, which undergo ES cell differentiation dependent DNA methylation. In the second portion of our study, we focused our attention on a single genomic locus corresponding to a newly identified gene, adenine nucleotide translocase 4 (*Ant4*). We showed that DNA methylation plays a

primary role in *Ant4* regulation, both in an ES cell differentiation model and in an adult animal model.

In conclusion, the overall goal of our study was to identify genomic targets of ES cell associated epigenetic machinery, mainly DNA methylation. The knowledge of such genomic targets would broaden our understanding concerning the unique epigenetic state of ES cells (82).

Table1-1 Molecular mechanisms of *de novo* DNA methylation

---

<b>Recruitment Model (via)</b>	
c-myc	Fuks <i>et al.</i> , 2005
siRNA	Taira & Kawasaki, 2004
PRL-RAR	Pellici <i>et al.</i> , 2002
PWWP domain	Li <i>et al.</i> , 2004
Dnmt3L	Li <i>et al.</i> , 2002
NSRF/REST	Rosenfeld <i>et al.</i> , 2002

---

<b>Protection Model (via)</b>	
Lac Repressor	Hsieh <i>et al.</i> , 2001
Promoter activity	Boon <i>et al.</i> , 2004
CTCF	Bartolomei <i>et al.</i> , 2004

## CHAPTER 2 METHODS AND MATERIALS

### ***In Vitro* ES Cell Differentiation**

ES cells (R1, J1, Dnmt3a Dnmt3b double null) were maintained on a gelatin-coated dish in ES maintenance medium containing 1000 U/ml recombinant mouse LIF (ESGRO; Chemicon International, Temecula, CA) as described previously (83). ES cells were differentiated using a hanging drop method. Briefly, ES cells were trypsinized, washed twice with ES differentiation medium and suspended in the same medium at a concentration of 100 cells/ml. Approximately fifty drops were plated on the lid of a 10 cm petri dish and cultured as hanging drops. After 72 hours, 25 drops containing embryoid bodies (EBs) were collected and cultured in a 6-well plate. Media was then replaced every other day. ES differentiation medium consists of Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 300 µM monothioglycerol.

### **Methylation Sensitive Restriction Fingerprinting (MSRF)**

The MSRF method was performed according to the original method established by the Huang group (84). Briefly, ES cells and EBs were harvested by gentle cell scraping followed by a 5 minute centrifugation in a bench-top centrifuge. Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Extracted DNA was digested with a methylation-insensitive restriction enzyme, MseI, either by itself or in combination with a methylation-sensitive restriction enzyme, BstUI,

at 10 units / 1 mg of DNA for each enzyme. Digestion with BstUI was performed for 2 hours at 60°C, followed by overnight incubation with MseI at 37°C. The PCR reaction was performed in 20 µl reaction mixtures containing 2 µl of digested DNA (50 -100 ng), 0.4 µM primers, 1.25 U of HotMasterTaq DNA polymerase (Eppendorf, Hamburg Germany), 200 µM deoxynucleotide triphosphates, 1 mCi/µl [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol, Amersham, Piscataway, NJ), and 2 µl of 10X reaction buffer. The primers were the following: Bs-1 5'-AGCGGCCGCG-3', Bs-2 5'-GCCCCCGCGA-3', Bs-3 5'-CGGGGCGCGA-3', and Bs-4 5'-ACCCACCCG-3'. Following mixing of PCR reagents, samples were overlaid with mineral oil. The PCR reaction consisted of initial denaturation step for 5 minutes at 94°C. Following thirty cycles were made up of denaturation for 2 minutes at 94°C, annealing for 1 minute at 40°C, and extension for 2 minutes at 72°C. The final extension step lasted for 8 minutes. After PCR amplification, 5 µliters of each sample was mixed with 1 µliter of 6X loading dye solution. 2.5 µliters duplicates of each sample were loaded using extrafine ("sequencing type") gel tips. A 4.5 % non-denaturing polyacrylamide gel with sharktooth combs to separate each loading lane were used. Sequencing aparata used were either BRL Gibco Models S2 or SA. Samples were ran for approximately 4 hours at 200 Volts and 25 miliAmps. All reactions were run as duplicates to account for loading error. Wet gels were laid on 3M filter paper cut slightly larger than the gel itself. Gels were then wrapped with plastic wrap and exposed to Kodak X-OMAT film for 24-48 hours at -80°C.

### **MSRF Cloning and Sequencing**

Positive fragments from MSRF screening were excised from polyacrylamide gels using a sterile scalpel. DNA was eluted by incubation of gel fragments in 50 ml sterile deionized water for 10 minutes at 100°C. Eluted DNA was re-amplified by the identical

primers and PCR conditions as used in the MSRF method. Re-amplified DNA fragments were then ran on a 2% agarose gel and excised from the gel. Fragments were ligated into a TA-cloning vector using pCRII-Topo Cloning Kit (Invitrogen), and sequenced. To determine the identity of resulting DNA sequences, searches were performed against mouse genomic databases: [www.ensembl.org](http://www.ensembl.org), <http://genome.ucsc.edu>, and <http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html>.

### **5'RACE**

RNA from undifferentiated ES cells (R1 strain) was used as a template for 5'RACE experiments. A 5'RACE reaction was performed using FirstChoice™ RLM-RACE Kit according to the manufacturer's instructions (Ambion, Inc.). Primers used were Ant4 gene specific primer 1 (5'-TCCAGAAGCCAGGTTTGCTA-3'), and Ant4 gene specific primer 2 (5'-AGTCCAGCATGCCCTTGTAG-3').

### **Construction and *in vitro* Methylation of Reporter Vectors**

Deletion fragments of the mouse *Ant4* promoter were PCR amplified from the mouse ES cells derived genomic DNA with a common antisense primer that spans -105 bp from the translational start site, (5'-CTCCCAGCATCCTCAGCGCCC-3'). We introduced a *HinDIII* restriction enzyme at the 3' end of this primer. We made various sense primers into which an *XhoI* restriction site was introduced (-2186 bp, 5'-GCAGGCTTGTTGAGCTGAGC-3'; -1166 bp, 5'-ACACTCTAGAGGCCGTGATGGG-3'; and -645 bp, 5'-ACACCAATTAAACAAACCAAC-3, -503bp, 5'-CAACAAGTCCAGGGTGACT-3', and -285bp, 5'-GCAGGAGCACTCCGCGC-3'). We constructed an Ant4 fragment lacking the predicted promoter region using (-2186 bp, 5'-GCAGGCTTGTTGAGCTGAGC-3', and -503bp, 5'-TAGTACCCTGGACTTGTTG -3'). The PCR products were digested with *HinDIII*

and XhoI restriction enzymes, gel extracted and ligated into the pGL2-Basic (Promega) promoterless and enhancerless vector. Reporter vectors were *in vitro* methylated using Sss I methylase (CpG methylase) according to manufacturer's instructions (New England Biolabs, Ipswich, MA).

### **Chromatin Immunoprecipitation**

The Chromatin Immunoprecipitation protocol is essentially a modification of the Upstate Company protocol. Briefly, cells were treated with a 1% formaldehyde solution for 10 minutes at room temperature, with gentle rotation. Formaldehyde crosslinking was quenched by addition of glycine solution to 0.125M glycine final. Following glycine addition, cells were left on a rotating plate for 5 minutes. Cells were then gently scraped, resuspended in ice cold PBS with protease inhibitors ( 0.1 PMSF, 1 $\mu$ g/ml aprotinin and leupeptin). Cells were pelleted by 5 minute centrifugation at 1500 g. Supernatant was discarded and cell pellets were resuspended in 200-300  $\mu$ liters of RIPA cell lysis solution, with protease inhibitors. Cells were kept on ice for 20 minutes, and cells were resuspended by pipetting and brief vortexing, intermittently. Nuclei were pelleted by 5 minute centrifugation at 5000 rpms, at 4°C. Nuclear pellets were resuspended in Upstate Lysis buffer, supplemented with protease inhibitors. Pellets were generally resuspended in 1.5-2 mls Upstate lysis buffer, as such volume was empirically determined to cause the least bubbling during a subsequent sonication step. Following a 10 minute incubation on ice, nuclear resuspensions were sonicated by two 30 second sonications, at power 7, using Sonic Dismembrator model 100, from Fisher Scientific Company. Such sonication conditions would consistently yield smear bands between 300-1000 bp size. A 200  $\mu$ liters of sonicated chromatin was aliquoted into separate tubes, and further processed according to the Upstate protocol as total input chromatin. An additional

aliquot was used to quantify the amount of DNA per sample by hoefer dye based flourometry. Following DNA concentration adjustment, equal amounts of chromatin were used for the immunoprecipitation step. Usually 12.5-15  $\mu$ g of sonicated chromatin was used. Dilution of sonicate DNA was done by using 2X volume of Upstate Dilution buffer solution. Next, 80  $\mu$ liters of salmon sperm protein-A-50% slurry was added to each sample. The sample was then incubated on a rotating platform for 2 hours at 4°C. After brief centrifugation, the supernatant was taken and added to 5  $\mu$ l of anti-H3 L9 acetylated antibodies or 5  $\mu$ l of anti-H3 L9 dimethylated antibodies. The sample was incubated on a rotating platform overnight at 4°C. Next day, 60  $\mu$ l of protein-A slurry was added to precipitate bound chromatin. After brief centrifugation ( 1 minute at maximum speed in a table-top microcentrifuge), supernatant was discarded and bound chromatin pellet washed with following washes: one time with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), one time with high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), one time with LiCl buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl), and two times with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Every wash lasted for 10 minutes on rotating platform, at 4°C. One-minute centrifugation was performed using Ultrafree-MC 45  $\mu$ m pore, Millipore column to separate bound chromatin slurry from wash solutions. This prevented uneven separation of bound chromatin between samples. Elution of bound chromatin was done with elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>). Samples were placed on a rotating platform at room temperature for 15 minutes, and intermitently vortexed. Protein-DNA crosslinks were reversed by treatment with 5M NaCl for 4 hours at 60°C. Samples were

further treated with proteinase solution (0.5 M EDTA, 1M Tris-HCl pH 6.5, Proteinase K 10mg/ml) for one hour at 45°C. DNA was extracted by Quiagen PCR purification kit according to manufacturer's instructions.

### **FLAG Tagged Ant4 Protein Overexpression**

Full length Ant4 cDNA was amplified using following the primers: pTYF\_ant3.f 5'-CGCGGATCCATGTCGAACGAATCCTCCAA-3' and pTYF\_ant3.r 5'-CTAGACTAGTTTAATCTCCTGATGAACTAC-3'. The DNA fragment corresponding to the predicted size of Ant4 was digested with BamHI and SpeI restriction enzymes, and the fragment was cloned into a pTYF-EF vector. The pTYF -EF vector contains a constitutive CMV promoter and 2XFLAG sequences in the N-terminal region. Transient transfection into NIH3T3 mouse fibroblast cells was performed using Fugene 6 reagent according to manufacturer's instructions (Rosche, Inc). Two days following transfections cells were collected by centrifugation.

### **Untagged Ant4 Protein Overexpression**

J1 ES cell RNA was used as a template for reverse transcription polymerase chain reaction to obtain the cDNAs of mouse Ant isoforms: Ant1.f (5'-ATAAGAATGCGGCCGCAGCATGGGGGATCAGGCTTTGAG-3'), Ant1.r (5'-GCGGGATCCTTACACATATTTTTTGTATCTCATC-3'), Ant2.f (5'-ATAAGAATGCGGCCGC<sup>T</sup>TTCAACATGACAGATGC-3'), Ant2.r (5'-GCGGGATCCTTATGTGTATTTCTTGATCTCATC-3'), Ant4.f (5'-CCGCTCGAGAACATGTCGAACGAATCCTCC-3'), and Ant4.r (5'-GCGGGATCCTTAATCTCCTGATGAACTACCTCC-3'). Full length cDNAs were cloned into the pcDNA3.1-Hygromycin(-) vector (Invitrogen). The DNA sequencing was used to confirm positive clones.

### **Western Blotting**

Cell pellets are dissociated in RIPA buffer (1M KPO<sub>4</sub>, 250 mM EDTA, pH8.0, 200 mM EGTA, pH8.0, 1 M MgCl<sub>2</sub>, 1 M B-GP, 10 % NP-40, 0.1 M Na<sub>3</sub>VO<sub>4</sub>) with protease inhibitors (1M dTT, 17.4 mg/ml PMSF, 2 mg/ml aprotinin, 2 mg/ml pepstatin) for 20 minutes at 4°C. Cells were then briefly spun and the pellet was discarded. Following the addition of loading buffer, supernatant was boiled for 5 minutes. Protein was transferred onto a nitrocellulose membrane using the BioRad transfer apparatus. The membrane was preincubated in blocking solution (4 % BSA or 5 % nonfat dry milk), on a rotating platform, for one hour at room temperature. Two µl of primary mouse anti-FLAG antibodies in 10 mls of 1X TBST were added to the membrane, which was left on a rotating platform for one hour at room temperature. Next, five brief rinses in 1 X TBST solution (1 % Tween 20) were performed. Two µl of secondary anti-mouse antibody was added, and the membrane was rotated for 30 minutes at room temperature. Finally, five brief rinses with 1X TBST were performed, and the membrane was developed using Kodak X-OMAT film in a dark room.

### **Immunohistochemistry**

The full length Ant4 cDNA was amplified using the following primers: forward (5'-CGCGGATCCATGTCGGAACGAATCCTCCAA-3'), and reverse (5'-CTAGACTAGTTTAATCTCCTGATGAACTAC-3') and cloned into pTYF-EF-FLAG, which possesses two repeats of FLAG-tagged sequences under the control of the EF-1 promoter. The vector was co-transfected with pTK-Hyg to isolate stable NIH3T3 clones expressing N-terminally FLAG-tagged Ant4 protein. Stably transfected cells were washed with ice cold PBS and fixed with pre-chilled 100% methanol for 10 min. After the fixation, cells were washed with PBS, and incubated with primary antibodies (anti-

FLAG, Stratagene, Inc; anti-HSP60 Santa Cruz, Inc) at room temperature for 1 hour. After the first incubation, cells were washed with PBS, and incubated with secondary anti-mouse or goat IgG antibodies conjugated with rhodamine or fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min at room temperature. Immunostained cells were examined using inverted fluorescence microscopy (Olympus, Inc.).

### **Bisulfite Sequencing and Combined Bisulfite Restriction Analysis (COBRA)**

Genomic DNA was extracted from ES cells, EBs and adult tissues using the DNA Wizard Genomic DNA Purification Kit (Promega). Mouse testis were decapsulated and seminiferous tubules were collected for analysis. A bisulfite reaction was performed using EZ DNA Methylation Kit (Zymo Research, Orange, CA). A genomic DNA, 2 µg, was used for conversion with the bisulfite reagent. Approximately 80 ng of precipitated bisulfite converted DNA was used as a template for each PCR analysis. Primers used for Ant4 cobra analysis and for bisulfite sequencing were 5'-TTGTTGTGTATTGATTGAGTATG-3' and 5'-ACACTAAAAAAACTAAAAAAC-3' (40 cycles). Primers used for extensive bisulfite mapping were: 5'-AAGGTGTGTGTTATTATTGTTTGATGT-3', 5'-TACCCCTCATCTATCATATCCCTA-3'; 5'-TTGAGGAGGAGTTAATAAGTTTAGGG-3', 5'-CCAAAAACACACTCTAACCAAATAC-3'; 5'-GTAGGTAAATTAATTGTGGATTAAATAGTA-3', 5'-TACACAACAACCTTTTACAAAAAAC-3'; 5'-AGTTGTTGTGTATTGATTGAGTATG-3', 5'-TCTTAAAAACTACTTCTTAAAAATTC-3', 5'-

TTTTTAAGAAGTAGTTTTTAAAGAAGG-3', 5'-  
 AAACAATCCAACATACCCTTATAAC-3'. Primers used for Rnf17 and Piwil2  
 bisulfite sequencing were: bsRnf17 (5'- GGGTTTAGTTTTTTTTGTTTTTTGATT-3', 5'-  
 ACCAATCCCAAACCTCCTCAC-3') and bsPiwil2 (5'-  
 TTTTAGGTTTGGTGTATTGAGTTT-3', 5'-  
 CCCACCCTTTTAAATTAACATCTAC-3'). PCR fragments were ran on a 2 % agarose  
 gel to check for presence of unique band. Fragments were subsequently cloned into  
 pCRII-TOPO cloning vector (Invitrogen) and individual clones were sequenced.

### **Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from ES cells and EBs using RNAqueous kit (Ambion, Austin, TX). Two mg of RNA was used as a template for RT reactions using SuperScript Synthesis for First Strand Synthesis Kit (Invitrogen). Sequences of forward and reverse primer pairs were as follows: Ant4 (5'-TGGAGCAACATCCTTGTGTG-3', 5'-AGAAATGGGGTTTCCTTTGG-3'), Oct4 (5'-TGGAGACTTTGCAGCCTGAG-3', 5'-TGAATGCATGGGAGAGCCCA-3'), Ttr (5'-CTCACCACAGATGAGAAG-3', 5'-CCGTGAGTCTCTCAATTC-3'), Fgf-5 (5'-AAAGTCAATGGCTCCCACGAA-3', 5'-CTTCAGTCTGTACTTCACTGG-3'),  $\beta$ -actin (5'-TTCCTTCTTGGGTATGGAAT-3', 5'-GAGCAATGATCTTGATCTTC-3'), Gapdh (5'-CCCTTCATTGACCTCACTACATGG-3', 5'-CCTGCTTCACCACCTTCTTGATGTC-3'), Hprt (5'-GCTGGTGAAAAGGACCTCT-3', 5'-CACAGG ACTAGAACACCTGC-3'), *Nr4A1* (5'-CACACGTGGTGCTCTTCTGT-3', 5'-AACCCCATCTCAACCTCTT-3'), *AK005608* (5'-GGTTGGTCAACGGTCGTATT -3', 5'-CATCACTGGTACCCCATTC-3'). Primers for Rnf17, Dazl, and Piwil2 transcripts were identical as in reference (85). The PCR reaction consisted of initial denaturation

step for 5 minutes at 94°C. Following thirty cycles (twenty five for  $\beta$ -actin transcript detection) were made up of denaturation for 1 minutes at 94°C, annealing for 1 minute at 40°C, and extension for 1 minutes at 72°C.

### **Northern Blotting**

Northern blotting was performed using multiple pre-made blots (Clontech, Palo Alto, CA; Rnway, South Korea). Briefly, the membrane was pre-incubated with 5 ml of ExpressHybrid Hybridization Solution at 68°C for 1 hour. Seventy five ng of the full length Ant4 or  $\beta$ -actin cDNA fragment was radio-labeled with [ $\alpha$ -32P] dCTP nucleotides. The Ant1 and Ant2 probes were PCR amplified using following primers: Ant1 (5'-GGCGCTACTTTGCTGGTAAC-3', 5'-GCAATCTTCCTCCAGCAGTC-3'), Ant2 (5'-CAGCTGGATGATTGCACAGT-3', 5'-CAAGCCCAGAGAATCTGTCC-3'). Unincorporated nucleotides were removed using ProbeQuant G-50 Micro Column (Amersham). Heat denatured and briefly chilled probe (~20 ng/ml) was added to the hybridization solution. The membrane was incubated with gentle shaking for 24 hours at 68°C, and washed twice for half an hour per wash in 2 X SSC/0.05% SDS at room temperature, and twice in 0.1 X SSC/0.1 % SDS at 50°C. The membrane was wrapped with plastic wrap and exposed to X-OMAT Kodak film at -80°C for 18-24 hours.

