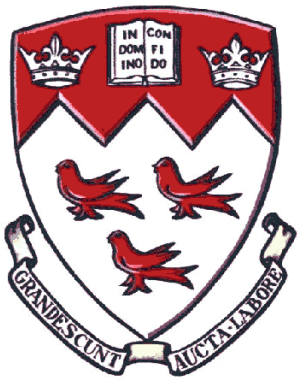

The role of DNMT1 regulation in cellular function.

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Dedicated to my wife and parents for their

love and support.

ABSTRACT

Disruption of the epigenome and its components is a hallmark of all forms of cancer. Typically observed in cancer is an alteration of the DNA methylation pattern, with silencing of tumour suppressor genes, as well as an increase in DNA methyltransferase 1 (activity or expression). However it has yet to be determined exactly how DNMT1 increases in cancer and how this increase might serve as therapeutic target. This thesis focuses on the regulation of DNMT1 in the cell cycle and the consequences of depleting DNMT1 in cancer cells.

During the cell cycle DNMT1 levels increase as the cell enters into S-phase. It has previously been shown that this cyclical regulation of DNMT1 occurs by destabilization of DNMT1 mRNA in G_0/G_1 through the action of a protein, identified to be the mRNA binding protein AUF1. AUF1 binds a regulator element located in the 3'UTR of DNMT1 mRNA and recruits the exosome, the RNA degradation complex, to degrade it.

When AUF1 is depleted in these cells, DNMT1 mRNA is stabilized which leads to increased DNMT1 protein levels, methyltransferase activity and genomic methylation. The changes of DNMT1 mRNA levels in the cell cycle were determined to occur as an inverse function of AUF1 protein levels. AUF1 levels were observed to decrease in S-phase which lead to increased stability in DNMT1 mRNA. This cell cycle regulation of AUF1 was determined to occur as a function of Rb. Rb actively stabilizes AUF1 protein. Indeed, upon elimination of Rb, AUF1 is degraded through the function of Hsp70 and the proteasome. This consequently leads to an elevation in DNMT1 protein levels which in turn increases genomic methylation levels. Elevated DNMT1 levels resulted in greater association with EZH2, which in turn leads to increased methylation of EZH2 targeted

promoters, including p16 and CNR1. This promoter hypermethylation occurred as a function of DNMT1 and EZH2.These observations indicate that regulation of DNMT1 is tied into the cell cycle function of Rb and upon disruption of this system, a characteristic of cancer, site-specific methylation occurs at tumour suppressors, another characteristic of cancer.

Furthermore, we examined the effect of depleting DNMT1 in cancer cells. Upon depletion of DNMT1, a signaling pathway known as the replication arrest/DNA damage checkpoint was induced. Activation of this pathway results in arrest of cell growth and cell cycle blockage and occurred independently of the catalytic activity of DNMT1 and instead responded to the absence of DNMT1. This supports a role for DMNT1 as a negative regulator of the replication arrest/DNA damage checkpoint through the action of interaction with an unknown protein. Moreover, suppression of the replication arrest/DNA damage checkpoint has been determined to be a necessary step in the proliferation of cancer cells. Taken together, the data from this thesis determined that common events in cancer, such as inactivation of Rb, lead to deregulation of DNMT1 mRNA, through AUF1, leading to site-specific methylation of tumour suppressors and could potentially serve to block growth arresting checkpoints like the replication arrest/DNA damage checkpoint. The novel functions of DNMT1, such as cell cycle regulation, site-specific methylation and role in the replication arrest/DNA damage checkpoint discovered in this thesis could serve to help better understand how cancer develops. The results of this thesis could serve to develop novel strategies to target these events and better treat cancer.

RESUME

L'altération de l'épigénome et de ses composants est une marque caractéristique de tous types de cancer. Une altération des profils de méthylation de l'ADN, associée à une inactivation de gènes suppresseurs de tumeurs ainsi qu'une augmentation de l'(activité/expression) de la méthyltransférase de l'ADN (DNMT1) sont largement observés dans les cancers. Cependant, les causes de cette augmentation de DNMT1 (expression/activité) dans le cancer et l'utilisation potentielle de cette augmentation comme cible thérapeutique n'ont pas encore été déterminées.

Cette thèse s'intéresse à la régulation de DNMT1 au cours du cycle cellulaire ainsi qu'aux conséquences d'une déplétion de DNMT1 dans les cellules cancéreuses.

Au cours du cycle cellulaire, le niveau de DNMT1 augmente dès lors que la cellule entre en phase S. Il a été montré précédemment qu'une régulation cyclique de DNMT1 se met en place grâce à une déstabilisation de son ARN messager en phase G0/G1 sous l'action d'une protéine non identifiée. Cette protéine a été identifié comme la protéine liant l'ARN, AUF1. AUF1 interagit avec un élément régulateur situé dans la partie 3'-UTR de l'ARNm de DNMT1 et entraîne la dégradation de cet ARNm en recrutant l'exosome, un complexe de dégradation de l'ARN. La déplétion d'AUF1 dans ces cellules stabilise l'ARNm de DNMT1 ce qui conduit à une augmentation de l'expression de cette protéine, de son activité méthyltransférase ainsi que de la méthylation du génome. Il a été également montré que le niveau d'expression de l'ARNm de DNMT1 au cours du cycle cellulaire est inversement corrélé à celui de la protéine AUF1. Ce niveau d'AUF1 est diminué en phase S ce qui traduit par une stabilité accrue de l'ARNm de DNMT1. Il a été montré que cette régulation d'AUF1 au cours du cycle cellulaire est fonction de la protéine Rb. Rb stabilise activement la protéine AUF1. En effet, en réponse à une déplétion de Rb, AUF1 est dégradée par l'intermédiaire de la protéine Hsp70 et du protéasome. Cette dégradation a pour conséquence une augmentation du niveau

d'expression de DNMT1 lequel conduit à une augmentation du niveau de méthylation du génome. De plus, cette augmentation de DNMT1 résulte en une plus grande association avec la protéine EZH2 entraînant une hyperméthylation de promoteurs de gènes ciblés par EZH2 (ex : p16, CNR1 et PCNA). Ces observations démontrent que la régulation de DNMT1 est étroitement liée aux fonctions de Rb dans le cycle cellulaire. Caractéristique dans les cancers, une rupture de cette relation DNMT1-Rb, entraîne ainsi une méthylation site-spécifique de gènes suppresseurs de tumeurs, une autre caractéristique des cancers.

En parallèle, nous avons étudié l'effet d'une déplétion de DNMT1 dans des cellules cancéreuses. Suite à une déplétion de DNMT1, une voie de signalisation connue comme un point de contrôle de l'arrêt de la réplication/lésions de l'ADN est induite. L'activation de cette voie de signalisation entraîne l'arrêt de la croissance cellulaire et le blocage du cycle cellulaire. L'activation de cette voie répond à l'absence de DNMT1 et de façon indépendante de son activité catalytique. Ceci est en faveur d'un rôle pour DNMT1 de régulateur négatif du contrôle de l'arrêt de la réplication/lésions de l'ADN *via* l'interaction avec une protéine qui reste encore à identifier. De plus, la suppression des points de contrôle de l'arrêt de la réplication/lésion de l'ADN a été montré comme étant une étape nécessaire à la prolifération des cellules cancéreuses. L'ensemble des données de cette thèse démontre que des événements communs aux cancers, telle que l'inactivation de Rb, peuvent conduire à la dérégulation, *via* AUF1, de l'ARNm de DNMT1, laquelle entraîne la méthylation site-spécifique de gènes suppresseurs de tumeurs. Cette dérégulation de DNMT1 pourrait potentiellement servir à bloquer les points de contrôle d'arrêt du cycle cellulaire/lésions de l'ADN.

Les nouvelles fonctions de DNMT1, telles que la régulation du cycle cellulaire, la méthylation site-spécifique et le contrôle de la réplication/lésions de l'ADN découverts dans cette thèse devraient permettre de mieux comprendre le développement cancéreux et de développer de nouvelles stratégies thérapeutiques.

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STATEMENT OF CONTRIBUTIONS

This thesis is written in manuscript format as permitted by the McGill University Faculty of Graduate Studies and is comprised of three manuscripts. The contribution of each author is described below.

Chapter 4: AUF1 cell cycle variations define genomic DNA methylation by regulation of DNMT1 mRNA stability. Molecular and Cellular biology (2007), 27(1): 395-410. Jerome Torrisani, Alexander Unterberger, Sachin R.Tendulkar, Keisuke Shikimi and Moshe Szyf.

Figure 1: All experiments were done by Jerome Torrisani

Table 1 was compiled by Jerome Torrisani

Figure 2 A, B, C, D were done by Jerome Torrisani

Figure 2 F, G were done by Alexander Unterberger

Figure 3 B, C were done by Jerome Torrisani and Sachin Tendulkar

Figure 3 A, D were done by Alexander Unterberger

Figure 4 A, B, E, F, G, H, I, J and K were done by Alexander Unterberger

Figure 4 C, D were done by Jerome Torrisani

Figure 5 A, B, C, D, F and G were done by Alexander Unterberger

Figure 5 E were done by Jerome Torrisani

Figure 6 A, B, E, F were done by Alexander Unterberger

Figure 6 C, D were done by Jerome Torrisani

Figure 7 B, D, E and I were done by Jerome Torrisani

Figure 7 A, C, F, G, H and J were done by Alexander Unterberger

Supplemental Figures S1, S4, S6 and S7 were done by Alexander Unterberger

Supplemental Figures S2 and S3 were done by Jerome Torrisani

Supplemental Figure S5 was done by Keisuke Shikimi

The text of the manuscript was prepared by Jerome Torrisani, Alexander Unterberger and Moshe Szyf.