### ***In situ* RNA Hybridization**

Digoxigenin labeled Ant4 riboprobes were prepared from linearized plasmid DNA using a DIG RNA labeling kit (Roche, Basel, Switzerland). The resulting 600 bp sense and antisense riboprobes were quantitated by OD260 and checked for digoxigenin incorporation by dot blot. Liver pieces and whole testes were harvested from adult male mice and quickly frozen in OCT (Global Medical Instrumentation, Ramsey, MN). Tissues were cut at 10 mm and immediately fixed for 15 minutes in 4%

paraformaldehyde made in DEPC treated 1XPBS. Sections were pre-hybridized for 3 hours in 50% formamide, 5x SSC and 40 mg/ml salmon sperm DNA prior to addition of either Ant4 sense or antisense riboprobe at a final concentration of 500 ng/ml. Hybridizations were performed overnight at 55°C under glass cover slips sealed with rubber cement. Post hybridization washes consisted of 30 minutes in room temperature 2x SSC, one hour in 2x SSC at 65°C and one hour in 0.1x SSC at 65°C. Following equilibration in Buffer 1 (Tris 100mM / NaCl 150mM, pH 7.5), slides were blocked in Buffer 1 containing 5% normal sheep serum for 30 minutes at room temperature. This was replaced with 1:5000 alkaline phosphatase coupled anti-digoxigenin (Roche) in fresh blocking solution and incubated for 2 hours. Slides were washed in Buffer 1 twice for 15 minutes before equilibration in freshly prepared detection buffer (Tris 100 mM / NaCl 100 mM / MgCl 50 mM, pH 9.5). Slides were transferred to chromagenic substrate (100 mg/ml NBT / 50 mg/ml BCIP X-Phosphate / 3.6 mg/ml levamisole in detection buffer) and monitored for color development. The reaction was stopped after 4 hours by submerging the slides in TE buffer (Tris 10 mM / EDTA 1 mM, pH 8.0) for 15 minutes. In order to remove non-specific background, slides were placed in 95% EtOH for one hour and rinsed for 15 minutes in water. Sections were counter-stained in nuclear fast red and mounted in Vectamount (Vector Labs, Burlingame, CA). Images were captured on an Olympus BX 51 microscope equipped with an Optronics Magnafire digital camera system.

#### **Immunostaining for Ant4**

Mouse ovary, testes and liver were harvested from 3 months old BALB/c mice, and immediately frozen into OCT (Optimal Cutting Temperature) blocks (Tissue-Tek, Torrance, CA). Frozen sections were cut at 5 µm, air dried for 2 hours then fixed in

acetone for 10 minutes, before being rehydrated, and blocked for endogenous peroxidase activity. Following a serum blocking step, affinity purified rabbit anti-Ant4 (Sigma Genosys, The Woodlands, TX) was applied at 2 µg/ml and incubated overnight at 4°C. Staining was achieved using the EnVision+ HRP kit (DakoCytomation, Glostrup, Denmark) following the manufacturers directions. Positive signal was detected with DAB+ (DakoCytomation) and slides were counterstained using Light Green SF Yellowish (Sigma, St. Louis MO).

### **Primordial Germ Cell Preparation**

Primordial germ cells were obtained from timed matings of B6C3F1 mice purchased from Jackson Labs. Noon of the day on which a mating plug was first observed is taken to be 0.5 days post coitus (dpc). The PGCs were immunomagnetically purified using anti-SSEA1 antibody from isolated urogenital ridges as described (16). The 12.5 dpc genital ridges were sex separated by visual inspection for testis cords. The immunodepleted fraction refers to cells not retained on the magnetic column.

### **Targeted Disruption of Mouse *Ant4* gene**

A targeting construct was designed to replace exons 2 through 5 of the mouse *Ant4* gene with an IRES (internal ribosome entry site)-*B geo-Neo'* cassette of pNF-SIBN targeting vector. A 2.1 kb fragment containing exon 1 and a 5.3 kb fragment containing exons 5 and 6 were amplified from mouse ES cells (R1 strain) genomic DNA and used as the 5' and 3' homologous arms of the targeting construct. Targeting arms were amplified by LA Taq PCR system (Takara, Madison, WI) with following primers Ant45.f (5'-CCGCTCGAGCTCTCATTGTTTTAACTGGATACGTG-3'), Ant45.r (5'-GCGTGTCGACTGGCCCTGCACATTCTCCAAAACACC-3'), Ant43.f (5'-CCGCTCGAGGAGCCATCGCCCCGTCATATACTCTG-3') and Ant43.r (5-

CCCGCTCGAGTAATTGGTGACTTTAAGTGGGTC-3'). Either targeting arm was individually cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). Following excision from pCR2.1-TOPO vectors, the 5' targeting arm was ligated into a unique XhoI site, and a 3' targeting arm was ligated into a unique Sall site, of pNF-SIBN targeting vector. The resulting targeting vector was linearized with Sall digestion and introduced into ES cells by electroporation. Genomic DNA from G418-resistant colonies was screened for homologous recombination by Southern blot analysis.

## CHAPTER 3 RESULTS

### **Introduction**

It is becoming increasingly clear that epigenetic modifications play a critical role in the regulation of gene expression in many cellular processes (86,87). Studying changes in epigenetic modifications during stem cell differentiation will help us to understand how cells maintain or lose differentiation potentials. In the present study, we attempted to identify differentially methylated loci that are hypomethylated in undifferentiated ES cells and become hypermethylated after differentiation. Murine embryonic stem (ES) cells are originally derived from the inner cell mass of a developing blastocyst and have the ability to differentiate into all cell types of an adult animal (88). Pluripotency of ES cells can be maintained *in vitro* when the cells are cultured in a serum-containing medium supplemented with leukemia inhibitory factor (LIF) (89). When LIF is removed from the medium, the ES cells begin to differentiate *in vitro* into all three embryonic germ layers.

An *in vitro* ES cell differentiation system serves as an excellent model to study the regulation of gene expression required for stem cell self-renewal and pluripotency (17-19). Recent studies on molecules involved in epigenetic modifications have revealed a unique expression pattern of DNA methyltransferases (90), histone deacetylases (23), and methyl-binding proteins (91) in ES cells. ES cells also have a differential genome wide DNA methylation pattern when compared to their descendant differentiated cells (20,21). However, the exact genomic loci of such differentially methylated regions remain unknown.

Using methylation-sensitive restriction fingerprinting (MSRF) method to screen for genome wide changes in DNA methylation patterns during ES cell differentiation, we identified 10 differentially methylated genomic loci. One of the differentially methylated regions corresponds to a promoter region of a novel gene encoding an adenine nucleotide translocase homolog that is specifically expressed in undifferentiated embryonic stem cells and germ cells. Furthermore, we show that DNA methylation, but not the availability of transcription factors, is the main restricting factor of gene expression.

### **Identification of Differentially Methylated Genomic Regions**

#### **A Survey of DNA Methylation Screening Methods**

In a search to identify exact genomic position of differentially methylated loci during ES cell differentiation we first underwent a literature search for a suitable screening method. The first method, restriction landmark genomic scanning (RLGS), entails global, genome wide screen for differentially methylated NotI-EcoRV-Hinfi digested fragments. The RLGS method has several advantages: (i) differentially methylated NotI sites are enriched in the CpG island containing fraction of DNA, (ii) highly reproducible banding patterns can be obtained (ie, tissue specific, tumor specific), (iii) RLGS is a high throughput method, allowing for simultaneous identification of around ~ 1000 differentially methylated fragments (92). However, due to the low DNA concentration of differentially methylated DNA, the RLGS method cannot be used for nucleotide sequencing of newly identified fragments (84). Recently, the Plass group developed improved RLGS cloning conditions, allowing for nucleotide sequencing of differentially methylated fragments (93).

The second method, originally developed by the Huang group, is a PCR based methylation sensitive restriction fingerprinting (MSRF) method for the identification of

differentially methylated regions (84). Two DNA samples suspected of containing differential genome wide DNA methylation patterns (ex. normal vs disease, wild type vs knockout sample) are subjected to a two step process, which allows for a genomic assignment of differentially methylated regions. Similarly to RLGS, the DNA sample is first digested with MseI/BstUI restriction enzymes. Then next step entails PCR amplification of digested DNAs with CpG rich ten-mer primers under low stringency and low cycle number PCR conditions. The PCR amplified DNAs are subsequently separated on an acrylamide gel, allowing for visualizations of amplified DNAs. The MSRF method has been used to identify differentially methylated regions in a variety of carcinomas, including breast carcinomas (84), prostate carcinomas (94), and nasopharyngeal carcinomas (95). Some MSRF limitations include: (i) fewer absolute numbers of newly identified differential regions, mostly 1 to 3 per study and (ii) a high rate of false positive signal. In spite of these limitations, the MSRF method was the only method that could assign a genomic location to differentially methylated fragments.

### **Methylation Sensitive Restriction Fingerprinting (MSRF) Method**

The DNA samples were prepared from either undifferentiated mouse ES cells or differentiated embryoid bodies (Figure 3-1A). Mouse ES cells were differentiated in a culture medium without LIF using a hanging drop method. DNA was extracted from undifferentiated ES cells (day 0) and differentiated embryoid bodies (EBs) (days 5 and 10). The DNA was then digested by a combination of methylation-sensitive (BstUI) and insensitive (MseI) restriction enzymes, and amplified using CpG-rich 10-mer primers (Figure 3-1B). Following the original method established by the Huang group, we used a combination of 4 different CpG-rich primers. Primer pairs used in our study included combinations of different sets of primers, single primer combinations, or a combination

of three primers simultaneously. The PCR amplification step entails the use of radioactively labeled dCTP nucleotides, allowing for simultaneous and highly sensitive detection of PCR generated fragments. We underwent careful visual inspections of radiograph sensitive films and identified a total of ten DNA fragments that exhibited differential methylation patterns during ES cell differentiation. Fragments of interest were then manually excised from gels, and cloned into pCR2.1-TOPO cloning vectors prior to sequencing. The nucleotide sequences of eight out of ten fragments could unambiguously be assigned to distinct genomic locations with the use of web-based publicly accessible mouse genome sequence servers (Table 3-1). We used three mouse genomic databases: [www.ensembl.org](http://www.ensembl.org), <http://genome.ucsc.edu>, and <http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html>. Using these genomic databases methylated fragment (MF) 1 and MF 6 could not be localized to a single genomic site. MF 1 corresponded with 100% nucleotide identity to two genomic sites: (i) located on mouse chromosome 13 (genomic location of the first nucleotide is chr13\_random - 570600) and (ii) located on mouse chromosome 3 (genomic location of the first nucleotide is chr3 + 40460682). According to genomic databases, a region approximately 250 kb in size, surrounding MF 1, is duplicated, with 100% sequence conservation, on both mouse chromosome 13 and 3. Since the conservation of such extended genomic region appears unlikely, we underwent a literature search to look for genes that were included in this 250 kb region. We found 4 validated genes, two of which are well studied and characterized. The literature search revealed that one of these well studied genes, serine/threonine-protein kinase (*PLK4*), has been assigned to mouse

chromosome 13 (96). Thus, we conclude that MF 1 corresponds to a distinct genomic locus on mouse chromosome 13.

The MF 6 nucleotide sequence had greater than 98% nucleotide sequence identity with multiple genomic sites across the mouse genome (genomic location of the first three hits is chr9: 26109765, chr3: 10936716, and chr8: 72282211). The MF 6 nucleotide sequence spanned 209 nucleotides overlapping the LTR region of highly repetitive LINE element. The remainder of differentially methylated MFs were localized to unique single copy genomic regions in the mouse genome (Table 3-1).

All MFs were randomly distributed with respect to their chromosomal position, tissue specific expression and general gene architecture of cognate neighboring genes. In agreement with genome wide *de novo* DNA methylation by gastrulation, the majority of MFs were found in differentiated cells rather than in ES cells. Curiously, MFs 1, 9, and 10, spanned across the CpG island region of testis-specific genes. As predicted, we observed a non-random distribution of MFs with respect to their genomic location, with an apparent propensity for these differentially methylated regions to be positioned in the 5' end proximity of cognate neighboring genes. We found considerable variation between individual MFs and between the genomic distances to cognate neighboring genes. Five out of ten MFs were localized within a CpG island region, and six out of ten MFs were localized in the 5' end vicinity of cognate neighboring genes.

### **Identification of DNA Methylation Controlled Genes**

To further investigate the significance of MF's differential methylation during ES cell differentiation, we conducted an *in silico* analysis of cognate neighboring genes. We collected data regarding the expression pattern of neighboring genes by careful analysis of expressed sequence tags (EST). This data was available on the University of California

genome web browser. As mentioned previously, tissue specific expression of neighboring genes varied considerably. We concluded that the majorities of genes are expressed in various adult tissues, during multiple stages of embryonic development, and are potentially controlled by independent mechanisms.

Next, we analyzed the expression patterns of cognate neighboring genes using semi-quantitative RT-PCR analysis with template cDNAs from undifferentiated ES cells, and day 5 and 10 embryoid bodies (referred to as differentiated cells). As an internal control for this experiment, we analyzed *β-actin* mRNA levels, which were found to be constant during ES cell differentiation. To confirm the timely differentiation of ES cell cultures, we analyzed mRNA levels of *Oct4*, a well characterized ES cell marker and *Brachyury*, a mesodermal marker (data not shown). We were successful in correlating the appearance of differentiation and a loss of the cognate neighboring gene for 3 out of 10 MFs (Figure 3-2A and B). The mRNA expression for the remainder of cognate neighboring genes was found to be independent of MF methylation.

Curiously, MFs 1 (*RikencDNA 1700034J06*) and 10 (*AK005608*) were associated with testis-specific genes that followed a similar expression pattern. Both testis-specific genes were found to be expressed in undifferentiated ES cells and downregulated following ES cells differentiation. We considered whether the relative abundance of testis-specific genes as MF cognate neighboring genes, signifies a general tendency for all testis-specific genes. Analysis of literature revealed that well characterized germ cell genes, such as deleted in azoospermia like (*Dazl*) gene, mouse ortholog of piwi domain containing (*Piwil2*) gene, and non-bHLH-ZIP (*Rnf17/Mmip2*) gene are known to be expressed in undifferentiated ES cells and downregulated following ES cell

differentiation (97) and (Figure 3-3A). To our knowledge, there were no studies showing that the expression of such germ cell-specific genes is regulated by DNA methylation during ES cell differentiation. To approach such a question, we examined the expression patterns of germ cell-specific genes during ES cell differentiation in *Dnmt3a* and *Dnmt3b* double null ES cells. To our surprise, downregulation of *Dazl*, *Piwil2* and *Rnf17* genes was inhibited in *Dnmt3a* and *Dnmt3b* double null ES cells (Figure 3-3B). To further solidify evidence that the expression of germline-specific genes in an ES cell model is DNA methylation dependent, we treated day 8 embryoid bodies with a generalized DNA methylation inhibitor drug, 5-aza-2'-deoxycytidine (5-aza-dC). After 48 hours of 5-aza-dC treatment, mRNA samples were collected from day 10 embryoid bodies. Our data indicates that 5-aza-dC relieved downregulation of germ cell-specific genes (Figure 3-3C). Using NIH3T3 somatic cell line treated with varying concentration of 5-aza-dC we detected re-expression of of *RikencDNA 1700034J06*, *Dazl*, *Piwil2* and *Rnf17* transcripts (Figure 3-4). However, 5-aza-dC failed to relieve downregulation of the *Oct4* gene, which is highly expressed in testis in adult mice, indicating that not all germ cell-specific genes are controlled by DNA methylation during ES cell differentiation (Figures 3-3C and 3-4).

Next, we analyzed DNA methylation levels of several germ-specific promoters during ES cell differentiation. We detected uniform increase in DNA methylation levels following ES cell differentiation (Figure 3-5). Thus, we conclude that during ES cell differentiation a subset of germ cell-specific genes is repressed in a DNA methylation dependent manner.

### **Identification of a Novel ES and Germ Cell-Specific Gene Using the MSRF Method**

In order to carefully examine changes in DNA methylation at a single genomic locus, we had to choose between the newly identified MFs. A 199-bp MF 1 (Figure 3-6B) was evaluated by MSRF analyses and was found to be hypomethylated in undifferentiated ES cells when compared to day 5 and day 10 embryoid bodies (Figure 3-6A). Database searches revealed that MF 1 (Figure 3-7), mapped to the exon 1/intron 1 boundary of a previously uncharacterized gene. Computational sequence analyses showed that the identified MF 1 is part of a putative 543-bp CpG island with a GC content of 63.4% and an observed/expected CpG ratio of 0.88. The MF 1 nucleotide sequence is identical to the exon1/intron 1 boundary of a novel, previously uncharacterized transcript, *RIKENcDNA 1700034J06*, with GenBank accession number, BC050810 (Figure 3-7).

Several reasons prompted us to focus our attention on the MF 1 cognate neighboring gene, *RIKENcDNA 1700034J06*. First, *in silico* analysis of tissue-specific expression revealed that *RIKENcDNA 1700034J06* is expressed at various stages of embryonic development and in adult mice, *RIKENcDNA 1700034J06* expression is testis-restricted. A recent breakthrough paper on molecular mechanisms of ES cell maintenance by the Yamanaka group, identified nine genes whose expression is high in murine ES cells (98). Eight out of nine genes identified by Yamanaka are also found to be expressed in mouse germ line (unpublished, personal communication). Second, the *RIKENcDNA 1700034J06* predicted amino acid composition, revealed that *RIKENcDNA 1700034J06* encodes a novel member of nucleus encoded ADP/ATP carrier proteins. Members of the ADP/ATP carrier gene family are known to play an essential role in the

maintenance of ADP/ATP influx/efflux equilibrium and mitochondrial dependent apoptosis (99). Lastly, *RIKENcDNA 1700034J06* belongs to a small group of candidate neighboring genes whose expression pattern correlates inversely with MF DNA methylation, thus lending itself to a more detailed analysis.

### ***RIKENcDNA 1700034J06* Computational Analysis**

The MF 1 BLAT analysis, indicated that a cognate neighboring gene, *RIKENcDNA 1700034J06*, is a novel, and previously uncharacterized member of the mitochondrial carrier (MC) family of genes. Most MCs are localized in the inner mitochondrial membrane, where they play an essential role in providing a solubility link between the cytosol and mitochondrial matrix for a variety of solutes, including H<sup>+</sup>, aspartate, glutamate, carnithine, and ATP. All MCs form homodimers and exhibit a sequence structure of around 300 amino acids. Such a primary amino acid sequence is in essence, a triple repeat of an 100 amino acid domain, such that each domain contains two highly hydrophobic amino acids (Figure 3-8C).

We named *RIKENcDNA 1700034J06* adenine nucleotide transporter 4 (*Ant4*), since a third Ant isoform has previously been reported in humans. The Ant4 protein also contains the signature sequence motif, P-h-D/E-X-h-K/R-X-R/K-(20–30 residues)-D/E-G-(4 residues)-a-K/R-G, where *h* represents a hydrophobic and *a* represents an aromatic residue. Such signature motifs are characteristic of all adenine nucleotide transporters (Figure 3-8A). The Ant4 amino acid sequence exhibits significant similarity to two other known mouse isoforms, adenine nucleotide transporter 1 (Ant1) and adenine nucleotide transporter 2 (Ant2) (Figure 3-8A). Amino acid similarity is roughly equally distributed across the entire amino acid sequence structure. However, the Ant4 has an extended N-terminal region (first 17 amino acids) and C-terminal region (last 5 amino acids) both of

which are absent in Ant1 and Ant2 isoforms. Using publicly available genome sequences, we identified the presence of Ant4 orthologous sequences in rat, dog, donkey, and chimpanzee genomes (data not shown). The Ant4 human ortholog is present in human chromosome 4, which has previously been reported to be in synteny with mouse chromosome 13 (96). The human ANT4 deduced amino acid sequence shares 85.9% overall amino acid identity with mouse Ant4 (Figure 3-8B). In addition, the genomic architecture of 6 exons is conserved between mouse and human (data not shown). It is notable that the human genome contains at least seven *ANT* pseudogenes on the X chromosome (100). However, in contrast to *Ant4*, such pseudogenes do not reveal the conserved exon/intron architecture or translatable coding regions. All known mammalian Ant isoforms (unlike plant adenine nucleotide translocases) lack an N-terminal mitochondrial localization sequence, yet they all localize to the mitochondria (101). In agreement with this observation, Ant4 also does not contain a classical mitochondrial localization sequence; however it does localize to mitochondria when N-terminal FLAG-tagged Ant4 is expressed in NIH3T3 fibroblasts (Figure 3-8D). In conclusion, we report the identification of a novel member of the MC family of genes, *Ant4*.

### 5'RACE

The *Ant4* promoter region did not contain a classical TATA box sequence in its 5'-flanking region. To further characterize the 5' end region of the *Ant4* gene, we mapped the transcription start site (TSS) of *Ant4* mRNA by employing a 5'RACE protocol. One antisense extension primer, gene-specific primer 1, 5'-TCCAGAAGCCAGGTTTGCTA-3', was designed complementary to position +399 relative to the translation start site. Another primer, gene-specific primer 2, 5'-AGTCCAGCATGCCCTTGTAG-3', was designed complimentary to position +208 downstream of the translation start site. Gene-

specific primer 2 was also used to generate the final PCR product. An initial PCR generated fragment was obtained by gene-specific primer 1 and 5'RACE adapter primer in the presence of total RNA from undifferentiated ES cells. Gene-specific primer 2 and 5'RACE adapter primer were then used to generate the final PCR product. Gel analysis of our PCR product indicated a smeared band roughly 350 bp in size. PCR products were subsequently cloned into a pCR-II cloning vector, and individual clones were sequenced. In accordance with the sequencing results, the TSS was mapped to multiple sites at -209, -129, -118, -103, and -77 relative to the translation start site, indicating that the CpG island of *Ant4* gene spans the putative promoter region, exon 1, and part of intron 1. Figure 3-9 depicts the presence of multiple TSS relative to the translation start site.

#### ***Ant4* Promoter Transcription is Inhibited by DNA Methylation**

We used the Proscan program (<http://thr.cit.nih.gov/molbio/proscan/>) that predicts promoter regions based on scoring homologies with putative eukaryotic Pol II promoter sequences. Using the 5' end region of the *Ant4* locus, the Proscan program predicted that the promoter region spanned from -285 to -35 nucleotides relative to the translation start site. We also used a TFSEARCH computer program (<http://www.cbrc.jp/research/db/TFSEARCHJ.html>) to identify putative binding sites for any transcription factors located in the *Ant4* 5' end region. It is noteworthy that an imperfect myeloblastosis associated oncogene (*c-myb*) binding sequence, 5'-CGCCAAGG CCGTTGGGCG-3', was located immediately upstream beginning at -184 relative to the translation start site. We also detected a myelocytomatosis associated oncogene (*c-myc*) binding sequence, beginning at 5'-AA ACACGTGTT-3', located -217 relative to the translation start site. We could not identify a classical TATA binding sequence. To confirm the predicted *Ant4* promoter region and to establish that the

repression of the *Ant4* proximal promoter could be mediated by DNA methylation, we generated several *Ant4* promoter reporter constructs. Using PCR technology, we amplified DNA fragments corresponding to various *Ant4* upstream regions. We created several PCR products: (i) starting at nucleotide positions -2186 to -105 (named pGL2-2), (ii) -1166 to -105 (named pGL2-1), (iii) -645 to -105 (named pGL2-0.5), (iv) -503 to -105 (named pGL2-0.3), (v) -285 to -105 (named pGL2-0.1), (vi) and no promoter control construct, -2186 to -503 (pGL2-no promoter). Each *Ant4* promoter fragment was ligated into a pGL2-basic vector, which lacks SV40 promoter and enhancer region, using XhoI and HindIII restriction enzymes. Such a ligation approach placed the *Ant4* promoter fragments in the immediate upstream position relative to the luciferase reporter gene (*luc*). These reporter constructs were introduced into undifferentiated ES cells and into NIH3T3 somatic cell line by means of Fugene 6 dependent transient transfection. To further define the presence of any ES specific enhancer elements in the *Ant4* 5' end region, we compared the expression of various promoter constructs in ES cells and NIH3T3 cells. We were able to detect that the pGL2-0.1 fragment was able to enhance luciferase activity in undifferentiated ES cells, but not in NIH 3T3 cells, relative to the pGL2-basic vector (Figure 3-10). Using a reporter construct lacking the predicted promoter region, the pGL2-no promoter, we confirmed that a region up to -503 nucleotides upstream of the translation start site, serves as the *Ant4* promoter (Figure 3-10). We cannot exclude the possibility that other promoter regions exist in the downstream region of the *Ant4* locus (ex. promoter in intron 1). However, in light of our northern blot analysis (we detected only a single band ~1.6 kb) such possibility seems unlikely.

Next, we examined whether methylation of the *Ant4* 5' end region affects the transcriptional activity of the *Ant4* gene. An *in vitro* DNA methylation of the *Ant4* promoter reporter constructs showed that DNA methylation of the CpG residues suppressed transcription (Figure 3-11). Our findings indicate that methylation of the *Ant4* associated CpG island could potentially serve as an important transcriptional regulatory mechanism in the *Ant4* promoter region.

### ***Ant4* Genomic Locus Undergoes Epigenetic Restructuring During ES cell Differentiation**

To carefully analyze DNA methylation changes associated with the *Ant4* genomic locus, we undertook a comprehensive bisulfite sequencing analysis of the *Ant4* 5' end region. We also examined changes in a covalent modification of the nucleosomal histone proteins in the vicinity of *Ant4* multiple transcription start sites. Our data indicates extensive and selective DNA methylation of the *Ant4* promoter region following ES cell differentiation. To determine the DNA methylation patterns across the *Ant4* promoter locus during ES cell differentiation, genomic DNA from ES cells or day 10 EBs were treated with bisulfite reagent and individual clones were subjected to sequence analysis. We analyzed the *Ant4* promoter region that encompasses a total of 47 CpG dinucleotides, from -516 bp upstream of the translation initiation site to the 3'-end of exon 1, +212 bp downstream of the translation initiation site. Sequencing of individual bisulfite-converted genomic DNAs revealed that the *Ant4* promoter and associated CpG island region were mostly unmethylated in undifferentiated ES cells (Figure 3-12). In contrast, EBs at day 10 showed significant hypermethylation around the *Ant4* promoter region indicating that following ES cell differentiation the *Ant4* promoter undergoes *de novo* DNA methylation (Figure 3-13). A further upstream region (>1kb) of the *Ant4* promoter

outside of the CpG island revealed cytosine hypermethylation regardless of the differentiation status of ES cells (Figures 3-12 and 3-13). The *Ant4* promoter region, from -483 to -239 nucleotides relative to the translation start site, was found virtually devoid of any methylated residues in undifferentiated ES cells. However, following differentiation, the percentage of methylated CpGs rose to 70-80%.

Next, we employed a chromatin immunoprecipitation protocol to analyze covalent modification of nucleosomal histones during ES cells differentiation. We detected a binary pattern of histone modification of the *Ant4* promoter region (Figure 3-14A). In undifferentiated ES cells, *Ant4* promoter was associated with hyperacetylated histone 3 (H3) at lysine 9 (Figure 3-14B), while in differentiated cells *Ant4* promoter region was associated preferentially with hyper methylated H3 at Lys 9 (Figure 3-14B). In conclusion, we detected that the *Ant4* promoter region and its surrounding chromatin undergo dynamic restructuring during ES cell differentiation, and this includes extensive *de novo* DNA methylation of the *Ant4* promoter region as well as differential covalent modification of the surrounding histone molecules.

#### **Semi-quantitative RT-PCR Analysis of the *Ant4* Transcript During ES cells Differentiation**

We next measured the levels of *Ant4* mRNA using a semiquantitative RT-PCR to determine the association between silencing of *Ant4* transcription and the appearance of *de novo* DNA methylation within the *Ant4* genomic locus. Using semiquantitative RT-PCR, relative levels of *Ant4* mRNA were found to be high in undifferentiated ES cells (detectable after 24 cycles), but downregulated (detectable after 30 cycles) following ES cell differentiation (Figure 3-15A). In parallel, we measured transcript levels of two other known genes, *Oct4* and  $\beta$ -*actin*, in order to determine whether the loss of *Ant4*

expression is a gene-specific event. As expected, the relative mRNA levels of the *Oct4* transcript decreased following ES cells differentiation (Figure 3-15B), while  *$\beta$ -actin* relative mRNA levels remained unchanged (Figure 3-15B). Thus, we conclude that *Ant4* transcript levels correlate inversely with the appearance of DNA methylation during ES cell differentiation. Our results are in agreement with the widely accepted principle that single copy gene DNA methylation in eukaryotes correlates with transcriptional inactivation.

#### **5-aza-dC Treatment Reactivates *Ant4* Transcript Expression**

Analysis of transcript levels showed only trace amounts of *Ant4* mRNA expression in differentiated embryoid bodies. However, after 2-day treatment with either 0.1, 1, or 10 micromolar of 5-aza-2'-deoxycytidine (5-aza-dC), *Ant4* mRNA was reexpressed in these differentiated cells (Figure 3-15B). On the contrary, 5-aza-dC treatment did not have any measurable effect on the level of *Oct4* and  *$\beta$ -actin* mRNA expression in differentiated embryoid bodies. In parallel, we analyzed the effect of 5-aza-dC treatment on *Ant4* transcript levels in the NIH3T3 mouse somatic cell line. Similarly, 5-aza-dC treatment reactivated *Ant4* transcription in this cell line (data not shown). Our results indicate that DNA methylation could play a dominant role in *Ant4* transcriptional repression in both ES cells and in somatic cells.

#### **Dnmt3 Activity Is Required for *Ant4* Repression**

DNA methyltransferase 3a and 3b (Dnmt3a, Dnmt3b) has been proposed to play a primary role in *de novo* methylation during murine embryonic development (10). In order to investigate the role of the Dnmt3 proteins in the establishment of DNA methylation of the *Ant4* promoter during ES cell differentiation, we utilized ES cells homozygously deleted for both the Dnmt3a and Dnmt3b genes. The Dnmt3a Dnmt3b

double null ES cells were subjected to the same differentiation protocol as parental (wild type) J1 ES cells. In all of the null cells, we detected aberrant downregulation of the *Ant4* transcript following ES cells differentiation (Figure 3-16A). Of interest, *Ant4* expression was not suppressed, but rather increased in differentiated double null cells (Figure 3-16A). It is noteworthy that Dnmt3a and Dnmt3b single null cells, but not Dnmt3a Dnmt3b double null cells, exhibited partial downregulation of the *Ant4* transcript. Such downregulation could be due to partial or low *de novo* DNA methylation levels across the entire *Ant4* 5' end region. In contrast, the pluripotent ES cell marker *Oct4* was downregulated, while the primitive ectoderm marker, fibroblast growth factor (*Fgf-5*) and endoderm marker, transthyretin (*Ttr*) were upregulated following ES cell differentiation. These results indicate that Dnmt3a Dnmt3b double null ES cells proceed with a normal differentiation pattern. The COBRA assays confirmed that there was an absence of DNA methylation at the *Ant4* promoter region in the double null ES cells (Figure 3-16B).

Using a stable lentiviral expression vector we re-expressed Dnmt3a and Dnmt3b proteins in Dnmt3a Dnmt3b double null ES cells. We detected partial recovery of *de novo* DNA methylation activity following such reconstitution (Figure 3-17). These results indicate that functional Dnmt3a and Dnmt3b are required for repression of the *Ant4* gene during ES cell differentiation. We conclude that Dnmt3 activity is responsible for DNA methylation-dependent transcriptional repression of the *Ant4* promoter region during ES cell differentiation.

### ***Ant4* is a Germ Cell-specific Gene**

To determine whether the *Ant4* transcript is specifically expressed in undifferentiated ES cells, we initially used a BLAST search against EST databases. An *in silico* analysis of available EST data (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed

that *Ant4* expression is highly restricted to male germ cells. We used full-length *Ant4* cDNA as a query sequence and found a total of 8 EST derived cDNA clones (with scores of >200 bits). All clones identified were from adult testis. We then investigated expression patterns of *Ant4* in adult mouse organs using northern blot analysis. The *Ant4* mRNA was found specifically in testes, at the predicted ~1.6 kb transcript size (Figure 3-18), while the previously identified *Ant* isoforms, *Ant1* and *Ant2*, were expressed in all other non-germ cell organs, but absent in testes. More significantly, our data indicates that *Ant4* could be the dominant ADP/ATP carrier in the male germ line. The *Ant4* mRNA was undetectable in other organs including stomach, small intestine, skeletal muscle, ovary, thymus, uterus, and placenta (data not shown). In conclusion, *Ant4* expression as analyzed by northern blot, indicates a male germ cell-specific expression of the *Ant4* gene.

We then examined which cells within the testes expressed the *Ant4* transcript using *in situ* RNA hybridization (Figure 3-19). The results obtained from *in situ* hybridization with riboprobes confirmed the presence of *Ant4* mRNA within testicular tissue. The strong cytoplasmic staining of spermatogonia, spermatocytes and spermatids within the seminiferous tubules, coupled with the lack of signal in interstitial, capillary and capsular cells suggests that *Ant4* transcripts are localized in testicular germ cells. Of interest, it appears that *Ant4* expression is absent in mature sperm. Staining performed on liver sections was negative, and the sense probe did not hybridize to either tissue type as expected. These data indicate that *Ant4* is specifically expressed in murine testicular germ cells.

Northern blot analyses using whole ovaries did not detect any *Ant4* gene expression (described above). To evaluate *Ant4* expression in the developing female germ cells, mainly primordial germ follicles, we performed immunohistochemistry in adult mice ovaries. Ovaries were collected from female mouse animals, and fresh sections were frozen in liquid nitrogen. We stained ovary sections using *Ant4* purified antibodies, and found that the *Ant4* protein is selectively expressed in the cytoplasm of the growing oocytes (Figure 3-20). In contrast, staining performed on liver sections was negative. Additionally, we did not detect any *Ant4* signal in oocyte's nuclei nor in any other cell type within the ovaries (ex. follicular cells, fibrocytes, endothelial cells). The *Ant4* staining was readily detected in primordial follicular, multilaminar primary follicular, and secondary follicular stages of oocyte development. In conclusion, we found that *Ant4* is strongly expressed in developing gametes in both testis and ovary. Similar data were obtained using *in situ* RNA hybridization with riboprobes against *Ant4* transcript (data not shown).

Additionally, *Ant4* was found to be expressed in pluripotent primordial germ cells (PGCs), obtained from E11.5 and 12.5 days post coitum (dpc) genital ridges and purified using anti-SSEA1 magnetic beads (Figure 3-21). This indicates that *Ant4* is expressed in premeiotic fetal germ cells.

#### ***Ant4* Promoter DNA Methylation in Normal Adult Organs**

Since CpG-rich promoters are typically hypomethylated in all adult cells, it is important to find out whether the *Ant4* promoter region is also hypomethylated in normal adult tissues, which do not express the *Ant4* gene. To that end, we used bisulfite sequencing to analyze the *Ant4* promoter for methylation patterns between nucleotides

-250 to -55 relative to the translation start site. We collected DNAs from four adult mouse organs. Our findings demonstrate that the *Ant4* promoter sequences were uniformly and heavily methylated in the majority of adult somatic tissues (Figure 3-22). However, *Ant4* promoter sequences obtained from testis were nearly completely unmethylated, while a small minority was heavily methylated. This minority could potentially represent testicular somatic parenchymal cells, such as Sertoli cells or fibroblasts. Since the adult murine testicular tissue contains a majority of germ line cells and a minority (<15%) of somatic cells (102), our findings are in agreement with the notion that *Ant4* is selectively expressed only in germ line cells, and unmethylated only in germ cells. Using a combined bisulfite restriction analysis (COBRA) method, we detected a uniform, albeit lower methylation of the methylation sensitive HhaI restriction site located in the *Ant4* promoter (Figure 3-23). To conclude, our findings are consistent with the notion that DNA methylation is the primary mechanism controlling *Ant4* expression in adult mouse organs.

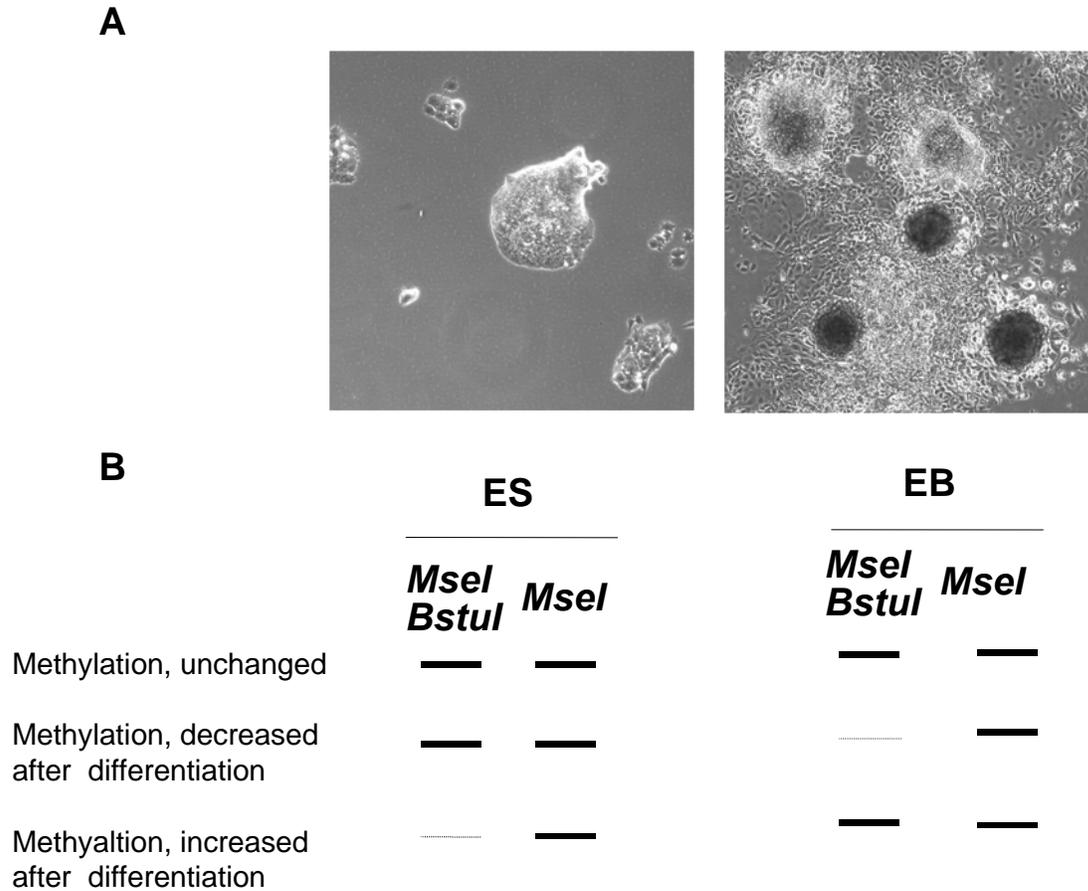


Figure 3-1 Morphology of ES cells and methylation sensitive restriction fingerprinting (MSRF) method outline. A, The ES cells grow as uniform compact colonies, while differentiated cells form cystic outgrowths with many cell types. B, MSRF outline: genomic DNA is either digested with *Bstul* or *Bstul/MseI* restriction enzymes. Diagram depicts several outcomes that can be screened for using the MSRF method.

Table 3-1 Characteristics of MSRF derived differentially methylated fragments in ES differentiation model.