Chapter 5: Gene specific methylation is defined by Rb regulation over AUF1 and DNMT1. Submitted to Molecular and Cellular Biology. Alexander Unterberger, Flora Pui-Chi Chik, Jerome Torrisani, and Moshe Szyf.

All experiments were performed by Alexander Unterberger except figure 1E, F which were performed by Flora Pui-Chi Chik.

The text of the manuscript was prepared by Alexander Unterberger and Moshe Szyf, and edited by Jerome Torrisani.

Chapter 6: DNA methyltransferase 1 knockdown activates a replication-stress checkpoint. Molecular and Cellular Biology (2006), 26(20); 7575-86. Alexander Unterberger, Stephen D Andrews, Ian C.G. Weaver and Moshe Szyf.

All experiments were performed by Alexander Unterberger except figure 5F, which was performed by Stephen D. Andrews.

The text of the manuscript was prepared by Alexander Unterberger and Moshe Szyf, and edited by Ian C.G. Weaver.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this thesis I have presented the following original results:

1. The mRNA binding protein AUF1 binds DMNT1 mRNA in a manner dependent upon the presence of the 3'UTR.
2. AUF1 binding decreases DNMT1 mRNA stability and recruits the exosome complex to aid in destabilizing the mRNA.
3. Depletion of AUF1 leads to increased DNMT1 mRNA stability, increased DNMT1 protein levels, increased DNA methyltransferase activity and genomic hypermethylation without affecting DNMT1 promoter activity or the stability of other DNMTs in the cell.
4. AUF1 protein levels were inversely correlated with DNMT1 mRNA levels throughout the cell cycle and the decrease of AUF1 protein was dependent on proteasome activity.
5. Depletion of Rb in untransformed human fibroblasts lead to increased AUF1 protein stability without altering AUF1 mRNA levels. Stabilization of AUF1 protein occurred independently of alterations in cell cycle, gene transcription or protein translation, however function of the proteasome was required.
6. Hsp70 mediated destabilization of AUF1 protein upon depletion of Rb.
7. Depletion of Rb leads to increased DNMT1 protein levels and genomic hypermethylation.
8. Depletion of Rb lead to increased binding of DNMT1 to EZH2
9. Depletion of Rb lead to site-specific increases in methylation of EZH2 targets p16, CNR1 and KCNA in a manner that was dependent on DNMT1 and EZH2 presence.
10. Depletion of DNMT1 in cancer cells lead to activation of the replication arrest/DNA damage checkpoint through phosphorylation of Chk1, Chk2 and γH2A.X foci formation.

11. Induction of the pathway was dependent on ATR function.
12. Induction of the pathway was observed to occur independently of DNMT1 catalytic function.
13. Presence of DNMT1 in the cell acts as a negative regulator of the replication arrest/DNA damage checkpoint.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the McGill University. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of McGill University.

The dissertation has not been presented to any other University for examination either in Canada or overseas.

SIGNED: **DATE:**

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LIST OF ABBREVIATIONS

Units

bp – base pair

kDa – Kilodalton

^{o}C – degrees centigrade

h – hour

l – liter

µl – microliter

g – gram

µg – microgram

M – molar

mM - millimolar

µM - micromolar

% - percentage

Chemicals, molecules and miscellany

3'UTR – 3' Untranslated region

5-**azaC** – 5-azacytidine

5-aza-CdR – 5-azadeoxycytidine

5mC – 5-Methyl Cytosine

A - Adenine

APC – Adenomatous polyposis coli

ARE - AU-rich element

ATM - Ataxia telangiectasia mutated

ATP – Adenosine triphosphate

ATR - Ataxia telangiectasia and Rad3 related

AU - Adenine and uridine

AUF1 - ARE/poly-(U) binding/ degradation factor 1

C - Cytosine

CBP – Creb binding protein

CDC – Cell division cycle

ChIP – Chromatin immunoprecipitation

Chk – Checkpoint homologue

CpG – Cysteine phosphate guanosine

dATP – Deoxy adenosine triphosphate

ddH_20 – Double deionised water

DNA – Deoxyribonucleic acid

DNMT- DNA Methyltransferase

DNMT1 – DNA Methyltransferase 1

DNMT3a – DNA Methyltransferase 3a

DNMT3b – DNA Methyltransferase 3b

dNTP – Deoxynucleoside triphosphate

G - Guanine

GADD45a – Growth arrest and DNA-damage-inducible protein 45 alpha

H2A – Histone 2A

H2A.X – Histone 2A.X

H2B – Histone 2B

H3 – Histone 3

H4 – Histone 4

HAT – Histone Acetyltransferase

HDAC – Histone Deacetylase

HMT – Histone Methyltransferase

hnRNP – Heterogeneous ribonuclear proteins

HPRT - Hypoxanthine-guanine phosphoribosyltransferase

Hsp – Heat Shock Protein

K - Lysine

LINE – Long Interspersed Element

Maldi-Tof - Matrix Assisted Laser Desorption Ionization Time of Flight

MBD – Methyl-CpG binding domain

mDIP – Methylated DNA immunoprecipitation

MeCP2 – Methyl-CpG-binding protein 2

mRNA – Messenger ribonucleic acid

NuRD - nucleosome remodeling and deacetylation complex

p – Probability

PCNA – Proliferating cell nuclear antigen

PHD – Plant Homeo Domain

R – Arginine

Rb- Retinoblastoma

RT- Reverse transcriptase

SAM – S-adenosyl methionine

SINE- Short Interspersed Element

siRNA – Small interfering RNA

T – Thymine

TDA – Thymidine Glycosylase

TSA – Trichostatin A

uPA – Urokinase-type plasminogen activator

α - Alpha

β - Beta

γ - Gamma

Chapter 1

Introduction

One of the greatest mysteries in understanding cancer is delineating the route that normal functioning cells have to traverse in order to convert into a rapidly growing and aggressive cancer. The answer to this question could possibly be found in the intricate pathways that program gene expression. It is clear that the conversion of a normal cell to a transformed cell involves a coordinated widespread reorganization of gene expression programming.

Cancer is a disease commonly marked with a dramatic alteration in the epigenome, the regulator of programmed gene expression (define). The epigenome is a layer of information in the cell that allows for heritable changes in gene expression without modifications of the genomic sequence. The epigenome is composed of chromatin – histone proteins which associate with DNA, and a covalent chemical modification of the genome itself – DNA methylation. These elements work in tandem to control gene expression through the creation of active and inactive regions of the genome, through the ability to recruit negative regulatory complexes to the genome and by preventing access to transcription-activating regulatory complexes.

DNA methylation is the covalent addition of a methyl group to cytosine residues in the genome. Typically, gene activity is regulated by methylation of cytosine residues in regulatory regions of a gene. A methylated promoter is usually associated with a silent transcriptional state, while an unmethylated promoter is associated with an active transcriptional state. Methylation occurs in mammalian genomes at the CpG dinucleotide sequence. However, not all CpG dinucleotides are methylated. Different

CpG dinucleotides are methylated in different cells, thus generating a pattern of distribution of methylated residues in the genome that is cell specific. DNA methylation is established by members of the DNA methyltransferase (DNMT) family. DNMTs establish and maintain methylation patterns throughout the genome. A characteristic of cancer is dysfunction of the epigenome whereby the genomic methylation pattern is altered. In a cancer cell, most of the genome is hypomethylated relative to normal cells yet several specific regions are hypermethylated. Genes that are typically silenced by methylation in cancer include tumour suppressor genes. This silencing of tumor suppressor genes may lead to tumorigenesis. Furthermore, the methyltransferase DNMT1, traditionally held responsible for homeostatically maintaining methylation patterns, is commonly upregulated in cancer. Several studies have shown that DNMT1 levels vary throughout the cell cycle are highest in S-phase. Interestingly, DNMT1 was shown to be regulated during the cell cycle through the action of an unidentified protein binding to a regulatory element in DNMT1 mRNA. When this regulatory element was removed it was observed that DNMT1 levels greatly increased and the cells underwent transformation. Studying the mechanisms involved in regulating DNMT1 levels in the cell cycle may help lead to a better understanding of how deregulation of DNMT1 plays a role in the development of cancer. Elevated DNMT1 levels in cancer have been used as a potential therapeutic target. Studies that targeted DNMT1 by specific antisense oligonucleotides have revealed that depletion of DNMT1 resulted in replication arrest and activation of a genotoxic stress checkpoint (Knox, Araujo et al. 2000; Milutinovic, Zhuang et al. 2003). Understanding how DNMT1 depletion induces replication arrest may contribute to development of improved therapeutic strategies for targeting DNMT1, as well as other DNMTs and epigenome regulatory enzymes.

The review of the literature for this thesis will lay out the background information regarding DNA methylation and the epigenome, link cancer to dysfunctions of the epigenome and describe how DNMT1 regulation plays a role in development of a cancer state. The review will also examine how targeting the epigenome is a viable

method of cancer treatment, and will examine possible directions for new strategies in targeting the enzymes responsible for maintaining the epigenome.

Chapter 2

Review of the Literature

2.1. Cancer as an epigenetic disease

Cancer is the leading cause of death due to disease (Jemal, Siegel et al. 2008) in the western world. Cancer is a disease that arises from uncontrolled growth of cells that have overcome internal and environmental boundaries set in place to prevent this. Cells growing at an uncontrolled pace can grow into a mass of cells called a tumor. Tumors can be either benign or malignant. Benign tumors are slow growing and do not display the malignant properties of cancer, including rapid growth, tissue invasiveness and metastasis. These cancers are easier to treat than rapid growing cancers that metastasize and invade tissues. Death due to cancer is commonly associated with metastasis and invasion of other tissues by malignant tumors. This requires cancer cells to overcome their environmental boundaries, disassociate from their local environment and survive independent of their primary tissues. These cells can migrate to other tissues, invade them and develop into a secondary tumor. Malignant tumors are more difficult to treat due to their distribution throughout the body and as well as their mobile nature.