MF	ES	EB 5	EB 10	Chromosomal Position of BstUI	Homology	GenBank Accession number	Context	CpG island
1				chr13_random: 570600	RIKENcDNA 1700034J06	BC050810	5'end	Yes
2				chr2: 158,677,844	Viaat	BC052020	5'end	Yes
3				chr5: 105028251	Stx18	AK007700	Body	Yes
4				chr19: 54784281	Vti1a	BC019386	Body	Yes
5				chr15: 101767270	Nr4A1	BC004770	3'end	Yes
6				chr9: 26109765 chr3: 10936716 chr8: 72282211	-	-	-	-
7				chr9: 61676565	Tle3	AK122524	5'end	No
8				chr6: 85732846	EST	AK129466	Body	Yes
9				chr1: 22511570	EST	BC055294	Body	No
10				chr15: 83722470	EST	AK005608	Body	Yes

BLAT search results using April 2005 freeze. MF, methylated fragment. White squares, no methylation. Black squares, high methylation. 5'end, the MF is found in the 5'end region of a gene or in its first exon. 3'end, the MF is found in the 3'end region of a gene or in its last exon. Body, the MF is found within the genomic structure of a gene. -, data not available.

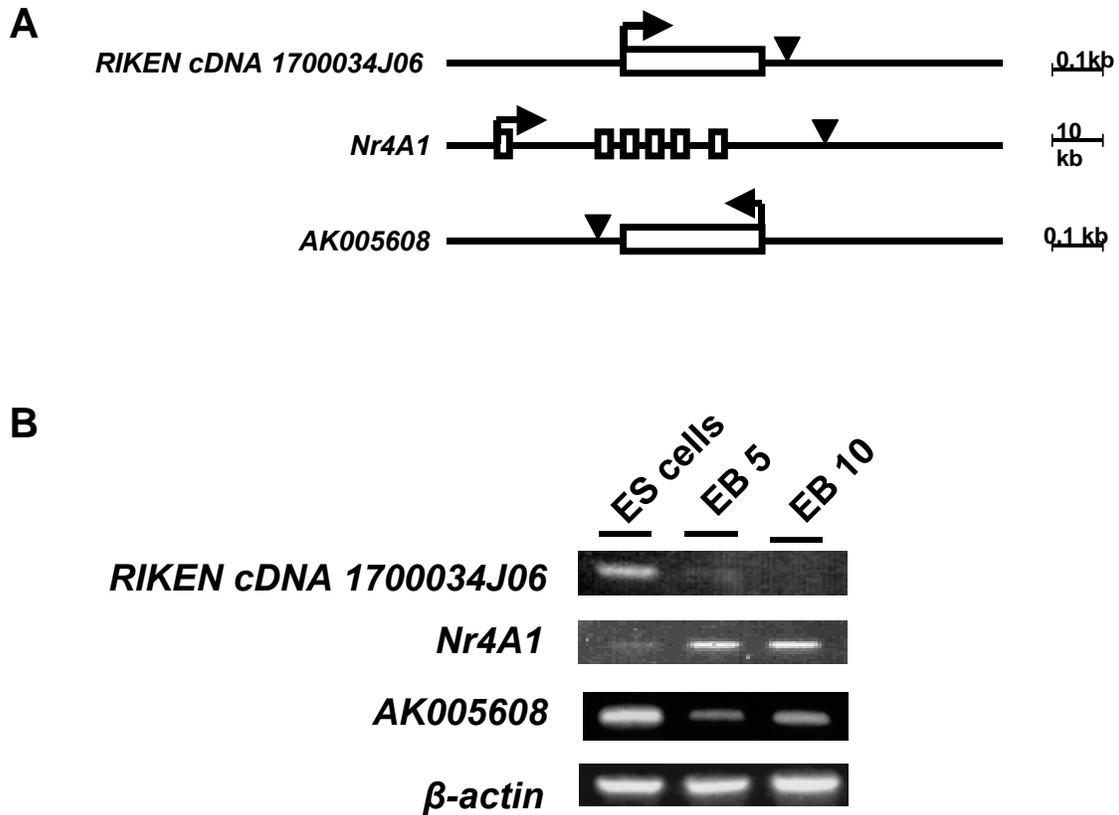


Figure 3-2 RT-PCR analyses of levels of *RIKENcDNA 1700034J06*, *Nr4A1*, *AK005608*, and *β-actin* transcripts during ES cell differentiation. A, Schematic diagram of four genomic regions frequently methylated during ES cell differentiation. The open rectangular boxes indicate exons. Arrows indicate the MF fragments identified during MSRF analysis. B, RT-PCR analysis of *RIKENcDNA 1700034J06*, *Viaat*, *AK005608*, and *β-actin* transcripts following ES cell differentiation. Total RNA was extracted from undifferentiated ES cells and differentiating embryoid bodies (days 5 and 10).

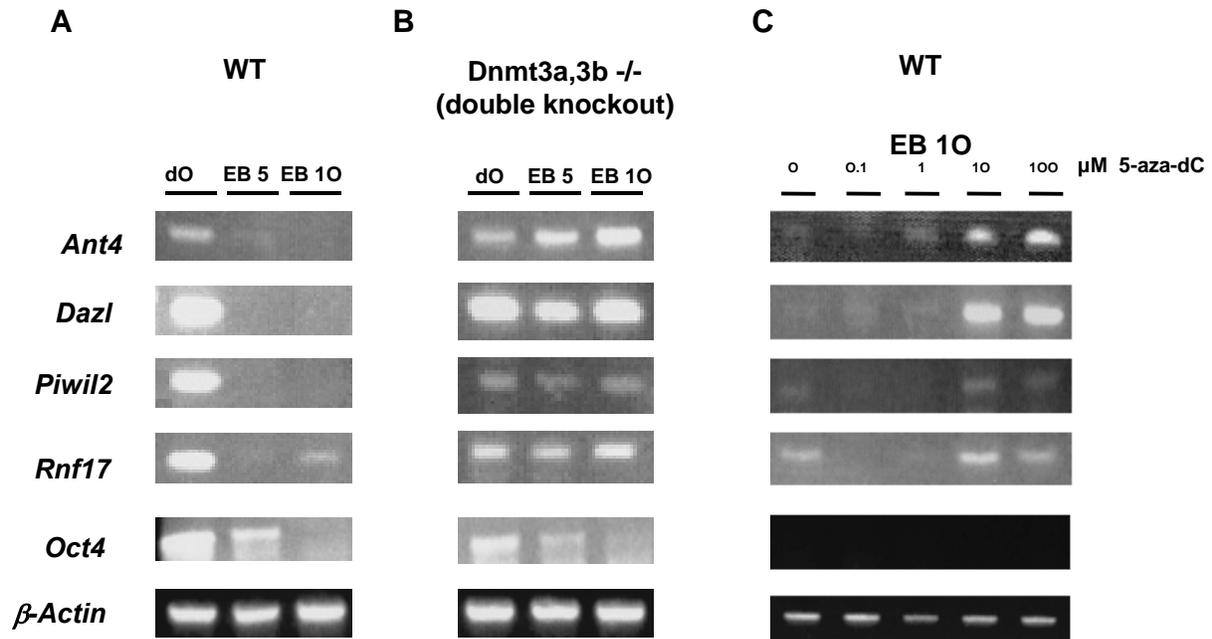


Figure 3-3 Germ cell-specific genes are repressed by DNA methylation during ES cells differentiation. A, Expression of the *Ant4*, *Dazl*, *Piwil2*, *Rnf17*, and *Oct4* genes in wild type ES cells and B, Dnmt3a Dnmt3b double null ES cells. *Oct4*, is a marker for undifferentiated ES cells. C, Germ cell-specific gene re-expression by 5-aza-dC. EBs were treated with various concentrations (0-10 $\mu$ M) of 5-aza-dC for 2 days prior to cell harvesting (day 10).

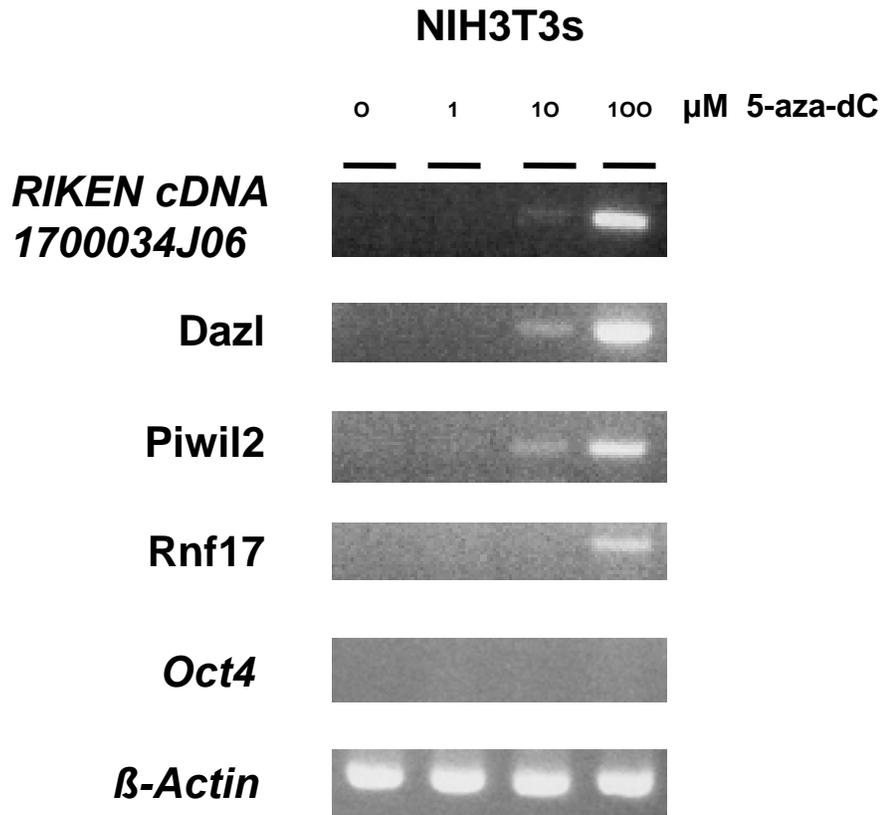


Figure 3-4 Germ cell-specific genes are repressed by DNA in NIH3T3 somatic cell line. Expression of the *RikencDNA 1700034J06*, *Dazl*, *Piwil2*, *Rnf17*, and *Oct4* genes in NIH3T3 somatic cell line. NIH3T3 somatic cells were treated with various concentrations (0-100 $\mu\text{M}$ ) of 5-aza-dC for 2 days prior to cell harvesting.

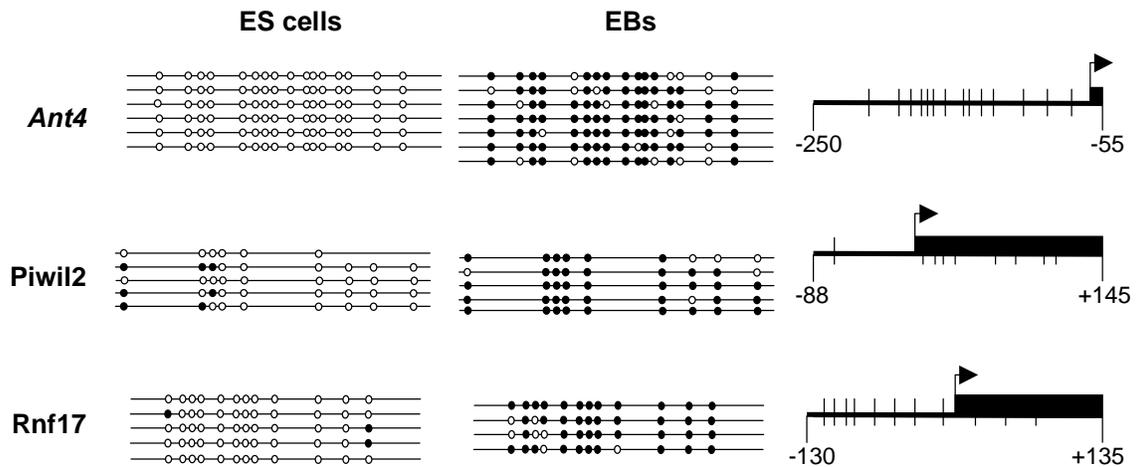


Figure 3-5 Germ cell-specific promoters are repressed by DNA methylation during ES cell differentiation. DNA methylation of the *Ant4*, *Piwil2* and *Rnf17* promoters in wild type ES cells and differentiated embryoid bodies (day 10) assayed by bisulfite sequencing of individual clones.

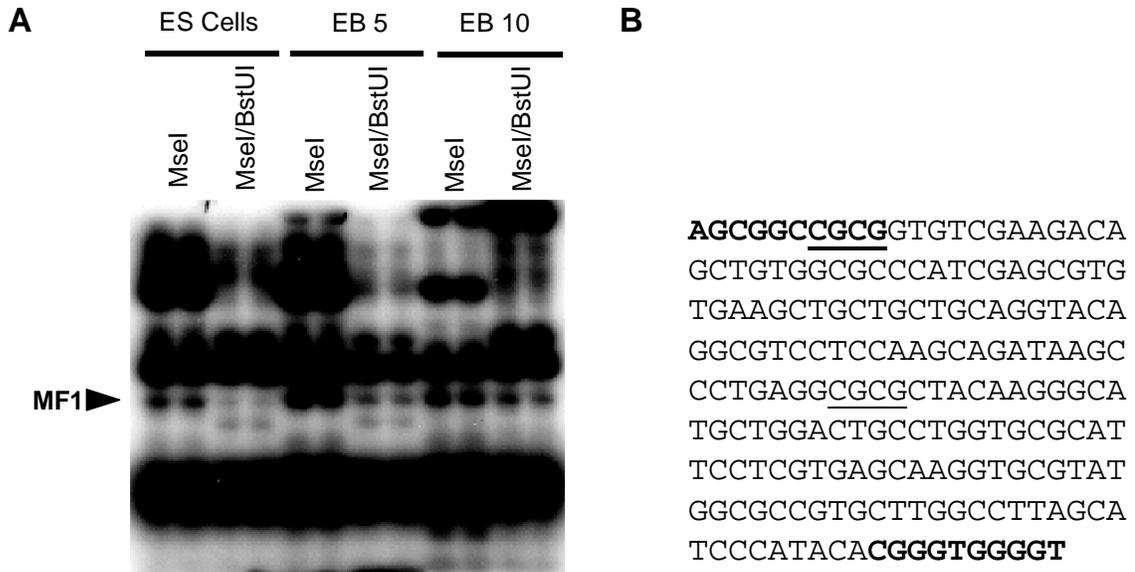


Figure 3-6 Identification of differentially methylated CpG-rich fragment in ES cells and EBs. A, Methylation sensitive restriction fingerprinting (MSRF): Genomic DNA was prepared from undifferentiated ES cells and *in vitro* differentiated to EBs (day 5 and day 10). DNA was digested either by MseI alone or MseI and BstUI, and subjected to PCR amplification using CpG rich 10-mer primers in the presence of radiolabeled dCTP. Amplified DNA fragments were separated in 4.5% polyacrylamide gels. A methylated fragment (MF1, arrowhead) appeared in EBs (day 5 and 10). B, Nucleotide sequence of MF1. Bold, primer sequence. Underlined, BstUI sites.

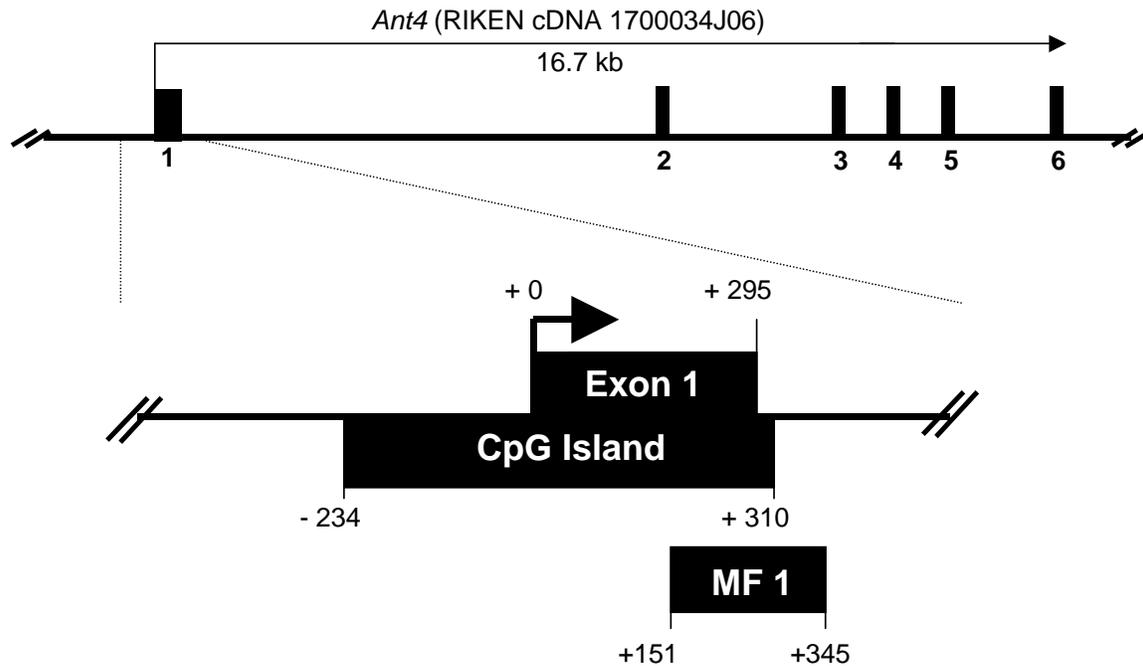
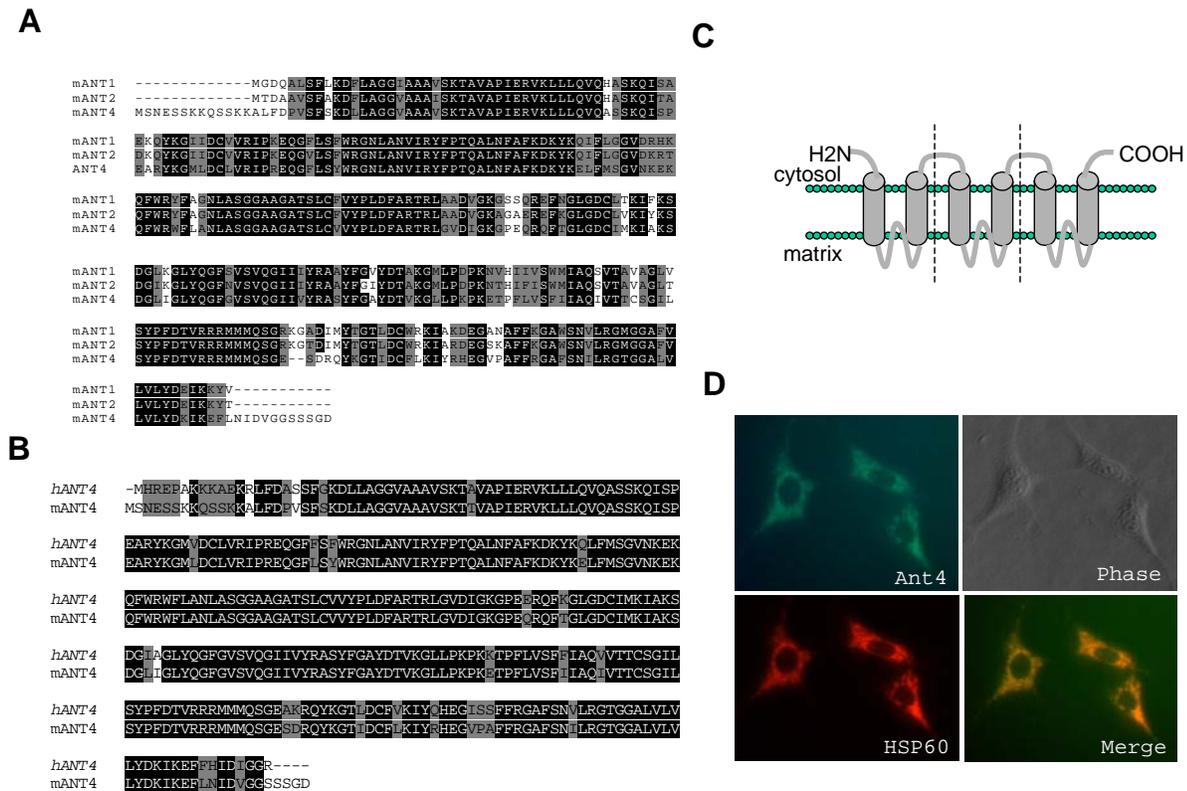


Figure 3-7 MF1 genomic localization. MF1 nucleotide sequence is identical to exon 1/intron 1 boundary region of a previously unknown sequence, *RIKEN cDNA 1700034J06*. Due to significant amino acid similarity with other adenine nucleotide translocase genes, the novel gene was termed, adenine nucleotide translocase 4 (*Ant4*). Arrow represents translation initiation site. BLAT search results using April 2005 freeze.



Rodic N et al., Stem Cells 2005 in press

Figure 3-8 RIKEN cDNA 1700034J06 encodes Ant4, a novel isoform of adenine nucleotide translocase. A, Deduced amino acid sequence of the mouse *Ant4* gene is aligned with previously identified mouse Ant proteins (Ant1 and Ant2). B, Deduced amino acid sequence of the mouse *Ant4* gene is aligned with ANT4 human ortholog. C, Topological diagram of mitochondrial adenine nucleotide translocases. Each Ant contains six helices that traverse the inner mitochondrial membrane with the N- and C-termini facing the cytosol. Sequence is divided into three domains with two transmembrane helices per domain. D, FLAG-tagged Ant4 was transfected into NIH3T3 cells and cell lysates were analyzed by immunostaining. HSP60, mitochondrial marker.

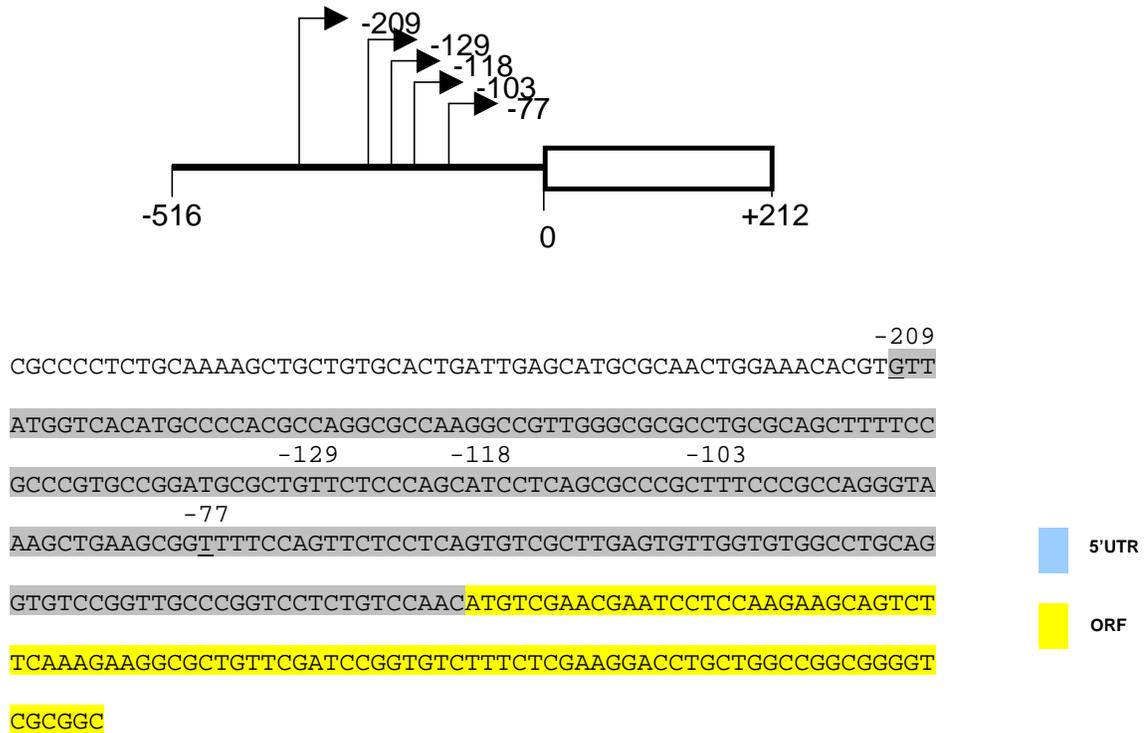


Figure 3-9 Transcription initiation site of the *Ant4* gene determination using 5'RACE sequencing. ES cell derived cDNA was used as a template for 5'RACE reaction. The number 0 represents the translation start site. Arrows represent multiple transcription initiation sites detected, with numbers relative to the translation start site.

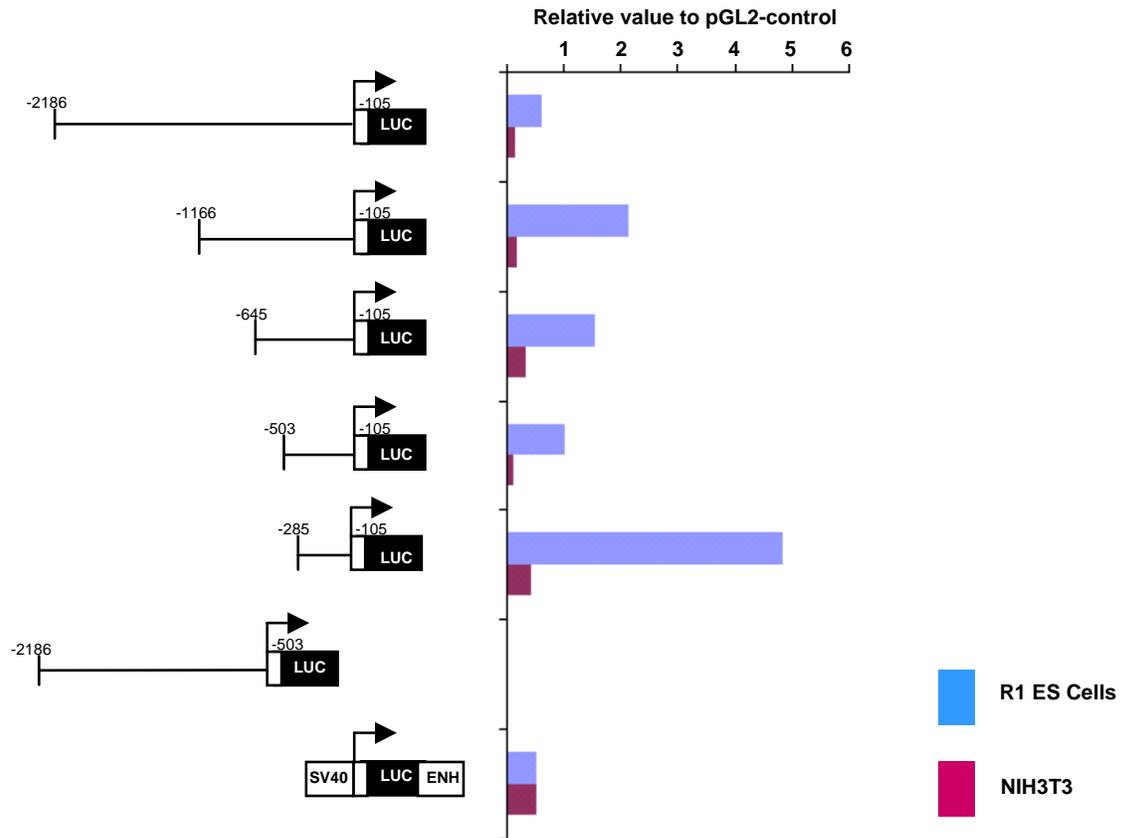


Figure 3-10 Analysis of *Ant4* promoter activity by a luciferase reporter assay in R1 ES and NIH3T3 cells. Different portions of the *Ant4* promoter were tested for luciferase activity. Relative luciferase activity is shown for each construct relative to the activity of the pGL2 control vector. Luciferase activity was assayed 24 hours after direct transfection of cells and is reported relative to the pGL2 control construct and Renilla Luciferase construct.

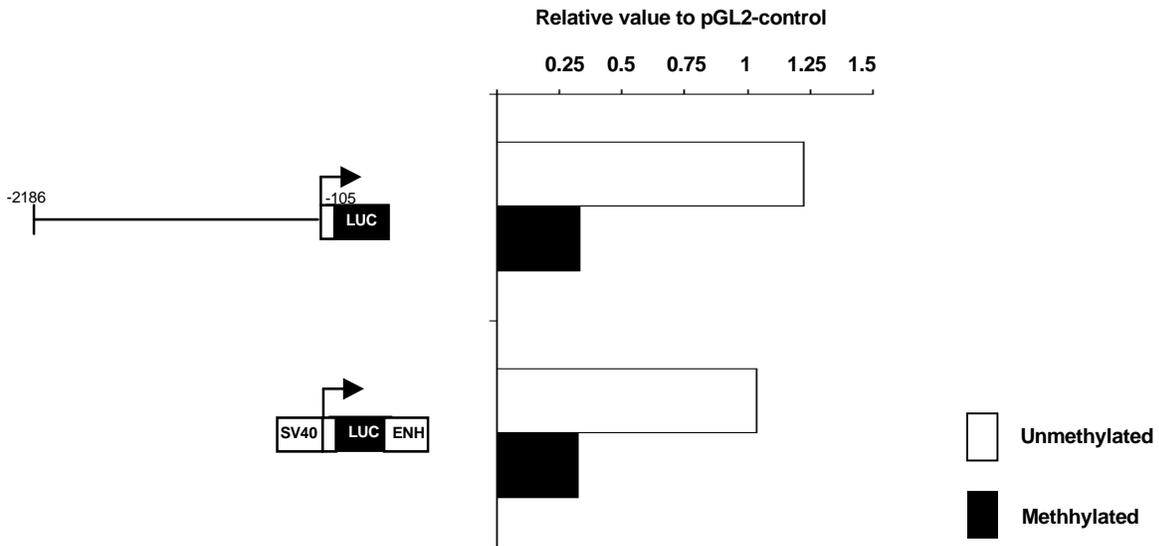


Figure 3-11 Promoter hypermethylation silences expression of *Ant4*. Analysis of *Ant4* promoter activity by a luciferase reporter assays in R1 ES cells. Relative luciferase activity is shown for 2kb *Ant4* construct relative to the activity of the pGL2-control vector. The promoter insert and pGL2 basic vector were treated with SssI methylase. Luciferase activity was assayed 24 hours after direct transfection of cells and is reported relative to the unmethylated (open rectangle) pGL2-control construct.

## ES Cells

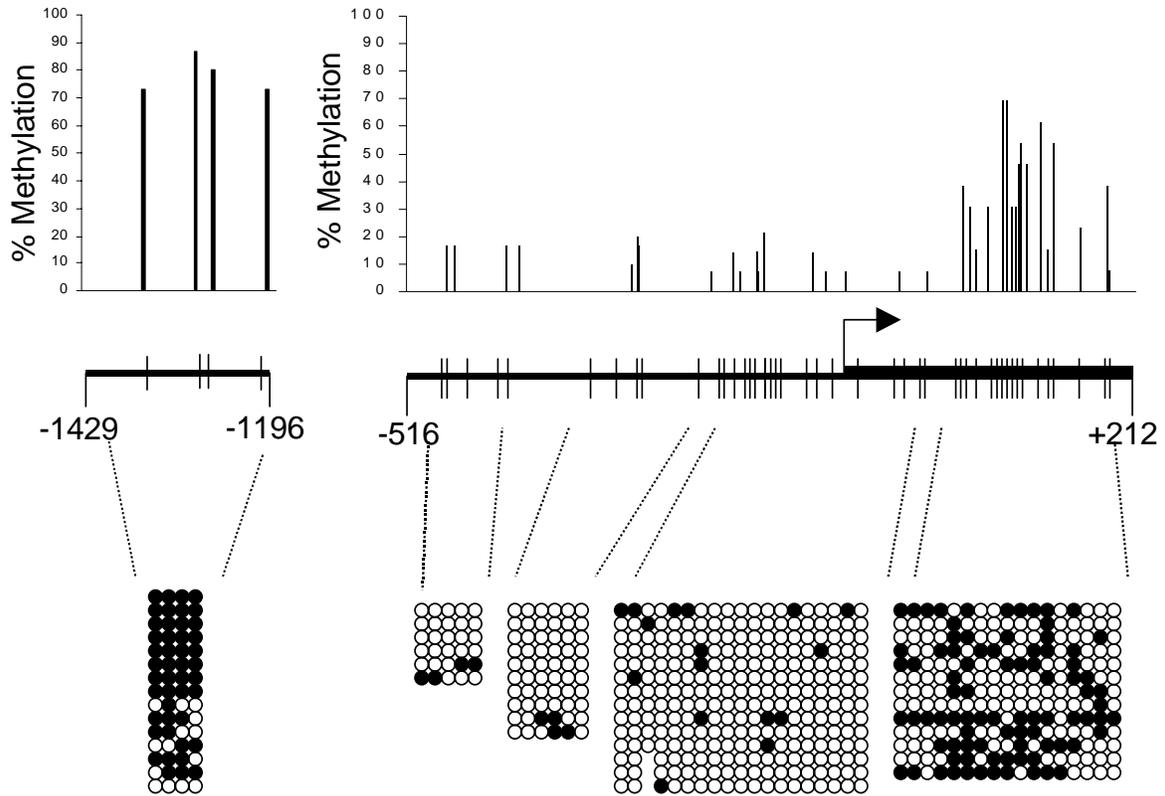


Figure 3-12 *Ant4* promoter region is hypomethylated in undifferentiated ES cells. Summary of *Ant4* methylation levels in ES cells is shown. For each primer pair, up to 14 clones were bisulfite sequenced to determine the percentage of methylation. The y-axis represents percentage methylation per CpG residue. Diagram at the bottom of the figure represents relative position of CpG residues within the 5'-end regulatory region of the *Ant4* gene. Arrow represents a major translation start site, black rectangle represents the first exon, and vertical lines mark the location of individual CpG residues.

## EB 10

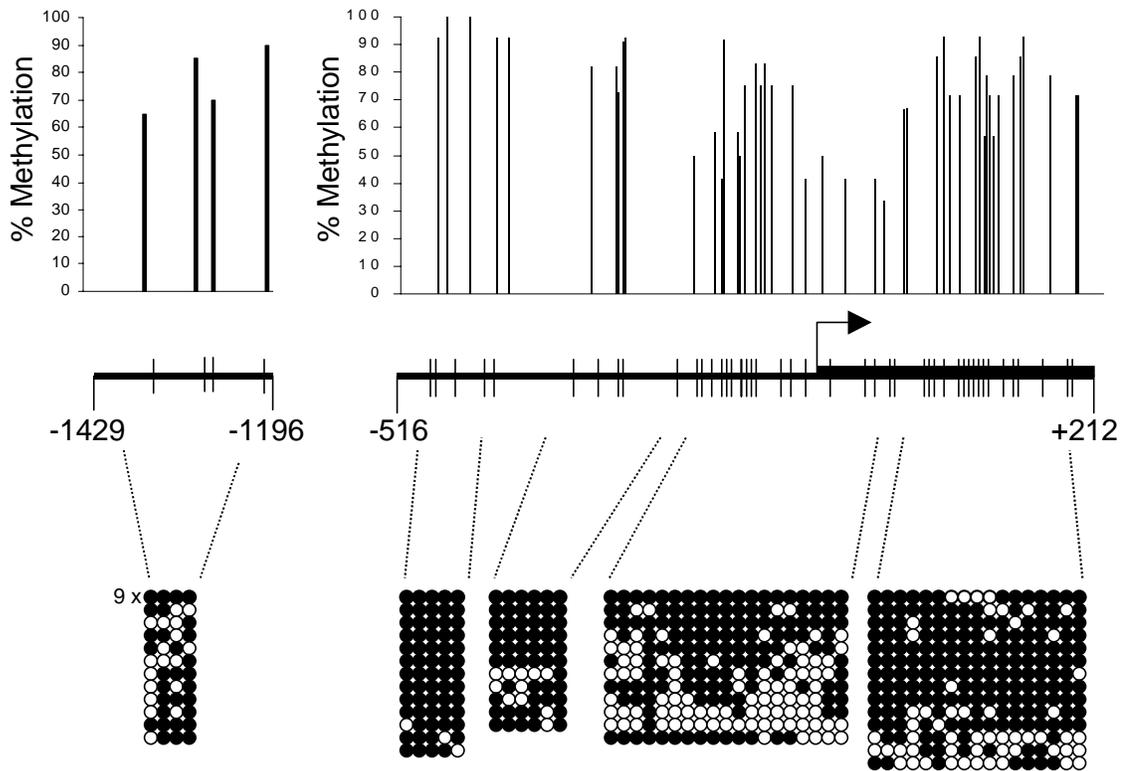


Figure 3-13 *Ant4* promoter region undergoes DNA methylation in differentiated EB cells. Summary of *Ant4* methylation levels in EB cells is shown. For each primer pair, up to 20 clones were bisulfite sequenced to determine the percentage of methylation. The y-axis represents percentage methylation per CpG residue. Diagram at the bottom of the figure represents relative position of CpG residues within the 5'-end regulatory region of the *Ant4* gene. Arrow represents a major translation start site, black rectangle represents the first exon, and vertical lines mark the location of individual CpG residues.

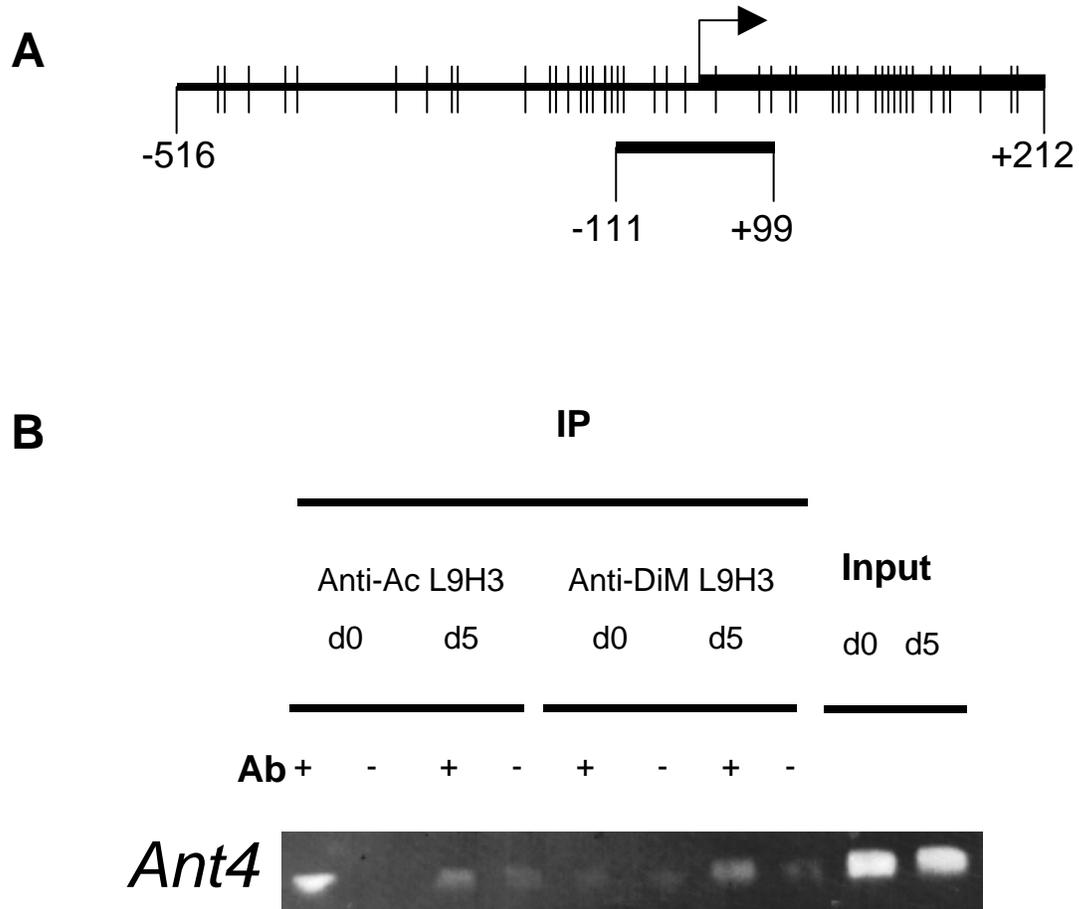


Figure 3-14 *Ant4* promoter region undergoes covalent histone 3 changes during ES cell differentiation. A, Diagram represents relative position of primer pair used for this study. B, Genomic DNA from either undifferentiated ES cells or EB (day 5) was processed according to chromatin immunoprecipitation (ChIP) protocol. Antibodies used in this study were either anti-acetylated lysine-9 histone-3 antibodies, or anti-dimethylated lysine-9 histone 3 antibodies. Input, non-immunoprecipitated genomic DNA.