The conversion of a cell from a normal to a cancerous state requires a fundamental alteration of the cell. This conversion must allow the cancer cell to have effectively limitless replicative potential, insensitivity to anti-growth signals, invasive

properties, sustained angiogenesis, self-sufficiency in growth signals and a loss of apoptotic capacity (Hanahan and Weinberg 2000). A change in a property of a cell requires an antecedent alteration in gene expression to facilitate this change. Multiple changes in gene expression allow the cell to acquire phenotypes required for carcinogenesis. Two classes of genes are involved in these levels of cellular regulation: proto-oncogenes/oncogenes and tumor-suppressor genes. Expression of proto-oncogenes is properly controlled in normal cells, however in cancer the regulation is altered or lost and these genes become constitutively active oncogenes. Oncogenes encode oncoproteins that can drive the cell toward unregulated cellular growth by 1) overriding inhibitory growth signals and cell cycle checkpoints and 2) through induction of transcription of other growth promoting factors. Conversely, tumor-suppressor genes are a class of genes that are normally expressed in healthy untransformed cells and serve to inhibit cell cycle progression, repair damaged DNA, regulate normal DNA replication and induce cell death when necessary. These genes can also produce cell adhesion molecules, preventing cells from migrating from their tissue of origin. In cancer, tumor-suppressor genes become inactivated which suppresses inhibitory growth signals to be suppressed, leading to unregulated cell growth and division. The combination of proto-oncogene activation and tumor-suppressor gene inactivation are necessary steps in cellular transformation and development of cancer.

The genome contains all the potential information required to manage a living organism. However, it must utilize different subsets of this information at different time points and in different tissue types. There must be a level of regulation that can control the differential expression of genomic information in a spatial and temporal manner. Although changes in gene expression could be caused by silencing mutations, promoter activating mutations, or increased protein stability and chromosomal translocation, this cannot be a mechanism for programmed gene expression during development. Similarly, the coordination of the events necessary for carcinogenesis requires

programmed changes in gene expression. The epigenome is responsible for regulation of the genome. In contrast to the genome, which is identical in different cell types and throughout life, the epigenome is dynamic and varies from cell type to cell type and from time point to time point in life. It is responsive to developmental, physiological, environmental, and pathological signals and confers both cell type and temporal identities of gene expression programs. DNA methylation patterns are a fundamental constituent of the epigenome. The review of the literature in this thesis will focus on the epigenetic changes in cancer, the nature of the epigenome, how the regulators of the epigenome can modulate the development of cancer, as well as understanding how the epigenome might serve as an effective anti-cancer target.

2.2. DNA Methylation

A primary component of the epigenome is DNA methylation. DNA methylation is a covalent modification of DNA and serves as a general transcriptional regulator, repressing gene expression (Razin and Riggs 1980). Some studies suggest that the main role of DNA methylation is to silence selfish genetic elements that have accumulated in the genome over the course of evolution (Yoder and Bestor 1998). However accumulating data suggests that it is involved in tissue specific gene expression, chromosome X inactivation, parental imprinting and responds to changing environments in early life and later. The role of DNA methylation as an interface between the dynamic environment and the genome is emerging as one of its most interesting functions (Szyf, Weaver et al. 2007; Szyf, McGowan et al. 2008). In vertebrates, DNA is methylated at the 5’ position of the cytosine rings in the sequence CpG(Razin and Riggs 1980). The DNA methylation reaction is catalyzed by the enzyme DNA methyltransferase (DNMT) (Section 2.4) and requires S-adenosyl-methionine (SAM) as a methyl donor. The enzymatic modification of cytosine with a methyl group consists

of the following events: 1) Projection of the cytosine base out of the double helix. 2) Nucleophilic attack of the 6th position of the cytosine pyrimidine ring by a cysteine residue in the catalytic domain of the DNMT. 3) Nucleophilic attack of the 5th position of the cytosine pyrimidine ring on the methyl group in SAM. 4) Release of S-adenosyl homocysteine and dissociation of the DNMT and the cytosine pyrimidine ring(Jeltsch 2002) (Figure 2.1). Although CpG is the most commonly methylated dinucleotide sequence in mammals, other dinucleotide residues have been shown to be methylated in early embryogenesis(Jeltsch 2002), including CpT , CpC and CpA(Haines, Rodenhiser et al. 2001; Dodge, Ramsahoye et al. 2002; Volpe 2005). Methylation at these sites occurs at much lower frequencies than CpG methylation. In total, 70-90% of all CpGs are methylated in mammals. In plants, DNA methylation occurs in CpG dinucleotide sequences as well as in CpNpG and CpHpH (H is any nucleotide except guanosine) trinucleotide sequences (Henderson and Jacobsen 2007; Vaillant and Paszkowski 2007).

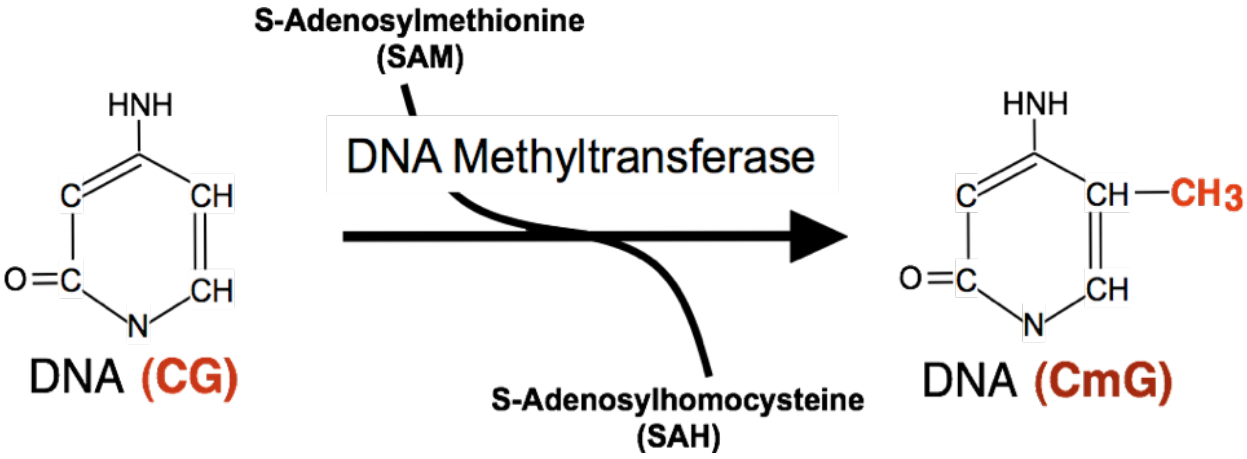

Figure 2.1 DNA Methylation Reaction. The transfer of a methyl group from S-Adenosylmethionine to the 5th position of the cytosine pyrimidine ring through the activity of the DNA methyltransferase enzyme. The reaction produces 5-methyl cytosine and S-Adenosylhomocysteine.

Studies have shown that CpG methylation in the plant occurs at a rate of 4 out of 5 CpGs(Gruenbaum, Naveh-Many et al. 1981). CpG methylation has been shown to serve as a transcriptional repressor, as well as protection against RNA viruses(Aufsatz, Mette et al. 2002). In bacteria, adenosine and cytosine residues are methylated at N6-methyladenine, and N4 or C5 methylcytosine. The methylated residues reside in specific sequences of the bacterial genome and serve as a protective mark against restriction endonucleases. These endonucleases serve to protect the cell from invading DNA bacteriophages. Other cellular processes involving DNA methylation in bacteria include DNA replication (Bakker and Smith 1989; Landoulsi, Malki et al. 1990), mismatch repair (Stambuk and Radman 1998), as well as control of gene expression (Oshima, Wada et al. 2002). The multicellular fungi, *Neurospora crassa* and *Aspergillus flavus* have been shown to have detectable levels of methylation in their genomes (Foss, Roberts et al. 1993; Gowher, Ehrlich et al. 2001). *N. crassa* in particular has been shown to require DNA methylation to control and inactivate proliferation of retrotransposon(Selker, Tountas et al. 2003).

Interestingly, unicellular fungi *S. cerevisiae* and *S. pombe* have been shown to have little or no methylation in their genomes(Selker, Tountas et al. 2003). Other model eukaryotes, including *Caenorhabditis elegans(Simpson, Johnson et al. 1986)* and *Drosophila melanogaster*(Lyko, Beisel et al. 2006), have also been shown to have no significant levels of DNA methylation. While some evidence disputes these claims and suggests that DNA methylation may serve as a retrotransposon regulator in *Drosophila*(Salzberg, Fisher et al. 2004), the fact that these four model systems function without DNA methylation suggests that another level of epigenetic gene transcription regulation may exist and can function independently of DNA methylation. This level of regulation exists at the level of chromatin (see section 2.6).

Studies have shown that CpG representation in the genome occurs at levels that are 20% of their expected frequency(Bird 1986), based on the expected mathematical proportion of CGs in the genome, and when they do occur they are usually heavily

methylated. However CpG distribution has been shown to be non-random and occurs at high frequency in promoter regions of genes(Saxonov, Berg et al. 2006). These regions are known as CpG islands and have been shown to have CpG content 5-10 fold higher than other areas in the genome(Gardiner-Garden and Frommer 1987). Gene promoters that contain unmethylated CpG islands include the promoters of housekeeping genes and genes that are expressed in most cell types(Razin and Szyf 1984).Tissue-specific genes on the other hand are sparsely occupied by CpG dinucleotides and are methylated in most tissues but are unmethylated and expressed in the proper cell type. Methylation of repetitive elements within the genome is also important for suppressing expression of transposons and endogenous retroviruses. CpG poor regions that are hypermethylated are typically found in repetitive sequences, such as satellite repeat, interspersed elements, long interspersed element (LINEs) and short interspersed element (SINEs). The gap between the frequencies of CpGs and methylation of CpGs is probably due to the deamination of methylated Cs to T. Whereas the product of cytosine deamination, uracil, is readily recognized as aberrant and is repaired, the deamination product of methylated cytosine is thymine, leading to transition mutations in the next round of replication(Duncan and Miller 1980). Other areas of the genome that have methylated CpG island regions include tissue-specific genes, silenced X chromosomes in females and parentally-imprinted genes.

Methylation patterns are established during germ cell and embryonic reprogramming, whereby waves of *de novo* methylation and demethylation sculpt the methylation pattern(Reik, Dean et al. 2001). Most of these patterns are maintained throughout life, specifically in the case of the silenced X chromosomes and parentally imprinted genes.

2.2.1. DNA Methylation and gene expression

The idea that DNA methylation was involved in the regulation of gene expression was around before the discovery of any functional DNMT proteins (See section 2.4). The notion that methylation status within the body of genes can be positively correlated with transcription was suggested by Simmen et al.(Simmen, Leitgeb et al. 1999) who proposed that methylation within coding regions can prevent the production of incorrectly initiated transcripts by silencing expression from spurious promoters. Initial studies showed that DNA rich in 5-methyl cytosine content (5mC) was more difficult to digest with nucleases than naked DNA(Razin and Cedar 1977). This implied that methylated DNA is associated with a more densely packed conformation of DNA. A landmark paper in 1980 by Razin and Riggs suggested that DNA methylation was involved in gene silencing, based on the observation that regulatory regions of inactive genes were often methylated(Razin and Riggs 1980). The role of DNA methylation in actively silencing expression, as opposed to serving as a passive marker of repression, has been examined in depth. Studies demonstrated that *in vitro* methylation of genes ectopically introduced into mammalian cells become suppressed(Stein, Razin et al. 1982; Vardimon, Kressmann et al. 1982). Other studies using DNMT inhibitors 5-azacytidine (5-azaC) or its deoxy analog 5-azadeoxycytidine (5-aza-CdR) have investigated the effect on gene expression when DNA methylation patterns are erased from gene promoters. Jones et al. observed that the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene, present on the heavily methylated and inactive X chromosome, was re-expressed upon DNMT inhibition by 5-azaC(Jones, Taylor et al. 1982). However, other early studies revealed that DNA methylation was not the sole negative regulator of gene expression. Treatment of adult chickens with 5-azaC led to complete demethylation of the embryonic *β-globin* gene, however there was no reestablishment of expression(Ginder, Whitters et al. 1983). Similar studies have shown that additional factors are required for expression of a silenced gene, in addition to the

removal of DNA methylation mark. For example, in C3H 10T1/2 mouse embryo fibroblast cells, treatment with 5-azaC led to stable removal of DNA methylation from the promoters of *c-fos* and *β-globin*, without a change in expression of those genes(Hsiao, Gattoni-Celli et al. 1984).

2.2.2. DNA Methylation silences gene expression by several mechanisms

How does DNA methylation silence gene expression? It is now clear that DNA methylation blocks gene expression by several mechanisms. The two most common mechanisms are direct interference with the binding of transcription factors to recognition elements and indirectly through recruitment of chromatin silencing proteins.

a. Direct mechanisms: Several mammalian transcription factors have CpG-rich binding sites and some have CpGs in their DNA recognition elements. A methyl group in the recognition element alters its chemical nature. There are a number of transcription factors that were shown to have their binding inhibited by methylation of their associated sequences, including c-AMP response element(Iguchi-Ariga and Schaffner 1989), Kruppel-like Factor 6(Guo, Lin et al. 2007), NGFI-A(Weaver, Cervoni et al. 2004), AP2(Comb and Goodman 1990), c-Myc(Prendergast, Lawe et al. 1991; Prendergast and Ziff 1991), ATF-like factor and retinoblastoma binding factor 1(Ohtani-Fujita, Dryja et al. 1997). In these studies *in vitro* binding assays suggested that methylation of DNA can physically interfere with transcription factor binding, and the corresponding *in vivo* experiments showed that this interference resulted in transcriptional repression. However, this mechanism cannot account for all the repressive effects of methylation since not all the transcription factors contain a CpG dinucleotide in their recognition

sequence, and because some transcription factors do not differentiate between methylated and unmethylated sites. One example is the ubiquitous transcription factor Sp1, which was shown to bind equally well to methylated and unmethylated DNA(Harrington, Jones et al. 1988; Holler, Westin et al. 1988). Recent data suggested that binding of Sp1 to its consensus sequence could be prevented by methylation(Mancini, Singh et al. 1999; Song, Ugai et al. 2003; Zhu, Srinivasan et al. 2003). The explanation for these conflicting reports may reside in the fact that methylation can indirectly interfere with Sp1 binding through the recruitment of other factors which can in turn preclude Sp1 binding(Kudo 1998).

b. Indirect mechanisms: DNA methylation serves as a negative regulator of genes by triggering the formation of inactive chromatin structure, causing gene silencing. The relationship between DNA methylation and chromatin is mediated by a protein family that is capable of 'reading and interpreting' DNA methylation signals. The first protein in this family, Methyl-CpG-binding protein 2 (MeCP2), was originally shown to recognize a single CpG site and to associate with inactive regions of the genome(Lewis, Meehan et al. 1992). These regions are distinguished by the presence of densely methylated DNA, as well as tightly arranged heterochromatin. MeCP2 belongs to the family of methyl-CpG binding domain (MBD) proteins. This family also includes MBD1, MBD2, MBD3 and MBD4(Nan, Meehan et al. 1993). Upon recognition and binding of methyl-CpGs, these proteins recruit multiprotein repressor complexes that modify chromatin by ATP-dependent nucleosome remodeling and histone deacetylase (HDAC) activities. Specifically, MeCP2 has been shown to recruit transcriptional repressor complex Sin3A/HDAC(Nan, Ng et al. 1998) through its transcriptional repressor domain, as well as to direct H3-Lys9 methylation through recruitment of histone methyltransferases (HMT) (Fuks, Hurd et al. 2003). MeCP2 interaction with HDACs and HMTs, leads to the removal of acetylation and the addition of methyl groups to histone tails, respectively,

promoting a tighter association between DNA and histones and a transcriptionally silent chromatin structure (For more information regarding histones, see section 2.6.1.).

Other MBDs act similarly. MBD1 has been shown to recruit histone deacetylase activity (Ng and Bird 2000) and SUV39H1 histone methyltransferase activity(Fujita, Watanabe et al. 2003). MBD2 and MBD3 have been shown to act as both gene expression activators(see section 2.5.1.) and repressors. As repressors, MBD2(Meehan, Lewis et al. 1989) and MBD3(Hendrich and Bird 1998; Zhang, Ng et al. 1999) have been shown to be integral components of the nucleosome remodeling and deacetylation (NuRD) complex. The NuRD complex mediates changes in chromatin structure, leading to inactive heterochromatin, via ATP-dependent nucleosome remodeling components, as well as histone deacetylase components(Tyler and Kadonaga 1999).

DNA methylation has roles other than silencing gene expression. DNA methylation has been shown to block aberrant recombination of repetitive sequences, such as transposons, endogenous retroviruses(Chen, Pettersson et al. 1998; Eden, Gaudet et al. 2003) and LINE-1(Ogino, Kawasaki et al. 2008). Methylation of centromeric sequences promotes stability of chromosomes(Miniou, Jeanpierre et al. 1994). The absence of DNA methylation in centromeric regions is observed in the Immunodeficiency, Centromeric instability and Facial abnormalities (ICF) disorder. This supports the hypothesis that hypomethylation of DNA could lead to chromosomal instability. The cause of hypomethylation in ICF is a mutation in the gene encoding DNA methyltransferase 3b (DNMT3b), one of the enzymes responsible for establishing the DNA methylation pattern at these centromeric sequences (Hansen, Wijmenga et al. 1999). Further support to this connection between chromosome stability and DNA methylation comes from the observation that mouse embryonic stem cells deficient for the *DNMT1* gene exhibit significantly elevated rates of genetic deletions(Chen, Pettersson et al. 1998).

2.3. DNA methylation in cancer

2.3.1. Gene silencing in cancer by aberrant methylation

A hallmark of cancer is a change in genomic methylation patterns(Szyf 1996). Upon cellular transformation, the genome undergoes global hypomethylation relative to normal cells, while specific gene promoters undergo hypermethylation. This regional hypermethylation is associated with silencing of tumor suppressor genes. One gene that is characteristically silenced in cancer is CDKN2A (cyclin-dependent kinase inhibitor 2A)/p16. p16 functions as a cell cycle inhibitor, preventing unrestrained entry into S-phase from G_1. When p16 expression is silenced in cancer, cells are able to bypass the G_1/S phase checkpoint and enter into unregulated growth and division. Familial cancers, such as hereditary malignant melanoma and pancreatic cancer, are associated with inactivating mutations of the p16 gene(Goldstein 2004; Pho, Grossman et al. 2006). However, in sporadic cancers, p16 silencing is commonly associated with promoter methylation, as observed in gastrointestinal(Luo, Zhang et al. 2006), and respiratory tract cancers(Wang, Lee et al. 2004). Additionally, cancer cell lines derived from tumors have also shown epigenetic silencing of p16 through DNA methylation(Gonzalez-Zulueta, Bender et al. 1995). Other genes that have been shown to be silenced by DNA methylation in cancer include DNA repair genes such as MLH1(Simpkins, Bocker et al. 1999) and BRCA1(Dobrovic and Simpfendorfer 1997), the cell cycle protein APC(Yang, Liu et al. 2006)and the mitosis inhibitor RASSF1A(Agathanggelou, Honorio et al. 2001; Astuti, Agathanggelou et al. 2001; Morrissey, Martinez et al. 2001; Song, Song et al. 2004). A common mutation in cancer is the functional inactivation of the Retinoblastoma (Rb)gene, eliminating the function of this tumor suppressor. Interestingly, this gene was the first tumor suppressor shown to be silenced by hypermethylation in cancer(Sakai, Toguchida et al. 1991). Thus in parallel to genetic

inactivation by sequence change, DNA methylation is an alternative mechanism for silencing tumor suppressor genes in the absence of a genetic change.