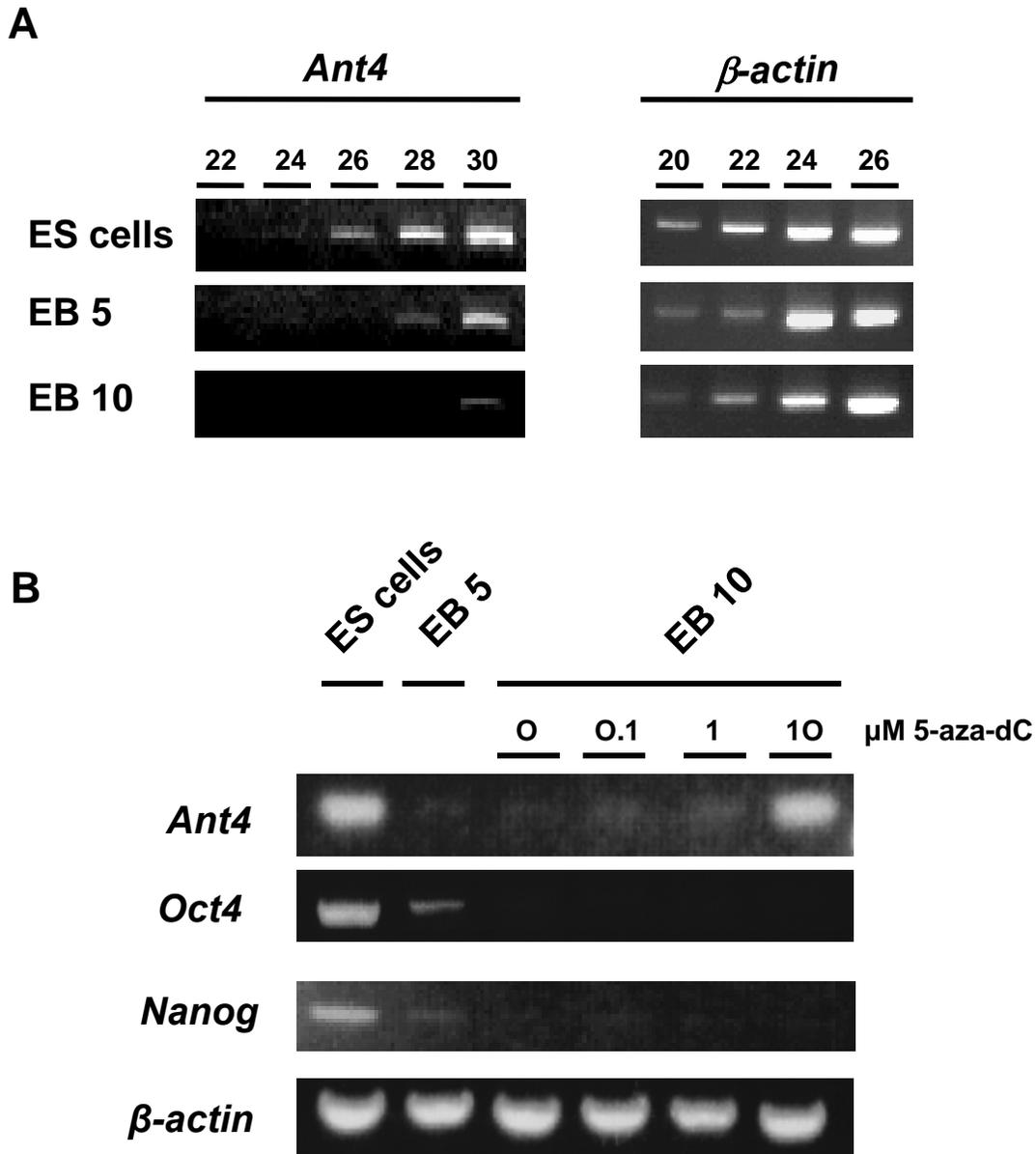


Figure 3-15 *Ant4* is repressed by DNA methylation during ES cell differentiation. A, Total RNA was extracted from ES cells and EBs. RNA expression levels of *Ant4* and *β-actin* were examined by RT-PCR (20 – 30 cycles). B, *Ant4* derepression by 5-aza-dC. EBs were treated with various concentrations (0-10μM) of 5-aza-dC for 2 days prior to cell harvesting (day 10). RNA expression levels of *Ant4*, *Oct4*, *Nanog* and *β-actin* genes were examined by RT-PCR.

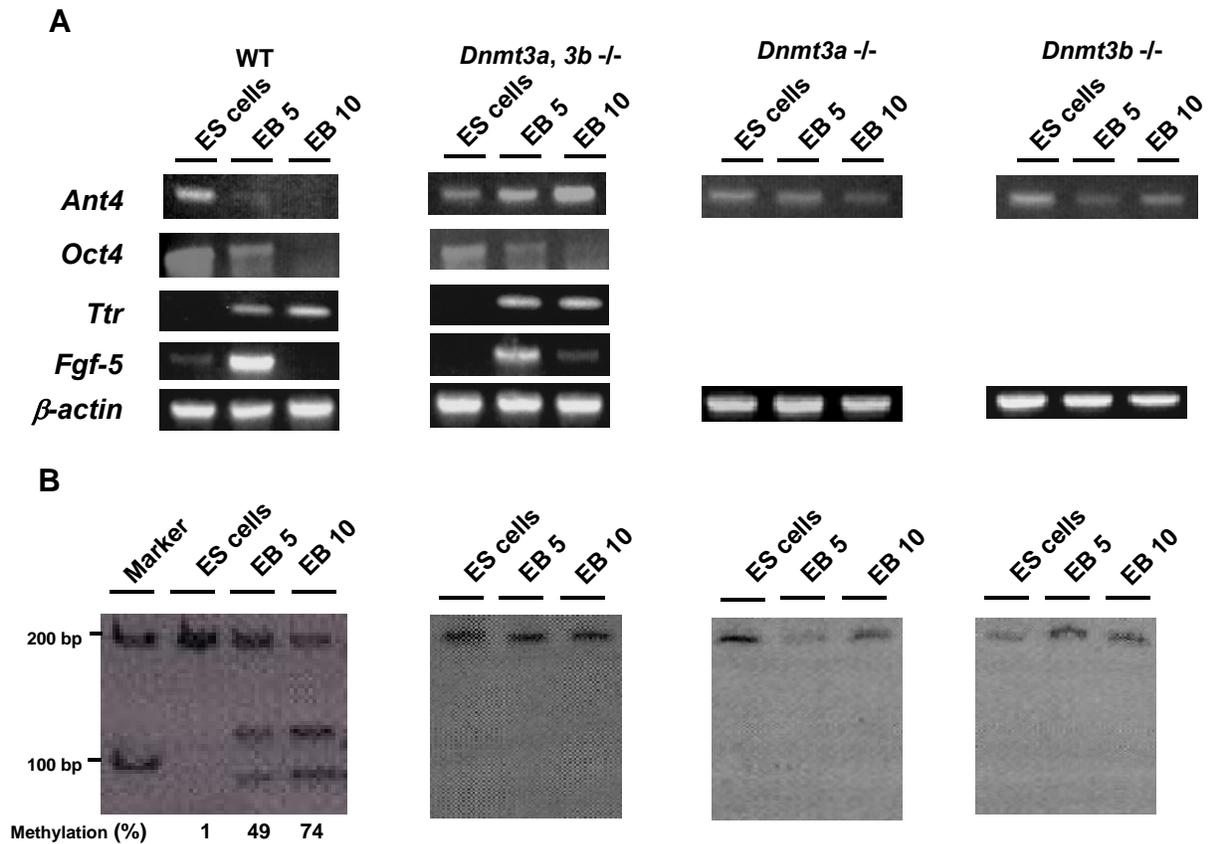


Figure 3-16 Dnmt3 is required for *Ant4* repression during ES cell differentiation. A, Expression of the *Ant4* gene in wild type ES cells, Dnmt3a Dnmt3b double null ES cells, Dnmt3a null ES cells and Dnmt3b null ES cells. *Oct4*, *Ttr*, *Fgf-5* are markers for undifferentiated ES cells, early visceral endoderm, and early ectoderm, respectively. B, *Ant4* promoter DNA methylation determined by COBRA assay. DNA was extracted from wild type ES cells and Dnmt3a Dnmt3b null ES cells and treated with bisulfite. The *Ant4* promoter region was amplified and subjected to overnight digestion with HhaI restriction enzyme, which cuts GCGC sites. DNA methylation of the CpG protects the site from bisulfite conversion. The digested DNA samples were separated in 4.5% polyacrylamide gels and visualized using a SyBr-green dye.

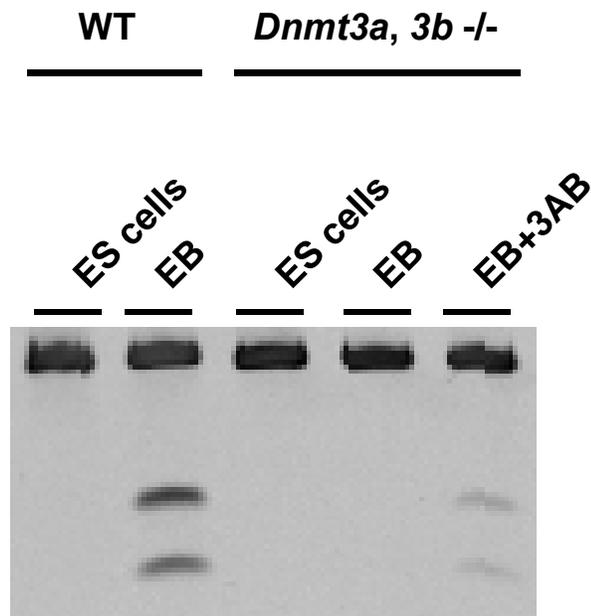


Figure 3-17 Dnmt3 is required for *Ant4* repression during ES cell differentiation. DNA was extracted from wild type ES cells, *Dnmt3a Dnmt3b* null ES cells, and *Dnmt3a Dnmt3b* null ES cells stably transfected with *Dnmt3a* and *Dnmt3b* lentiviral expression vectors. Extracted DNAs were treated with bisulfite reagent. The *Ant4* promoter region was amplified and subjected to overnight digestion with *HhaI* restriction enzyme, which cuts GCGC sites. DNA methylation of the CpG protects the site from bisulfite conversion. The digested DNA samples were separated in 4.5% polyacrylamide gels and visualized using a SyBr-green dye.

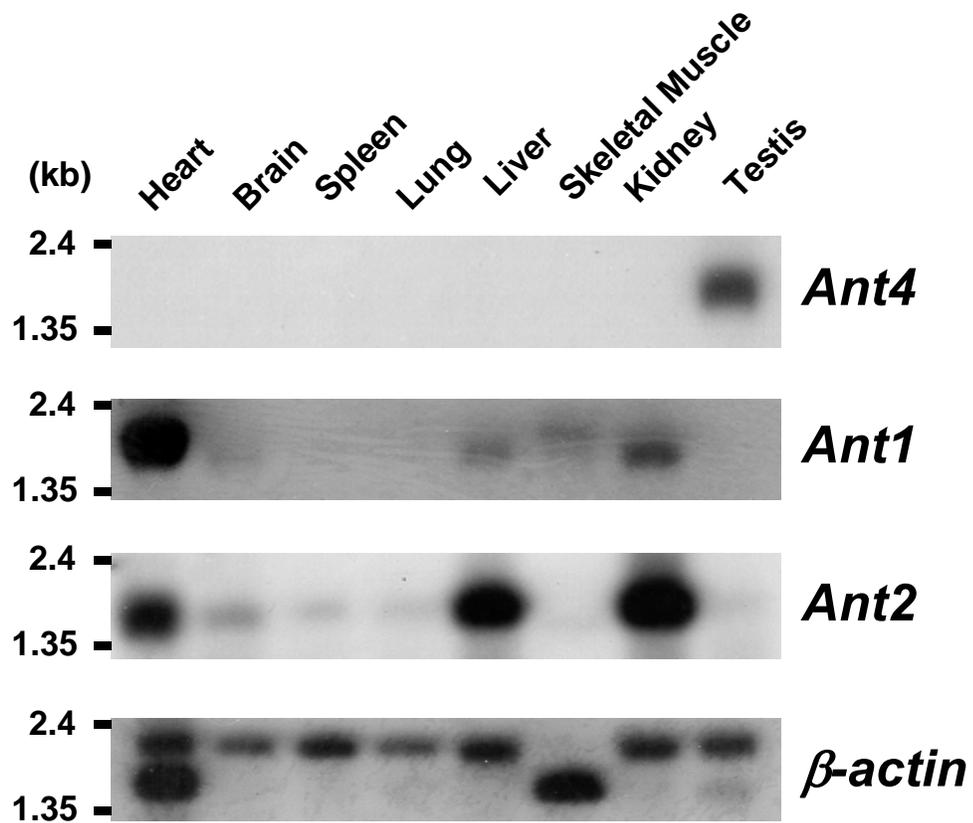


Figure 3-18 *Ant4* is expressed selectively in testis. Northern blot analysis of *Ant4* mRNA expression in various organs from adult mice (8 weeks old). The blot was hybridized to specific cDNA probes for *Ant4*, *Ant1*, *Ant2* and  $\beta$ -actin.

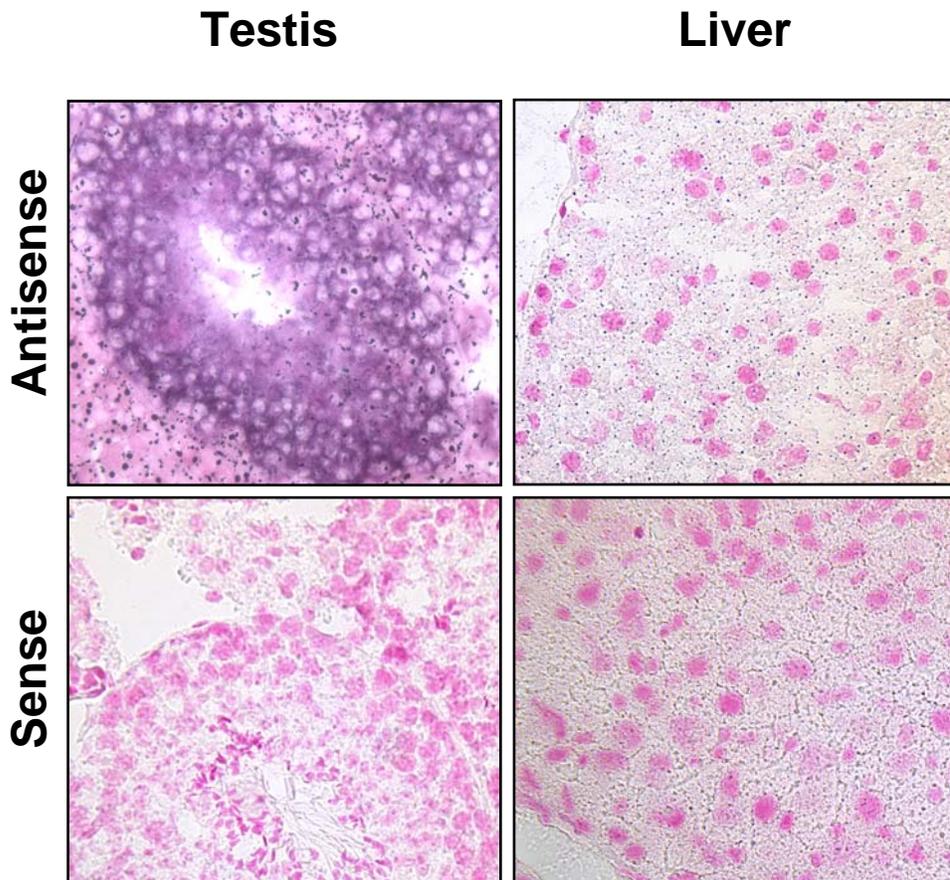


Figure 3-19 Localization of *Ant4* demonstrated by *in situ* hybridization. Digoxigenin-labeled riboprobes were applied to 10 micrometer freshly frozen murine testes and liver sections (8 weeks old). A positive signal is indicated by the presence of a blue deposit (BCIP-NBT) within the cytoplasm. Slides were counterstained in nuclear fast red (pink). Germ cells within the seminiferous tubules stained positive with the anti-sense probe but not with the sense probe. *Ant4* was not detected in any of the liver tissue, nor was it found to be in the interstitial cells, capillaries or capsules of the testes.

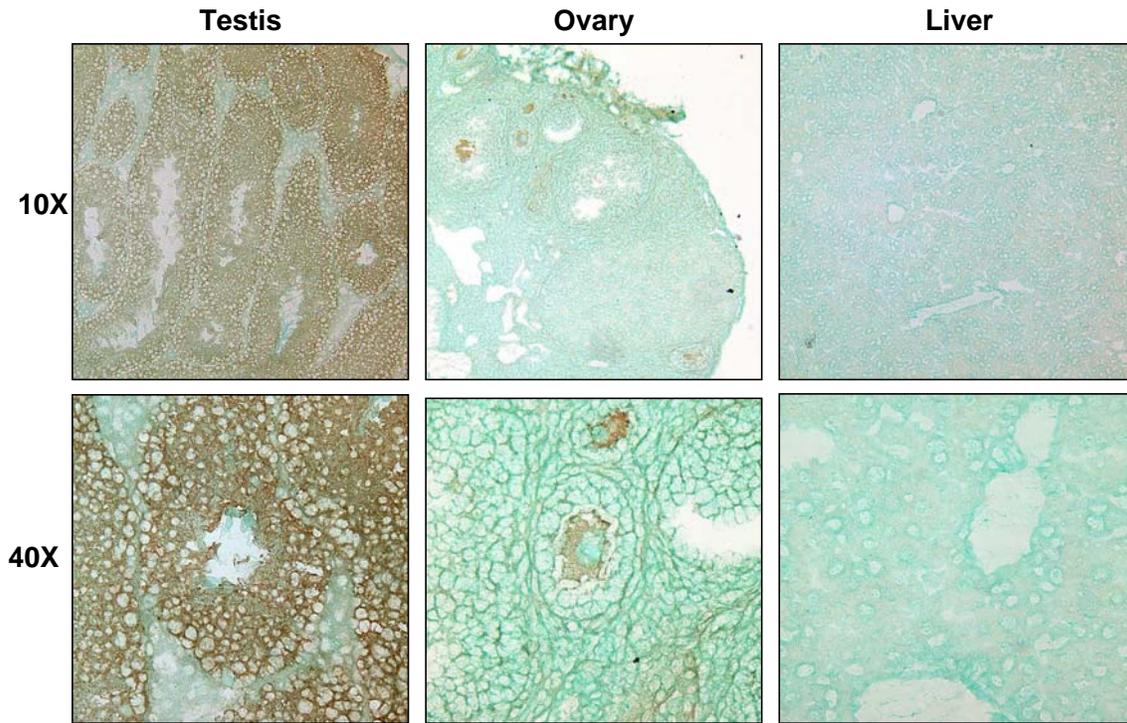


Figure 3-20 Ant4 is expressed in male and female germ cells. Immunohistochemical analysis of Ant4 expression in testis, ovary and liver. Mouse ovary, testis and liver were harvested from 3 month old mice, and frozen sections were stained with affinity-purified rabbit polyclonal anti-Ant4 antibodies raised against the N-terminal Ant4 peptide (1-MSNESSKKQSSKKALFD-17) and visualized using horse radish peroxidase (brown). Slides were counterstained using Light Green SF Yellowish.