2.3.2. Gene activation in cancer by aberrant hypomethylation

In cancer, certain oncogenes have been shown to be activated through promoter activating mutations, gene amplification, coding mutations that lead to increased protein stability or loss of negative regulatory domains. Importantly, in addition to these genetic mechanisms, epigenetic mechanisms are involved in aberrant activation of gene expression in cancer. The first oncogene to be studied in terms of genetic and epigenetic regulation in cancer was Ras. Ras protein serves as a signaling molecule that regulates cellular proliferation and migration, as well as apoptosis. Activating mutations of Ras in tumors and cancer cell lines are well established(Bos 1989). Increased expression of Ras in cancer may also be caused by decreased promoter methylation (Feinberg and Vogelstein 1983). Another example is the urokinase-type plasminogen activator (uPA) gene. This gene plays a critical role in cancer metastasis. uPA normally serves as a serine protease which facilitates degradation of the extracellular matrix. uPA levels are increased(Van Veldhuizen, Sadasivan et al. 1996) in cancer due to gene amplification (Helenius, Savinainen et al. 2006). However increases in uPA levels could also occur in response to a decrease in promoter methylation (Guo, Pakneshan et al. 2002). Increased promoter activity due to promoter hypomethylation can lead to a number of cellular transformation events, as well as increased invasiveness and metastasis of cancer cells.

2.4. DNA Methyltransferases

The DNA methylation reaction is catalyzed by the enzymatic action of the family of proteins called DNA methyltransferases (DNMTs). As previously discussed (section 2.2, figure 2.1), DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethionine (SAM), the biological methyl donor, to the 5th carbon position of the cytosine pyrimidine ring. It was initially proposed in 1975 that two main classes of DNMTs were required for two distinctly different methylation processes(Riggs 1975): *De novo* methylation, which establishes methylation patterns, as well as maintenance methylation, which copies the methylation pattern onto nascent DNA strands arising from DNA replication. Over 700 methyltransferases have been identified in prokaryotes(Bestor 2000), however in mammals 3 families of DNMTs comprising 4 members have been found: Dnmt1, Dnmt2, Dnmt3A and Dnmt3B. A 5th member has been more recently discovered, DNMT3L, which has been shown to serve as a stimulator of the Dnmt3A and Dnmt3B enzymes(Hata, Okano et al. 2002). It is not yet known at what stage of evolution mammals lost this large number of methyltransferases. All the known mammalian DNMTs have a common structure of the catalytic domain and are characterized by the 10 conserved amino acid motifs implicated in the catalytic function. In addition, the Dnmt1 and Dnmt3 enzymes contain a large N-terminal regulatory domain (Figure 2.2).

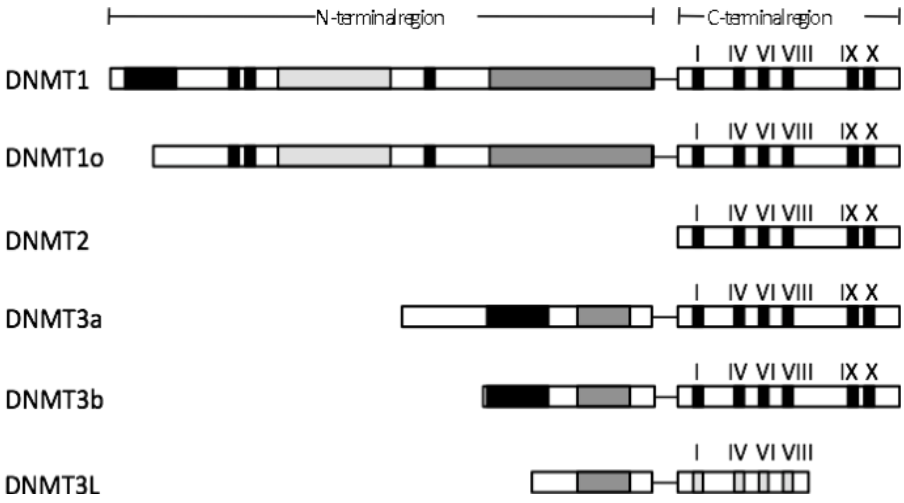

Figure 2.2 DNA Methyltransferase family. DNMT1 is 1620 amino acids in length and has a N-terminal regulatory domain and a C-terminal catalytic domain. DNMT1o lacks 114 amino acids in the N-terminal domain of DNMT1. DNMT2 is 415 amino acids in length and lacks the regulatory N-terminal region but contains a homologous C-terminal region. DNMT3a is 912 amino acids in length and contains a C-terminal catalytic domain as well as an N-terminal regulatory domain. DNMT3b is homologous to DNMT3a but is 853 amino acids in length. DNMT3L lacks a functional catalytic C-terminal domain.

2.4.1. DNA Methyltransferase 1

The first mammalian methyltransferase to be discovered was DNMT1 (Bestor 1988). DNMT1 has been observed to be the most abundant member of the DNMT family (Araujo, Croteau et al. 2001) and has been defined as the primary maintenance methyltransferase. DNMT1 has a large N-terminal domain with regulatory function and a smaller C-terminal catalytic domain. The large N-terminal regulatory domain is associated with multiple functions mediated by to many protein-protein interactions with motifs in this region. Included among the protein interacting motifs in this region are motifs for the DMAP1 transcriptional repressor protein(Rountree, Bachman et al. 2000), proliferating cell nuclear antigen (PCNA)(Chuang, Ian et al. 1997), Rb(Pradhan and Kim 2002), as well as Bromo adjacent homology domains for interaction with Polybromo-1 protein. Other regions in the N-terminal domain include a nuclear

localization signal (Fatemi, Hermann et al. 2001), a replication foci targeting region (Leonhardt, Page et al. 1992), as well as a cysteine-rich Zn^{2+} binding domain (Bestor 1992; Chuang, Ng et al. 1996). This Zn binding domain has been shown to be necessary for DNMT1 to bind DNA(Fatemi, Hermann et al. 2001). The N-terminal domain serves as a spatial regulator of DNMT1 entry into the nucleus, as well as catalytic function(Liu, Oakeley et al. 1998). The C-terminal region contains the catalytic domain necessary for the transfer of a donor methyl group on the 5^{th} carbon position of the pyrimidine ring. In this region there are 10 (I-X) motifs involved in the catalytic function, six of which are highly conserved(Posfai, Bhagwat et al. 1989) between the DNMTs. Motifs I and X are necessary for interaction with the methyl donor, motif IV and VI are required for the enzymatic function and motif IX plays a role in recognizing base-specific contacts in the major groove (Bestor and Verdine 1994)(Figure 2.2).

The most abundant member of the DNMT family, DNMT1, is a protein 1620 amino acids in length. Other forms of DNMT1 have been documented, including DNMT1b and DNMT1o. DNMT1b has 48 additional nucleotides between exons 4 and 5, producing a slightly larger protein than DNMT1(Hsu, Lin et al. 1999). DNMT1b is a functional DNA methyltransferase, however, its levels in the cell are 2-5% of the total abundance of DNMT1, making DNMT1b a minor contribution to methyltransferase activity(Bonfils, Beaulieu et al. 2000). DNMT1o is a short germ-cell specific isoform of DNMT1 identified in the mouse(Mertineit, Yoder et al. 1998; Howell, Bestor et al. 2001) that lacks 114 amino acids in the N-terminal domain (Figure 2.2). DNMT1o plays a role in genomic imprinting(Howell, Bestor et al. 2001) and embryonal tissue development(Cirio, Martel et al. 2008). Most recently a truncated form of DNMT1 was identified in HCT116 cells resulting from deletion in the DNMT1 gene(Egger, Jeong et al. 2006). This form lacked the N-terminal regulatory domain but retained the C-terminal catalytic domain and remained enzymatically functional.

Studies have examined the role of DNMT1 in DNA methylation by studying DNMT1 genetic knockouts. In 1992, Li and colleagues made a knockout of the *DNMT1*

gene in mice(Li, Bestor et al. 1992). While they were able to produce viable embryonic stem cells, deletion of DNMT1 in mice lead to abnormal development and embryonic lethality. They observed that stable deletion of DNMT1 lead to a dramatic decrease in genomic methylation, by which they concluded that DNMT1 was the primary methyltransferase in the cell. While other deletion studies have revealed that the absence of DNMT1 in human cancer cell lines may not affect genomic methylation significantly (Rhee, Bachman et al. 2002), it has been clearly established that DNMT1 functions as a maintenance methyltransferase.

DNMT1 is involved in multiple cellular functions due to the numerous protein interaction domains in its N-terminal regulatory region. DNMT1 has been shown to play a role in DNA replication, where it serves as a component of the replication complex(Vertino, Sekowski et al. 2002), interacts with PCNA(Chuang, Ian et al. 1997) and induces an arrest in replication when it is removed from cells(Knox, Araujo et al. 2000; Milutinovic, Zhuang et al. 2003). DNMT1 interaction with the replication complex allows DNMT1 to copy the methylation pattern of the paternal strand onto the newly synthesized non-methylated DNA strand. This was deduced when DNMT1 was observed to have a preference for hemimethylated DNA(Hsu, Lin et al. 1999; Hermann, Goyal et al. 2004). The association of DNMT1 with DNA replication is crucial for the maintenance of DNA methylation. DNMT1 plays a role in gene expression independent of the effect of DNA methylation. DNMT1 has been shown to directly recruit histone remodeling complexes and establish transcriptional repressor complexes. DNMT1 is known to specifically recruit HDAC1 and DMAP1 to regions in the genome, leading to the formation of a transcriptionally repressed chromatin(Rountree, Bachman et al. 2000). Other studies have shown that DNMT1 can associate with other transcriptional repressor complexes by interacting with LSH, a protein related to the SNF2 family of chromatin-remodeling ATPases, leading to transcriptional silencing of genes(Myant and Stancheva 2008). DNMT1 has also been shown to form a repressive complex with Rb, E2F and HDAC1, leading to the silencing of E2F specific promoters(Robertson, Ait-Si-Ali et al. 2000). Other repressor complexes that have been shown to interact with DNMT1

include the histone remodeling protein SUV39H1 (Fuks, Hurd et al. 2003), HDAC2(Rountree, Bachman et al. 2000) and HP1(Fuks, Hurd et al. 2003) and the methyl CpG binding proteins MBD2, MBD3(Tatematsu, Yamazaki et al. 2000) and MeCP2(Kimura and Shiota 2003). Through protein-protein interaction, DNMT1 can function as a transcriptional repressor by directly recruiting histone remodeling complexes to gene promoters.

While DNMT1 has been characterized as the maintenance methyltransferase, primarily due to its observed 15-40 fold preference for hemimethylated DNA over unmethylated DNA (Flynn, Glickman et al. 1996; Pradhan, Bacolla et al. 1999; Fatemi, Hermann et al. 2001), other studies have suggested than DNMT1 may also function as a *de novo* methyltransferase. Overexpression of DNMT1 in human fibroblasts induced *de novo* methylation of *HIC-1*, *estrogen receptor*, α_1 *and* α_2 *globin*, *E-cadherin* and *somatostatin* genes (Vertino, Yen et al. 1996). The interaction between DNMT1 and DNMT3a can also lead to de novo methylation, in a manner that is initiated through novel methylation by DNMT3a and continued by DNMT1 (Fatemi, Hermann et al. 2002). Other studies have shown that interaction of DNMT1 and DNMT3a lead to *de novo* methylation of nucleosomal DNA (Gowher, Stockdale et al. 2005). Association of DNMT1 and DNMT3b in *de novo* methylation has also been investigated, implicating their relationship in *de novo* methylation (Kim, Ni et al. 2002). These results suggest that though DNMT1 acts as a maintenance methyltransferase, it is also possible that it can be involved in establishing novel methylation patterns (For more information regarding targeting DNA methylation see section 2.8.1.).

2.4.2. DNA Methyltransferase 2

DNMT2 was initially discovered in 1998 as a protein of 415 amino acids in length and is homologous to the C-terminal domain of DNMT1 (Yoder and Bestor 1998). It is ubiquitously expressed in most human tissues, as well as mouse embryonic stem cells

(Okano, Xie et al. 1998). While initial investigations of DNMT2 function revealed a distinct lack of DNA methyltransferase activity (Van den Wyngaert, Sprengel et al. 1998), recent studies have shown a weak but detectable methyltransferase function in humans (Hermann, Schmitt et al. 2003). The controversy around the DNA methyltransferase activity of DNMT2 has been resolved by knockout experiments where it was revealed that cells depleted in DNMT2 were viable and displayed normal levels of methylation at endogenous sequences (Okano, Xie et al. 1998). While DNMT2 may lack a DNA methylation function, DNMT2 is a tRNA methyltransferase, which methylates cytosine 38 in the anticodon loop of aspartic acid transfer RNA (Goll, Kirpekar et al. 2006).

2.4.3. DNA Methyltransferases 3a and 3b

The second group of DNA methyltransferases to be discovered, the *de novo* methyltransferases, were DNMT3a and 3b(Okano, Xie et al. 1998). DNMT3a and 3b were initially classified as *de novo* methyltransferases, due to their ability to methylate CpG dinucleotides without a preference for hemimethylated DNA (Okano, Xie et al. 1998; Gowher and Jeltsch 2001). Further evidence for DNMT3a and 3b playing a role as the *de novo* methyltransferases was provided in studies that detected *in vivo de novo* methylation of unmethylated exogenous plasmids transfected into human cells overexpressing DNMT3a or DNMT3b proteins (Hsieh 1999). Knockout studies of DNMT3a and 3b have further elaborated their role in DNA methylation and embryonic development. Disruption of these genes impaired *de novo* methylation, as well as post-gestational death in DNMT3a null mice and developmental defects and embryonic lethality in DNMT3b null mice (Okano, Bell et al. 1999). While the function of the DNMT3 family is to establish novel patterns of DNA methylation, there is also a body of evidence implicating DNMT3a and 3b in maintenance methylation, whereby they

cooperate in maintaining methylation patterns of LINE-1 repetitive elements (Liang, Chan et al. 2002) and the p16 tumor suppressor gene (Rhee, Bachman et al. 2002).

Structurally the DNMT3 family is similar to the DNMT1 protein. While smaller than DNMT1, with DNMT3a 912 amino acid in length and DNMT3b 853 amino acids in length, they are comprised of a regulatory N-terminal region and a catalytic C-terminal domain (Figure 2.2). The N-terminal domain of DNMT3a and 3b contain a Plant Homeo domain (PHD), as well as a PWWP (proline-tryptophan-tryptophan-proline) domain. The PHD domain is necessary to repress transcription, independent of methyltransferase activity (Bachman, Rountree et al. 2001; Fuks, Burgers et al. 2001). The PWWP domain acts as the DNA binding domain for the DNMT3 family as well as a chromatin-targeting module (Ge, Pu et al. 2004). The C-terminal catalytic domain bears a strong homology to the catalytic domain of DNMT1 and features similar motifs to DNMT1. However DNMT3a and DNMT3b have somewhat different targets (Gowher and Jeltsch 2002), with DNMT3a acting on smaller CpG poor areas, such as single copy genes (Hata, Okano et al. 2002), and DNMT3b has a more processive function in methylating CpG rich regions, such as pericentromeric repeats.

Different isoforms of DNMT3a and 3b have been identified, as well as a third DNMT3 subtype, DNMT3L. DNMT3a2 differs from DNMT3a by 223 amino acids in the N-terminal domain. While DNMT3a is ubiquitously expressed in all adult tissues, DNMT3a2 is the predominant form of DNMT3a in male germ cells during embryogenesis (La Salle and Trasler 2006) and is commonly localized to euchromatin, the active component of chromatin, while maintaining high methyltransferase activity (Chen, Ueda et al. 2002). DNMT3b has multiple isoforms, arising from alternative splicing of exons 10, 21 and/or 22 from the *DNMT3b* gene (Xie, Wang et al. 1999). These isoforms, DNMT3b2, 3b3, 3b4, 3b5 and 3b6, have been reported to be expressed at different levels during

development (Robertson, Uzvolgyi et al. 1999). Of these isoforms, DNMT3b and 3b2 are enzymatically active, while DNMT3b3 has been shown to lack a motif necessary for enzymatic activity (motif IX)(Aoki, Suetake et al. 2001). This does not disqualify DNMT3b3 from playing a role in DNA methylation, as it has been shown to lead to *de novo* methylation of the D4Z4 subtelomeric repetitive element in cells overexpressing DNMT3b3, suggesting that 3b3 may serve as a regulator of DNA methylation (Weisenberger, Velicescu et al. 2004). DNMT3b4 and b5 also lack the motifs necessary for a functional methyltransferase domain, however 3b4 has been shown to regulate DNA methylation, in part due to its ability to compete with DNMT3b, in a dominant negative manner leading to genomic hypomethylation (Saito, Kanai et al. 2002). The third DNMT3 subtype, DNMT3L, was first isolated in 2000 as a DNMT3 homologous protein (Aapola, Kawasaki et al. 2000) and was characterized as a regulator of genomic imprinting during gametogenesis (Bourc'his, Xu et al. 2001). This function occurs independently of catalytic activity since the C-terminal catalytic domain of DNMT3L is truncated and it has key catalytic residues mutated. It is suggested that DNMT3L mediates DNA methylation of parentally imprinted genes through its interaction with DNMT3a, DNMT3b and HDAC1 (Bourc'his, Xu et al. 2001; Deplus, Brenner et al. 2002; Hata, Okano et al. 2002) leading to chromatin remodeling and gene silencing.

The members of the DNMT3 family have been shown to interact with a number of proteins. As previously mentioned DNMT3a and DNMT3b have been shown to interact with DNMT1, as well as HDAC1 (Fuks, Burgers et al. 2001). Other proteins that have been shown to interact with DNMT3a include the histone methyltransferase Suv39H1(Fuks, Hurd et al. 2003), G9a(El Gazzar, Yoza et al. 2008), SETDB1(Li, Rauch et al. 2006) and hepatitis B virus X protein(Zheng, Zhang et al. 2009). These associations allow DNMT3a to participate in a complex that serves as a negative transcriptional regulator through chromatin remodeling.

2.5. DNA Demethylation

DNA methylation patterns, once established, were originally considered to be unchanging throughout life. However, it has been recently recognized that methylation patterns are dynamic and can respond to external signals, such as environmental signals, and internal signals, such as the endocrine system. The original process of DNA demethylation was thought to occur through the passive loss of DNA methylation patterns. This occurred through several rounds of DNA replication in absence of the maintenance methylation machinery leading to an overall decrease in DNA methylation. While this mechanism has been observed in particular cases (Matsuo, Silke et al. 1998), DNA demethylation has also been observed in replication-independent models, suggesting that both DNA methylation and DNA demethylation can occur actively.