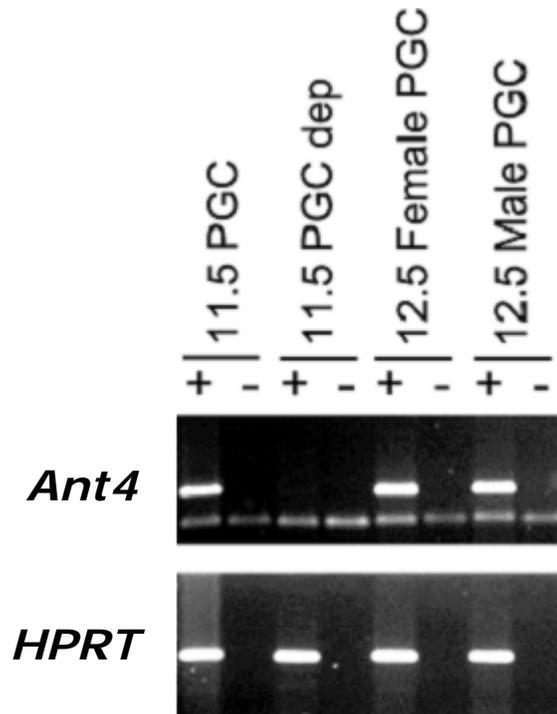


Figure 3-21 *Ant4* is expressed in primordial germ cells. *Ant4* mRNA is expressed in purified primordial germ cells. Primordial germ cells were obtained from E11.5 and 12.5 dpc genital ridges and purified using anti-SSEA1 magnetic beads. The immunodepleted fraction (dep) refers to cells not retained on the magnetic column. Individual samples were subjected to RT-PCR analysis. +/- indicates whether reverse transcriptase was added to reaction mixture.

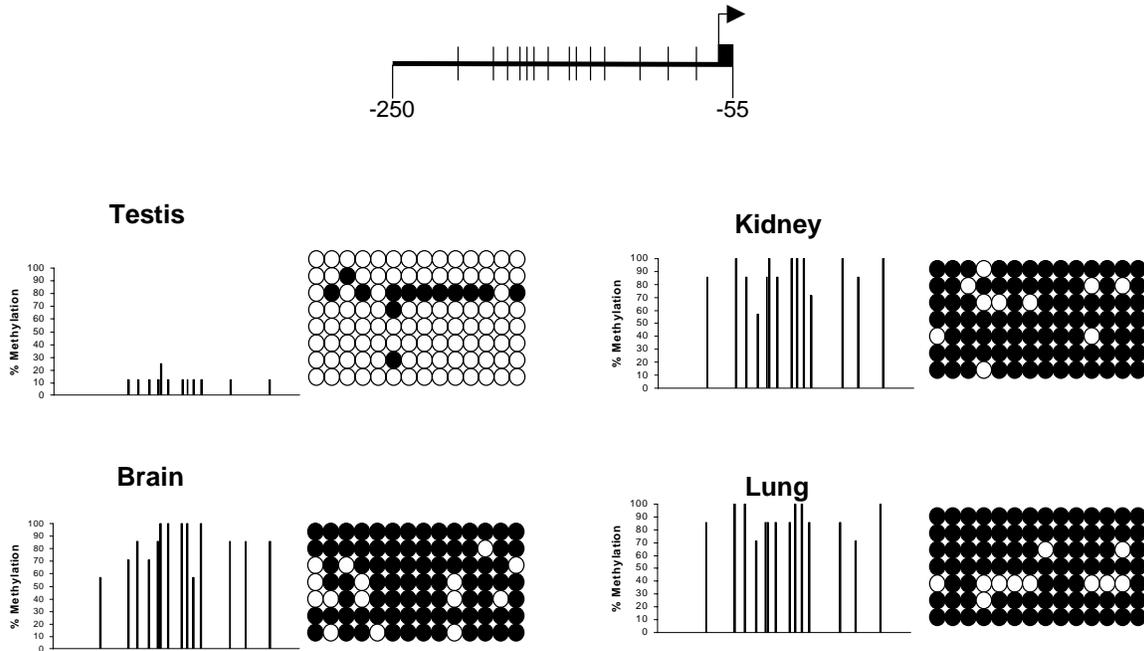


Figure 3-22 Bisulfite analysis of the *Ant4* promoter in various organs from adult mice (8 weeks old). DNA samples were extracted from the indicated mice organs and subjected to bisulfite conversion. Several clones, usually 7 to 8 individual clones were sequenced for each samples.

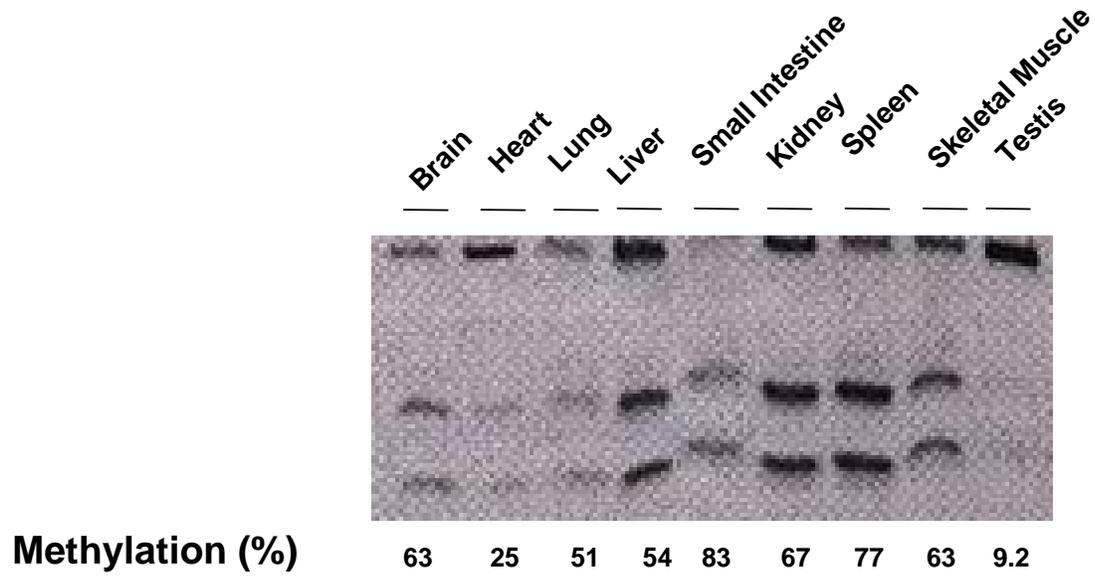


Figure 3-23 COBRA analysis of the *Ant4* promoter for DNA methylation levels in various adult mice organs. DNA samples were extracted from the indicated murine organs and subjected to bisulfite conversion.

## CHAPTER 4 DISCUSSION

### **On Newly Identified Differentially Methylated Genomic Regions**

Although previous DNA methylation studies in ES cells have accumulated considerable knowledge concerning the enumeration of differentially methylated regions and the cooperativity requirement for Dnmts, little is known about the genomic position of differentially methylated regions, and subsequently about the nature of target genes. We employed a powerful genome wide screening method, termed MSRF, to identify differentially methylated genomic regions during ES cell differentiation. In the past, MSRF has been used to isolate aberrantly methylated DNA fragments in prostate, breast, and nasopharyngeal cancers. Similar methods, such as methylation-sensitive arbitrarily primed PCR (AP-PCR), have been developed to determine how repetitive DNA elements become methylated (55). In our current study, we identified ten genomic loci that exhibit differential DNA methylation patterns between undifferentiated ES cells and differentiated embryoid bodies. On a genome wide scale, our data likely represents less than 5% of all differentially methylated regions in ES cells and differentiated embryoid bodies. According to earlier studies, 247 differentially methylated regions exist between ES cells and differentiated embryoid bodies (103). However, the data generated from such studies, due to assay limitations of utilizing only certain restriction enzymes (ie NotI), most likely underrepresented the absolute number of differentially methylated regions. The DNA methylation of selected genomic regions, such as CpG islands, requires the cooperative action of Dnmt1, Dnmt3a, and Dnmt3b enzymes in

undifferentiated ES cells. In absence of Dnmt1 or Dnmt3a and Dnmt3b proteins by genomic homozygous deletion, ES cells lose their DNA methylation in an identical set of 236 genomic loci. Similarly, methylation of the class II retroviral sequence CII-d and the type A repeats in the LINE repetitive elements require cooperativity between the DNA methyltransferases (55).

### **On Newly Identified DNA Methylation Controlled Genes**

To extend our MSRF findings, we studied the effects of MF associated differential methylation on transcriptional activity of neighboring genes. Using semi-quantitative RT-PCR, we established an inverse relationship between mRNA expression and hypermethylation of the 4 cognate neighboring genes. Although our findings are modest in light of the great number of differentially methylated fragments on a genome wide scale, current knowledge regarding genomic location of MFs is insufficient. To date, the vast majority of DNA methylation screening studies did not look at the effects of DNA methylation on cognate neighboring genes. To our knowledge, such an inverse relationship has been shown for only two other genes, *Sall3* (104), and *PMP4* (105). Our findings indicate that transcripts *RIKENcDNA 1700034J06*, *Nr4A1*, and *AK005608* expression correlates inversely with DNA methylation of neighboring differentially methylated regions. To that extent, we believe our findings are significant. Our findings also indicate that less than half of cognate neighboring genes is potentially regulated by DNA methylation. Such data indicates that there could be a high rate of false positive results associated with genome wide DNA methylation screening methods. In fact, a recent independent study points to a similar conclusion (93).

### **On the Link Between DNA Methylation and Germ-specific Genes**

Undifferentiated ES cells are known to be transcriptionally permissive to the expression of certain germ line cell specific genes (85). This is most likely due to a conserved mode of transcriptional regulation between ES and germ cells, but it might just reflect the fact that ES cells are derived from largely unmethylated pre-implantation embryos (ex. blastocyst). Our results indicate that DNA methylation plays a role in the regulation of a subgroup of germ cell-specific genes during ES cells differentiation. We confirmed that *Dazl*, *Piwil2*, and *Rnf17* transcripts undergo downregulation in a DNA methylation dependent fashion. We have also analyzed the expression of other germ cell-specific genes, such as cytochrome c (*Cyt cT*), melanoma associated antigens (*MAGE*), *Acrosin*, and *Sry* (data not shown). We found their expression to be either low in undifferentiated ES cells (for *Sry*) or upregulated after differentiation (for the remainder). We believe that at least two criteria are useful when determining whether germ cell-specific gene expression is controlled by DNA methylation in ES cell derived differentiated cells. First, the suspected gene must be expressed in ES cell. Second, the suspected gene must be expressed in both female and male germ cells.

DNA methylation acts as a primary mechanism to control transcription in a subset of testis specific genes. One such group is *MAGE* genes, which are selectively hypomethylated in testis, at the location where transcription from *MAGE* promoters occurs. In light of the fact that the majority of CpG islands are unmethylated in adult somatic cells, it is particularly unusual that *MAGE* promoters are hypermethylated in all but germ cells. DNA methylation of such germ cell-specific promoters maintains transcriptional repression, since 5-aza-dC treatment readily causes re-expression of the gene. The exact molecular mechanisms responsible for such widespread DNA

methylation are beginning to be elucidated. In a recent report, the Boon group showed that transcriptional activity, mediated by ubiquitous transcription factors, can protect *MAGE* promoters from DNA methylation (106). Such an elegant promoter-dependent mechanism would be consistent with the major role that DNA methylation exerts on *MAGE* promoters. More importantly, the presence of transcription factors as a ubiquitous cellular program is not testis-specific and thus fails to explain the tissue-restricted expression of *MAGE* genes. Nevertheless, it appears likely that a promoter-dependent mechanism could be activated following treatment by ubiquitous DNA methylation inhibiting agents, such as 5-aza-dC. After 5-aza-dC, demethylated promoters would remain unmethylated due to the transcription activity of ubiquitous transcription factors.

Recent reports also demonstrate that DNA methylation is involved in the transcriptional regulation of several other germ cell-specific genes, including testis-specific phosphoglycerate kinase 2 (*pgk2*) (107), pyruvate dehydrogenase alpha-subunit (*pdha-2*) (108), and testis specific gene, *Tact1/Actl7b* (109). Since *Ant4* is expressed exclusively in the testis and developing oocytes in the adult mouse, the regulation of *Ant4* transcription may be closely related to that of other germ cell-specific genes. Of interest, the promoter region of *Ant4* contains the chicken ovalbumin upstream promoter transcription factor (*COUP-TF*) binding element at -586 bp, which has been implicated in the selective silencing of the proto-oncogene, *c-mos*, in somatic cells (110,111). In addition, the *Ant4* promoter has multiple elements that potentially bind to *Sox/Sry* factors, which have also been implicated in germ cell-specific transcription. By elucidating the role of DNA methylation in the regulation of gene expression in pluripotent cells, we

may uncover common genetic regulatory pathways underlying the transition between pluripotent stem/germ cells and somatic cells.

#### **On the Newly Identified Adenine Nucleotide Translocase, *Ant4***

We have identified a novel isoform of the adenine nucleotide translocase genes, termed *Ant4*, using a screen of differential DNA methylation patterns in ES cells and embryoid bodies. The selective expression status of *Ant4* in undifferentiated ES cells, primordial germ cells, and germ cells, indicates that *Ant4* is a pluripotent cell specific isoform of the adenine nucleotide translocase family. This is in contrast with the previously identified isoforms, *Ant1* and *Ant2*, which are predominately expressed in somatic cells. The deduced amino acid sequence predicts *Ant4* as a functional adenine nucleotide translocase. Like the other *Ant* isoforms, the *Ant4* protein localizes to the mitochondria. Additionally, both coding sequences and genomic architecture are conserved between mouse and human orthologs.

Concurrently with our own investigation, the Palmieri group has reported an independent discovery of the human *Ant4* gene. Human *Ant4* was shown to (i) catalyze obligatory antiport between ADP and ATP, (ii) catalyze electrophoretic exchange between ADP and ATP, (iii) and have tissue restricted expression with high expression in testis and liver, while *Ant4* mRNA was not detectable in any of the other tissues (112).

It is intriguing that ES and germ cells have a specific isoform for *Ant*. This finding might indicate a unique mitochondrial energy metabolism in such pluripotent cells. Developing spermatocytes are known to express testis-specific isoforms of pyruvate dehydrogenase complex E1a subunit, *Pdha-2* (108), testis specific cytochrome c, *Cyt cT* (113), and subunit VIb of cytochrome c oxidase (114). Interestingly, *Cyt cT* null mice exhibit early testicular atrophy and apoptosis. Male germ cells respire by oxidative

phosphorylation, mainly utilizing lactate as an energy source. Thus, *Ant4* could play a pivotal role in supplying ATP from the mitochondrial matrix to the cytosol in germ cells. Some major consequence of *Ant4* deletion could be (i) an accumulation of ATP nucleotides in mitochondria, (ii) hyperpolarization of the inner mitochondrial membrane, (iii) lactic acidemia within the testis, (iv) or a compensatory increase in mitochondrial biosynthesis. Our future studies utilizing *Ant4* null ES cells and *Ant4* null mice will elucidate the *in vivo* function of the *Ant4* protein in spermatogenesis and ES cell maintenance (Figure 4-1).

The present data indicate that *de novo* DNA methylation, mediated by Dnmt3a and Dnmt3b, plays a pivotal role in gene repression of *Ant4* during ES cell differentiation. In adult organs, as well as during *in vitro* ES cell differentiation, *Ant4* expression levels inversely correlate with the DNA methylation status of the gene. Further, *Ant4* is readily derepressed by the addition of the demethylating agent 5-aza-dC. In contrast, *Oct4*, a gene specifically expressed in pluripotent stem cells and primordial germ cells, is not derepressed by 5-aza-dC. This implies that transcription factors involved in *Ant4* gene expression are present and active in differentiated EBs, and that DNA methylation may play a primary role in the suppression of the *Ant4* gene in somatic cells. We confirmed derepression of *Ant4*, but not *Oct4*, by addition of 5-aza-dC into two other somatic cell lines. Further, this hypothesis was supported by the fact that a functional deletion of Dnmt3a and Dnmt3b led to failure of *Ant4* gene suppression, but not *Oct4*, during ES cell differentiation. Taken together, these data suggest that DNA methylation is required for the transcriptional repression of the *Ant4* gene.

Recent evidence suggests that there is a deregulation of the DNA methylation apparatus during cancer development. Indeed, the genome wide hypomethylation that is frequently observed in cancer cells can lead to the reactivation of genes silenced by DNA methylation (115). Members of the cancer-testis family of antigens or associated genes (CTAs) are encoded by genes expressed by cancers of diverse histological origin. These genes are typically absent in normal adult tissues, with the exception of male germ cells. Epigenetic events may represent the unique mechanism regulating the expression of CTAs in cancer cells, with DNA methylation playing a the major role. In fact, a correlation between hypomethylated CpG dinucleotides in *MAGE* promoters and their expression has been found in neoplastic cell lines (106) and tissues (116). Based on how *Ant4*'s expression parallels that of CTAs such as *MAGE*, we examined whether *Ant4* gene was also expressed in human cancer cell lines. The *Ant4* gene was indeed expressed in some cancer cell lines, implicating that the gene is aberrantly derepressed in malignant cells. Since accelerated ATP turnover would be beneficial to rapidly proliferating cancer cells, *Ant4* derepression might be involved in cancer progression. As a future direction, providing insights into how *Ant4* functions in cancer cells would be of significant benefit.