The most biologically significant example of active demethylation occurs during embryonic development. With hours of fertilization of the embryo, the paternal genome undergoes demethylation at a rate that is much faster than the maternal genome (Oswald, Engemann et al. 2000). This demethylation occurs within hours of fertilization before the genome is replicated, excluding the possibility that it occurs by a passive demethylation mechanism. Cellular differentiation also provides a clear example of active demethylation with the instance of erythroleukemia cell differentiation (Aujame, Craig et al. 1984; Razin, Szyf et al. 1986). A first example of a global replication-independent demethylation induced by a change in histone acetylation is the demethylation of the EBV virus in B cells in response to treatment by the HDAC inhibitor sodium butyrate (Szyf et al., 1985). Demethylation has also been observed to occur in a promoter-specific manner. T-cell lymphocyte differentiation leads to demethylation and activation of the *interleukin-2* promoter and activation of CD4+ T cells induce

demethylation and expression of the *IL-2* gene (Bruniquel and Schwartz 2003; Murayama, Sakura et al. 2006).

In all these examples DNA demethylation was an active process because it was observed that these cells were not dividing. The depth and detail of this process also highlights the specific nature of DNA demethylation, suggesting that DNA methylation plays a role in regulating genes in active and repressive manners rather than serving as a unidirectional repressive mark.

2.5.1. DNA Demethylases

Three different demethylation mechanisms have been proposed. The first mechanism is based on the action of nucleotide excision repair, whereby a section of DNA is removed and replaced with an altered modified sequence. This mechanism requires the removal of 2-30 nucleotides, leaving a single-stranded break which is subsequently filled in by a DNA polymerase. The DNA damage response protein growth arrest and DNA-damage-inducible protein 45 alpha (GADD45α) and DNA repair endonuclease XPG have been shown to induce active demethylation of the *Oct4a* promoter through nucleotide excision repair (Barreto, Schafer et al. 2007).

The second proposed mechanism is through base excision repair, where by a methylated base is removed and replaced with an unmethylated base. A glycosylase cleaves the N-glycosolytic bond, generating an apyrimidinic site. The remaining phosphate-sugar is removed by an apyrimidinic lyase or endonuclease and a 3'-5' DNA phosphodiesterase revealing a single nucleotide gap. The gap is filled in with an unmethylated cytosine by DNA polymerase. Proteins have been linked with DNA

demethylation through this mechanism including MBD4 (Bellacosa, Cicchillitti et al. 1999; Hendrich, Hardeland et al. 1999) and the unrelated protein thymidine DNA glycosylase (TDG)(Neddermann, Gallinari et al. 1996). Recently it was proposed that DNMT3a and DNMT3b participate in demethylation. DNMT3a and 3b possess in addition to their DNA methylation function a deaminase activity (Metivier, Gallais et al. 2008). Deamination transforms 5mC to thymine. This thymine is then excised by TDG, recruited by DNMT3a (Li, Zhou et al. 2007), leading to demethylation. These results provide an insight into the dynamic nature of methylation patterns through methylation and demethylation by a single enzyme, as observed in the rapid methylation cycling of the *ERα* promoter (Kangaspeska, Stride et al. 2008). However, these results are highly contested and remain a controversial subject (Ooi and Bestor 2008) due to a report that TDG function is so ineffective at removing 5mC that its involvement in DNA demethylation may be unlikely(Cortazar, Kunz et al. 2007).

These two mechanisms share the idea that loss of methyl groups from cytosine is impossible and therefore a repair activity of some sort removes the methylated base and replaces it with an unmethylated cytosine. The third mechanism proposes a direct removal of the methyl group from the cytosine through hydrolysis with a water molecule, or oxidation producing either methanol or formaldehyde and an unmethylated cytosine (Cedar and Verdine 1999; Hamm, Just et al. 2008)(Figure 2.3).

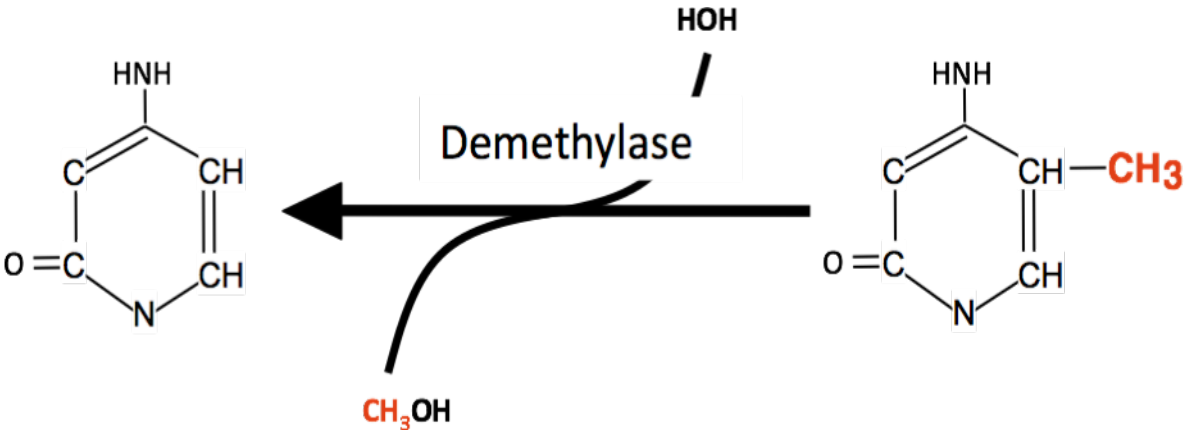

Figure 2.3. DNA demethylation. Removal of a methyl group from the 5th position of the cytosine pyrimidine ring through the activity of the DNA demethylase enzyme and a hydroxyl group from water. The reaction produces methanol.

The first direct DNA demethylase to be proposed was MBD2 (Bhattacharya, Ramchandani et al. 1999). This result has been contested since other groups reported that MBD2 played a role as a negative transcriptional regulator (Hendrich and Bird 1998) and since MBD2 knock-out mice did not exhibit overt differences in genomic methylation (Hendrich, Guy et al. 2001). Other demethylases proposed to act by removing of methyl groups include MBD3, another member of the methyl binding domain protein family, which has been shown to induce both global demethylation (Brown, Suderman et al. 2008) as well as site-specific demethylation of ribosomal RNA gene loci (Brown and Szyf 2007).

2.6. Chromatin

Aside from DNA methylation, the other component of the epigenome is chromatin. Chromatin is a structural combination of DNA and protein in the nuclei of cells which serves to package and organize DNA into smaller volumes, and control gene expression. Organization of DNA begins with the association of DNA with a histone

octamer core, made up of histones 2A and B, histone 3 and histone 4 (H2A, H2B, H3 and H4). This complex is a called a nucleosomal core. Nucleosomes are arranged in chromatin fibers that ultimately make up the structure of chromosomes. Chromatin exists in two states, heterochromatin and euchromatin. Heterochromatin is the densely packaged form of chromatin while euchromatin is the more loosely packaged and accessible form of chromatin. The role of chromatin in the epigenome is seen in light of its control of gene transcription. The formation of heterochromatin and euchromatin is controlled by chemical modifications of histones that alter their association with DNA. These modifications, acting in tandem with DNA methylation in many instances, serve as a method of controlling gene transcription.

2.6.1. Histone modification and the histone code

Histones serve as the organizational basis for arranging DNA into a compact, regulatory structure. As DNA is arranged around the histone octamer, the strength of the association dictates the nature of the structure, as either heterochromatin or euchromatin. Histones are highly conserved proteins that consist of a globular domain and a more flexible, amino terminus called the histone tail. Histone tails protrude from the nucleosomal core and are rich in positively charged side chains that interact with the negatively charged phosphate backbone of DNA. These interactions are regulated through neutralization or reversal of histone tail charges by a number of post-translational modifications. Among these modifications are acetylation, methylation, phosphorylation and ubiquitination. The modifications occur at specific amino acid at the N-terminal tail of histones, including lysine (K), serine(S), arginine(R) and threonine(T) residues (Berger 2007). The variety of modifications and sites of modification have served to create a “histone code” where the chemical modification has a consequence on gene expression. This introduction will review the best studied modifications: acetylation and methylation.

a. *Histone acetylation*. Acetylation occurs at lysine residues and has the impact of relieving a transcriptionally repressed state (Pogo, Allfrey et al. 1966). Acetylation of positively charged lysine residues masks the overall positive charge that interacts with the negatively charged phosphate backbone of DNA. This weakens the interaction between DNA and the histone octamer, creating an accessible area for transcription factor binding (Spotswood and Turner 2002). Typically histones 3 and 4 are acetylated at the following residues: H3 lysine 4, 9, 14, 18, 27, 36, 56 and 79, H4 lysine 5, 8, 13, 16 and 20 (D'Alessio and Szyf 2006). All these residues are found on the N-terminal tail of their respective histone. Histone acetylation also serves as a target for chromatin remodeling enzymes and transcription factors. Bromo-domain containing proteins are able to recognize and bind acetylated histones, stabilize the active structure and recruit other transcription factors(Fukuda, Sano et al. 2006). Histone acetylation is regulated by HATs and histone deacetylases (HDAC). HATs exist into two classes: type A HATs and type B HATs. Type A HATs are nuclear proteins that serve to acetylate histone and non-histone proteins. These HATs can be classified into GNAT, MYST and p300/Creb Binding Protein (CBP) proteins. GNAT proteins are involved in recruiting transcription factors to promoters (Vetting, LP et al. 2005), MYST proteins are bromodomain proteins that recruit transcription factors and proteins for DNA replication and repair (Utley and Cote 2003; Iizuka, Matsui et al. 2006). The p300/CBP HAT proteins are the stereotypical regulators of gene transcription (Fukuda, Sano et al. 2006). Type B HATs are cytoplasmic proteins that serve to acetylate newly synthesized histones before they are translocated into the nucleus (Hasan and Hottiger 2002).