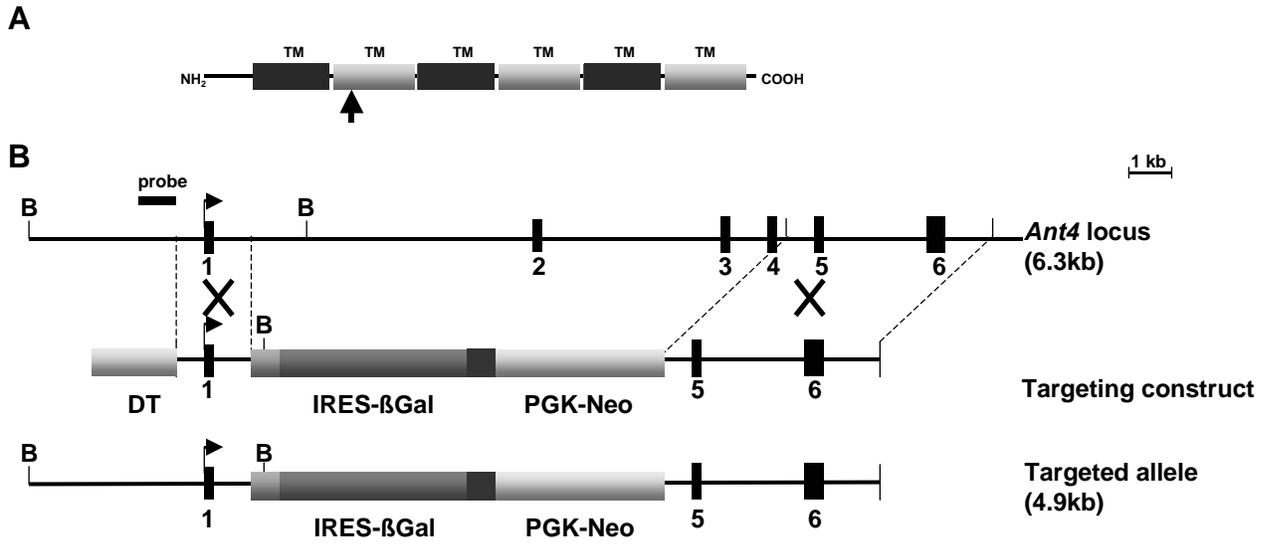


Figure 4-1 Disruption of *Ant4*. A, The *Ant4* protein, arrow indicates relative insertion sites of the targeting construct. Transmembrane (TM) domain. B, Targeted disruption of *Ant4*. DT, diptheria toxin; IRES- $\beta$ Gal, internal ribosomal entry site-beta galactosidase; PGK-Neo, phosphoglycerate kinase promoter-neomycin. B-BamHI site used for screening targeted allele.

## LIST OF REFERENCES

1. Morgenegg, G., Celio, M. R., Malfoy, B., Leng, M., and Kuenzle, C. C. (1983) *Nature* **303**, 540-543
2. Adams, R. L. P., and Burdon, R. H. (1985) *Molecular Biology of DNA Methylation*. Springer Series in Molecular Biology (Rich, A., Ed.), Springer-Verlag, New York
3. Kuo, T. T., Huang, T. C., and Teng, M. H. (1968) *J. Mol. Biol.* **34**, 373-375
4. Sager, R., and Kitchin, R. (1975) *Science* **189**, 426-433
5. Dodge, J. E., Ramsahoye, B. H., Wo, Z. G., Okano, M., and Li, E. (2002) *Gene* **289**, 41-48
6. Doerfler, W. (1984) *Curr. Top. Microbiol. Immunol.* **108**, 79-98
7. Ramsahoye, B. H., Biniszkievicz, D., Lyko, F., Clark, V., Bird, A. P., and Jaenisch, R. (2000) *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5237-5242
8. Li, E., Bestor, T. H., and Jaenisch, R. (1992) *Cell* **69**, 915-926
9. Lei, H., Oh, S. P., Okano, M., Juttermann, R., Goss, K. A., Jaenisch, R., and Li, E. (1996) *Development* **122**, 3195-3205
10. Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999) *Cell* **99**, 247-257
11. Szyf, M., Rouleau, J., Theberge, J., and Bozovic, V. (1992) *Journal of Biological Chemistry* **267**, 12831-12836
12. Maatouk, D. M., and Resnick, J. L. (2003) *Developmental Biology* **258**, 201-208
13. Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N., and Cedar, H. (1984) *Proc. Natl. Acad. Sci. U S A* **81**, 2275-2279
14. Gidekel, S., and Bergman, Y. (2002) *J. Biol. Chem.* **277**, 34521-34530
15. Reik, W., Dean, W., and Walter, J. (2001) *Science* **293**, 1089-1093
16. Durcova-Hills, G., Ainscough, J., and McLaren, A. (2001) *Differentiation* **68**, 220-226
17. Niwa, H., Miyazaki, J., and Smith, A. G. (2000) *Nat. Genet.* **24**, 372-376
18. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003) *Cell* **113**, 643-655

19. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) *Cell* **113**, 631-642
20. Shiota, K., Kogo, Y., Ohgane, J., Imamura, T., Urano, A., Nishino, K., Tanaka, S., and Hattori, N. (2002) *Genes Cells* **7**, 961-969
21. Kremenskoy, M., Kremenska, Y., Ohgane, J., Hattori, N., Tanaka, S., Hashizume, K., and Shiota, K. (2003) *Biochem. Biophys. Res. Commun.* **311**, 884-890
22. Ohgane, J., Hattori, N., Oda, M., Tanaka, S., and Shiota, K. (2002) *Biochem. Biophys. Res. Commun.* **290**, 701-706
23. Lee, J. H., Hart, S. R. L., and Skalnik, D. G. (2004) *Genesis* **38**, 32-38
24. Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., Biniszkiwicz, D., Yanagimachi, R., and Jaenisch, R. (2001) *Science* **293**, 95-97
25. Tweedie, S., Charlton, J., Clark, V., and Bird, A. (1997) *Mol. Cell. Biol.* **17**, 1469-1475
26. Chen, C., Yang, M. C. K., and Yang, T. P. (2001) *Journal of Biological Chemistry* **276**, 320-328
27. Santoro, R., and Grummt, I. (2001) *Molecular Cell* **8**, 719-725
28. Lorincz, M. C., Schubeler, D., Hutchinson, S. R., Dickerson, D. R., and Groudine, M. (2002) *Molecular and Cellular Biology* **22**, 7572-7580
29. Siegfried, Z., Eden, S., Mendelsohn, M., Feng, X., Tsuberi, B. Z., and Cedar, H. (1999) *Nature Genetics* **22**, 203-206
30. Hejnar, J., Hajkova, P., Plachy, J., Elleder, D., Stepanets, V., and Svoboda, J. (2001) *Proc. Natl. Acad. Sci. U S A* **98**, 565-569
31. Hashimshony, T., Zhang, J., Keshet, I., Bustin, M., and Cedar, H. (2003) *Nat. Genet.* **34**, 187-192
32. Schubeler, D., Lorincz, M. C., Cimbor, D. M., Telling, A., Feng, Y. Q., Bouhassira, E. E., and Groudine, M. (2000) *Mol. Cell. Biol.* **20**, 9103-9112
33. Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001) *Dev. Cell.* **1**, 749-758
34. Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G., and Rosenfeld, M. G. (2002) *Science* **298**, 1747-1752
35. Mal, A., and Harter, M. L. (2003) *Proc. Natl. Acad. Sci. U S A* **100**, 1735-1739

36. Ben-Shushan, E., Pikarsky, E., Klar, A., and Bergman, Y. (1993) *Mol. Cell. Biol.* **13**, 891-901
37. Hershko, A. Y., Kafri, T., Fainsod, A., and Razin, A. (2003) *Gene* **302**, 65-72
38. Tremblay, K. D., Saam, J. R., Ingram, R. S., Tilghman, S. M., and Bartolomei, M. S. (1995) *Nat. Genet.* **9**, 407-413
39. Hata, K., Okano, M., Lei, H., and Li, E. (2002) *Development* **129**, 1983-1993
40. Pant, V., Mariano, P., Kanduri, C., Mattsson, A., Lobanenkov, V., Heuchel, R., and Ohlsson, R. (2003) *Genes. Dev.* **17**, 586-590
41. Bakin, A. V., and Curran, T. (1999) *Science* **283**, 387-390
42. Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003) *Science* **300**, 455
43. Nguyen, C. T., Weisenberger, D. J., Velicescu, M., Gonzales, F. A., Lin, J. C., Liang, G., and Jones, P. A. (2002) *Cancer. Res.* **62**, 6456-6461
44. Liang, G., Gonzales, F. A., Jones, P. A., Orntoft, T. F., and Thykjaer, T. (2002) *Cancer. Res.* **62**, 961-966
45. Velicescu, M., Weisenberger, D. J., Gonzales, F. A., Tsai, Y. C., Nguyen, C. T., and Jones, P. A. (2002) *Cancer. Res.* **62**, 2378-2384
46. Di Croce, L., Raker, V. A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S., and Pelicci, P. G. (2002) *Science* **295**, 1079-1082
47. Zardo, G., Reale, A., Passananti, C., Pradhan, S., Buontempo, S., De Matteis, G., Adams, R. L., and Caiafa, P. (2002) *Faseb J.* **16**, 1319-1321
48. Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000) *Nat. Genet.* **25**, 269-277
49. Rhee, I., Bachman, K. E., Park, B. H., Jair, K. W., Yen, R. W., Schuebel, K. E., Cui, H., Feinberg, A. P., Lengauer, C., Kinzler, K. W., Baylin, S. B., and Vogelstein, B. (2002) *Nature* **416**, 552-556
50. Rhee, I., Jair, K. W., Yen, R. W., Lengauer, C., Herman, J. G., Kinzler, K. W., Vogelstein, B., Baylin, S. B., and Schuebel, K. E. (2000) *Nature* **404**, 1003-1007
51. Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999) *Cell* **99**, 247-257
52. Chen, T., Ueda, Y., Xie, S., and Li, E. (2002) *J. Biol. Chem.* **277**, 38746-38754
53. Weisenberger, D. J., Velicescu, M., Preciado-Lopez, M. A., Gonzales, F. A., Tsai, Y. C., Liang, G., and Jones, P. A. (2002) *Gene* **298**, 91-99

54. Fatemi, M., Hermann, A., Gowher, H., and Jeltsch, A. (2002) *Eur. J. Biochem.* **269**, 4981-4984
55. Liang, G., Chan, M. F., Tomigahara, Y., Tsai, Y. C., Gonzales, F. A., Li, E., Laird, P. W., and Jones, P. A. (2002) *Mol. Cell. Biol.* **22**, 480-491
56. Aapola, U., Lyle, R., Krohn, K., Antonarakis, S. E., and Peterson, P. (2001) *Cytogenet. Cell. Genet.* **92**, 122-126
57. Bourc'his, D., Xu, G. L., Lin, C. S., Bollman, B., and Bestor, T. H. (2001) *Science* **294**, 2536-2539
58. Chedin, F., Lieber, M. R., and Hsieh, C. L. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 16916-16921
59. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998) *Nat. Genet.* **19**, 187-191
60. Lembo, F., Pero, R., Angrisano, T., Vitiello, C., Iuliano, R., Bruni, C. B., and Chiariotti, L. (2003) *Mol. Cell. Biol.* **23**, 1656-1665
61. Festenstein, R., Sharghi-Namini, S., Fox, M., Roderick, K., Tolaini, M., Norton, T., Saveliev, A., Kioussis, D., and Singh, P. (1999) *Nat. Genet.* **23**, 457-461
62. Hwang, K. K., and Worman, H. J. (2002) *Biochem. Biophys. Res. Commun.* **293**, 1217-1222
63. Lo, W. S., Duggan, L., Tolga, N. C., Emre, Belotserkovskya, R., Lane, W. S., Shiekhhattar, R., and Berger, S. L. (2001) *Science* **293**, 1142-1146
64. Noma, K., Allis, C. D., and Grewal, S. I. (2001) *Science* **293**, 1150-1155
65. Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S. I. (2002) *Science* **297**, 2232-2237
66. Cai, S., Han, H. J., and Kohwi-Shigematsu, T. (2003) *Nat. Genet.* **34**, 42-51
67. Hamaguchi, I., Woods, N. B., Panagopoulos, I., Andersson, E., Mikkola, H., Fahlman, C., Zufferey, R., Carlsson, L., Trono, D., and Karlsson, S. (2000) *J. Virol.* **74**, 10778-10784
68. Brenner, C., Deplus, R., Didelot, C., Loriot, A., Vire, E., De Smet, C., Gutierrez, A., Danovi, D., Bernard, D., Boon, T., Pelicci, P. G., Amati, B., Kouzarides, T., de Launoit, Y., Di Croce, L., and Fuks, F. (2005) *Embo. J.* **24**, 336-346
69. Jeffery, L., and Nakielny, S. (2004) *Journal of Biological Chemistry* **279**, 49479-49487
70. Kawasaki, H., and Taira, K. (2004) *Nature* **431**, 878-878

71. Lin, I. G., Tomzynski, T. J., Ou, Q. L., and Hsieh, C. L. (2000) *Molecular and Cellular Biology* **20**, 2343-2349
72. Fedoriw, A. M., Stein, P., Svoboda, P., Schultz, R. M., and Bartolomei, M. S. (2004) *Science* **303**, 238-240
73. Robbins, P. B., Yu, X. J., Skelton, D. M., Pepper, K. A., Wasserman, R. M., Zhu, L., and Kohn, D. B. (1997) *J. Virol.* **71**, 9466-9474
74. Juttermann, R., Li, E., and Jaenisch, R. (1994) *Proc. Natl. Acad. Sci. U S A* **91**, 11797-11801
75. Fuso, A., Cavallaro, R. A., Orru, L., Buttarelli, F. R., and Scarpa, S. (2001) *FEBS Lett.* **508**, 337-340
76. Friso, S., and Choi, S. W. (2002) *J. Nutr.* **132**, 2382S-2387S
77. Poirier, L. A. (2002) *J. Nutr.* **132**, 2336S-2339S
78. Jones, P. A., Taylor, S. M., Mohandas, T., and Shapiro, L. J. (1982) *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **79**, 1215-1219
79. Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., 3rd, Biniszkiwicz, D., Yanagimachi, R., and Jaenisch, R. (2001) *Science* **293**, 95-97
80. Schreiber, S. L., and Bernstein, B. E. (2002) *Cell* **111**, 771-778
81. Richards, E. J., and Elgin, S. C. (2002) *Cell* **108**, 489-500
82. Pannell, D., Osborne, C. S., Yao, S., Sukonnik, T., Pasceri, P., Karaiskakis, A., Okano, M., Li, E., Lipshitz, H. D., and Ellis, J. (2000) *Embo. J.* **19**, 5884-5894
83. Oka, M., Tagoku, K., Russell, T. L., Nakano, Y., Hamazaki, T., Meyer, E. M., Yokota, T., and Terada, N. (2002) *Molecular Biology of the Cell* **13**, 1274-1281
84. Huang, T. H., Laux, D. E., Hamlin, B. C., Tran, P., Tran, H., and Lubahn, D. B. (1997) *Cancer Res.* **57**, 1030-1034
85. Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G. Q. (2004) *Nature* **427**, 148-154
86. Grewal, S. I., and Moazed, D. (2003) *Science* **301**, 798-802
87. Bird, A. P., and Wolffe, A. P. (1999) *Cell* **99**, 451-454
88. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., and Tada, T. (2001) *Curr. Biol.* **11**, 1553-1558

89. Evans, M. J., and Kaufman, M. H. (1981) *Nature* **292**, 154-156
90. Okano, M., Xie, S., and Li, E. (1998) *Nat. Genet.* **19**, 219-220
91. Huntriss, J., Hinkins, M., Oliver, B., Harris, S. E., Beazley, J. C., Rutherford, A. J., Gosden, R. G., Lanzendorf, S. E., and Picton, H. M. (2004) *Mol. Reprod. Dev.* **67**, 323-336
92. Yu, L., Liu, C., Vandeusen, J., Becknell, B., Dai, Z., Wu, Y. Z., Raval, A., Liu, T. H., Ding, W., Mao, C., Liu, S., Smith, L. T., Lee, S., Rassenti, L., Marcucci, G., Byrd, J., Caligiuri, M. A., and Plass, C. (2005) *Nat. Genet.* **37**, 265-274
93. Yu, L., Liu, C. H., Vandeusen, J., Becknell, B., Dai, Z. Y., Wu, Y. Z., Raval, A., Liu, T. H., Ding, W., Mao, C., Liu, S. J., Smith, L. T., Lee, S., Rassenti, L., Marcucci, G., Byrd, J., Caligiuri, M. A., and Plass, C. (2005) *Nature Genetics* **37**, 265-274
94. Wu, M. C., and Ho, S. M. (2004) *Oncogene* **23**, 250-259
95. Lo, K. W., Tsang, Y. S., Kwong, J., To, K. F., Teo, P. M. L., and Huang, D. P. (2002) *International Journal of Cancer* **98**, 651-655
96. Hudson, J. W., Chen, L. Y., Fode, C., Binkert, C., and Dennis, J. W. (2000) *Gene* **241**, 65-73
97. Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G. Q. (2004) *Nature* **427**, 148-154
98. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) *Cell* **113**, 631-642
99. Bauer, M. K. A., Schubert, A., Rocks, O., and Grimm, S. (1999) *Journal of Cell Biology* **147**, 1493-1501
100. Chen, S. T., Chang, C. D., Huebner, K., Ku, D. H., McFarland, M., DeRiel, J. K., Baserga, R., and Wurzel, J. (1990) *Somat. Cell. Mol. Genet.* **16**, 143-149
101. Mozo, T., Fischer, K., Flugge, U. I., and Schmitz, U. K. (1995) *Plant. J.* **7**, 1015-1020
102. De Smet, C., Lorient, A., and Boon, T. (2004) *Mol. Cell. Biol.* **24**, 4781-4790
103. Hattori, N., Abe, T., Suzuki, M., Matsuyama, T., Yoshida, S., Li, E., and Shiota, K. (2004) *Genome Res.* **14**, 1733-1740
104. Ohgane, J., Wakayama, T., Senda, S., Yamazaki, Y., Inoue, K., Ogura, A., Marh, J., Tanaka, S., Yanagimachi, R., and Shiota, K. (2004) *Genes Cells* **9**, 253-260
105. Wu, M., and Ho, S. M. (2004) *Oncogene* **23**, 250-259

106. De Smet, C., Lurquin, C., Lethe, B., Martelange, V., and Boon, T. (1999) *Mol. Cell. Biol.* **19**, 7327-7335
107. Zhang, L. P., Stroud, J. C., Walter, C. A., Adrian, G. S., and McCarrey, J. R. (1998) *Biol. Reprod.* **59**, 284-292
108. Iannello, R. C., Young, J., Sumarsono, S., Tymms, M. J., Dahl, H. H., Gould, J., Hedger, M., and Kola, I. (1997) *Mol. Cell. Biol.* **17**, 612-619
109. Hisano, M., Ohta, H., Nishimune, Y., and Nozaki, M. (2003) *Nucleic Acids Research* **31**, 4797-4804
110. Xu, W., and Cooper, G. M. (1995) *Mol. Cell. Biol.* **15**, 5369-5375
111. Lin, H. B., Jurk, M., Gulick, T., and Cooper, G. M. (1999) *J. Biol. Chem.* **274**, 36796-36800
112. Dolce, V., Scarcia, P., Iacopetta, D., and Palmieri, F. (2005) *FEBS Lett.* **579**, 633-637
113. Narisawa, S., Hecht, N. B., Goldberg, E., Boatright, K. M., Reed, J. C., and Millan, J. L. (2002) *Mol. Cell. Biol.* **22**, 5554-5562
114. Huttemann, M., Jaradat, S., and Grossman, L. I. (2003) *Mol. Reprod. Dev.* **66**, 8-16
115. Karpf, A. R., Lasek, A. W., Ririe, T. O., Hanks, A. N., Grossman, D., and Jones, D. A. (2004) *Mol. Pharmacol.* **65**, 18-27
116. Sigalotti, L., Coral, S., Altomonte, M., Natali, L., Gaudino, G., Cacciotti, P., Libener, R., Colizzi, F., Vianale, G., Martini, F., Tognon, M., Jungbluth, A., Cebon, J., Maraskovsky, E., Mutti, L., and Maio, M. (2002) *Br. J. Cancer.* **86**, 979-982

## BIOGRAPHICAL SKETCH

Nemanja Rodić was born in Sabac, Serbia, Eastern Europe. He immigrated to the United States of America in fall 1997. As an undergraduate student, he attended the University of Florida where he graduated in 2001 with the highest honors. He joined the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine in 2001 where he began his doctoral study under the guidance of Dr. Naohiro Terada in the Department of Molecular and Cell Biology.