HDACs are enzymes that remove acetyl group from lysines, reestablish the positive charge and lead to a repressed transcriptional state (Lusser 2002). The first HDAC identified, HDAC1, was cloned in 1996 (Taunton, Hassig et al. 1996). Since then over 18 non-redundant HDACs and Sir2-like deacetylases have been identified (Dokmanovic, Clarke et al. 2007). HDACs are classified to three families: class I, class II and the Sir2-like deacetylases. Class I is comprised of HDAC 1, 2, 3, and 8, are nuclear proteins that are ubiquitously expressed. Class II is comprised of HDAC 4, 5, 6, 7 and 9.

These are much larger proteins than class I HDACs, and are tissue-specific (Dokmanovic, Clarke et al. 2007). Functionally, class I and II HDACs are mechanistically similar and mediate the same deacetylase reaction (Verdone, Agricola et al. 2006). The Sir2-like deacetylases differ in their requirement of NAD^+ as a co-factor, as well as in their mechanism of action (Santos-Rosa and Caldas 2005). HDACs associate with transcriptional repressor complexes (as mentioned in Section 2.3.1.) further inhibiting transcription in addition to their primary role in inducing a closed chromatin state (Zhang, Ng et al. 1999). The association with transcriptional repressors plays a role in targeting the HDACs to specific sequences at precise points in time.

b. Histone methylation. Although histone acetylation uniformly serves as a gene expression activating mark, histone methylation can go either way and can serve as both an activating or repressive mark for transcription depending on the position of the methyl moiety. While methylation of histone residues may not alter the ionic interaction between DNA and histones, the methylated residue serves as a recruitment mark for transcription factors or repressors (D'Alessio and Szyf 2006). Histone methylation on lysine 9 recruits the heterochromatin protein HP-1, which condenses the chromatin into an inactive structure (Cao, Wang et al. 2002), while histone methylation at lysine 4 is associated with gene activation (Santos-Rosa, Schneider et al. 2002).

Addition and removal of methyl groups to and from histones is catalyzed by histone methyltransferases and histone demethylases. Histone methyltransferases catalyze the transfer of one to three methyl groups from SAM to lysine and arginine residues on histone proteins. Over 40 histone methyltransferases have been identified, including Suv39H1, Suv39H2 and EZH2 (Volkel and Angrand 2007). Common to all histone methyltransferases is the catalytic domain, or the SET domain. Suv39H1 and H2 were first identified to specifically methylate H3K9 (Rea, Eisenhaber et al. 2000) and act in tandem with transcriptional repressor and histone-remodeling complexes (Fuks, Hurd et al. 2003). EZH2 is a member of the polycomb-group family and methylates H3K9 and H3K27. EZH2 serves to mediate transcriptional silencing through recruitment of

transcriptional repressor NIPP1(Nuytten, Beke et al. 2008) as well as recruiting DNMT1 to methylate specific promoters (Vire, Brenner et al. 2006). In fact the H3K27 methylation by EZH2 has recently emerged as a major mark for *de novo* methylation of gene promoters in tumorigenesis (Schlesinger, Straussman et al. 2007) (This will be discussed further in section 2.8.1.). Histone methyltransferases that are associated with gene activation include coactivator-associated arginine methyltransferase 1, which was shown to induce expression of the pS2 gene through methylation of H3R17 (Bauer, Daujat et al. 2002).

Histone demethylases catalyze removal of methyl groups from mono-, di- and tri-methylation at lysine residues. LSD1 was the first histone demethylase to be identified. It was shown to remove methyl groups from activated H3K4, leading to transcriptional repression (Shi, Lan et al. 2004). Further studies have revealed that when coupled with different proteins, LSD1 can demethylate other histones, specifically H3K9, leading to activation of gene transcription(Metzger, Wissmann et al. 2005). Interestingly LSD1 has been shown to demethylate and stabilize DNMT1, leading to increased DNA methylation activity, further enhancing a repressed state (Wang, Hevi et al. 2009). Other histone methyltransferases that have been identified since include the JmjC domain demethylases JMJD2s and JDHM2/3. These enzymes have been shown to remove methyl groups from H3K9 and H3K27 (Huang, Fang et al. 2006).

Other modifications of histone residues include phosphorylation and ubiquitination. The functional repercussions of these modifications are more varied than just transcriptional regulation. Histone phosphorylation also plays a role in DNA repair and cell cycle control. Histone variant H2A.X is phosphorylated at serine 129 in response to DNA damage (Rogakou, Pilch et al. 1998) and serves as an anchoring point for DNA repair enzymes (Stucki, Clapperton et al. 2005). Phosphorylation of H2A.X is performed by the *Ataxia telangiectasia mutated* (ATM) and *Ataxia telangiectasia and Rad3-related* (ATR) kinases (Burma, Chen et al. 2001; Stiff, Reis et al. 2005) and occurs around areas

of double stranded DNA breaks or arrested replication complexes (Hunter, Borner et al. 2001; Furuta, Takemura et al. 2003). Other sites of phosphorylation are associated with transcriptional regulation. Phosphorylation of H3S10 is mediated by SNF and induces gene transcription (Lo, Duggan et al. 2001). Ubiquitination of histones has also been found to play a role in transcription initiation and DNA repair. Ubiquitination of histone 2B by Rad6 is necessary for RNA polymerase II-dependent transcription (Shilatifard 2006). Ubiquitination of histone 2A, like H2A.X phosphorylation, has been observed at areas of DNA breaks and is a requisite step in functional nucleotide excision repair (Bergink, Salomons et al. 2006; Bergink, Severijnen et al. 2006).

The different possible histone modifications can have a wide variety of effects on cellular function. From their primarily studied role in modulating gene transcription to DNA repair and cell cycle control, chromatin acts as a chaperone for genetic information, serving to control gene expression.

2.6.2. The role of chromatin in cancer and therapeutics

As mentioned in section 2.1., cancer is a disease distinguished by widespread alterations in gene expression profiles. Since gene expression programs are controlled by epigenetic states, it stands to reason that these could be changed in cancer and that these changes play a causal role in the disease. We discussed the alterations in DNA methylation in cancer in section 2.3.. Similar to DNA methylation, histone modification, as well as histone modifying enzymes, are altered in various types of cancer. Global levels of H4K16 acetylation and H4K20 trimethylation are decreased in cancer tissues as compared to the neighboring normal tissues (Fraga, Ballestar et al. 2005). It has also been observed that specific promoters also undergo changes in histone modifications upon transformation. For example, the tumor suppressor p21 is deacetylated and hypermethylated at histones 3 and 4 in cancer cells (Richon, Sandhoff et al. 2000). The changes in histone modifications are accompanied with changes in the enzymes that

mediate them. It has been shown that in breast and liver cancer there is a decrease in the expression of Suv4-20h2 histone methyltransferase and an increase in the expression of Suv39h1 histone methyltransferase (Pogribny, Ross et al. 2006). The role of Suv39h1 is further implicated in cancer since Suv39H1 null mice are prone to develop cancer (Richon, Sandhoff et al. 2000). Other histone modifying enzymes have been shown to play a role in tumorigenesis. For example, the deletion of the HAT CBP increases the frequency of cancer (Gibbons 2005), frameshift mutations of HDAC2 are found in a number of cancers (Ropero, Fraga et al. 2006) and inactivating mutations of HAT p300 have been isolated in a number of breast, colorectal and gastric carcinomas (Muraoka, Konishi et al. 1996; Gayther, Batley et al. 2000). The histone methyltransferase EZH2 has been shown to be overexpressed in a number of cancers (Visser, Gunster et al. 2001; Varambally, Dhanasekaran et al. 2002) and correlates with the aggressiveness of breast cancer (Kleer, Cao et al. 2003).

Since alterations in histone modifications play a role in cancer, therapeutics targeting those modifications are emerging as new cancer therapeutics. HDAC inhibitors are the most widely studied chromatin targeting drugs. HDAC inhibitors have been found to act exclusively on class I and II HDACs by acting on their homologous catalytic domain. The HDAC inhibitors currently clinically available are classified into five groups: hydroxamic acid (Trichostatin A, SAHA), cyclic tetrapeptides (Trapoxin B), benzamides, electrophilic ketones and aliphatic acids (valproic acid) (Drummond, Noble et al. 2005; Mehnert and Kelly 2007). HDAC inhibitors were shown to block cellular proliferation and induce apoptosis. While the mechanisms of action of these drugs are yet fully understood, HDAC inhibitors exhibit transcription dependent as well as transcriptional independent effects.

Inhibition of HDAC activity results in hyperacetylation and transcriptional activation of silenced tumor suppressor genes in cancer. Examples are the *p21, p57, E-CADHERIN* and *RAR*α genes (Komatsu, Kawamata et al. 2006; Kumagai, Wakimoto et al. 2007; Ou, Torrisani et al. 2007). Beneficial effects of re-expressing silent tumor suppressor genes include p21-dpendent reactivation of the G1/S phase checkpoint (Sandor, Senderowicz et al. 2000) and induction of the G0/G1 checkpoint by SAHA treatment (Komatsu, Kawamata et al. 2006). Transcriptionally-independent effects of HDAC inhibition include the increased dephosphorylation of Akt leading to Akt pathway downregulation and increased apoptosis (Chen, Weng et al. 2005). This occurs through the altered interaction of HDAC with Protein Phosphatase 1, causing increased dephosphorylation of Akt. HDAC inhibitors also inhibit deacetylation of non-histone proteins leading to increased acetylation, altering their function. For example, increased p53 acetylation in response to HDAC inhibition leads to increased p21 expression (Roy, Packman et al. 2005).

2.7. DNMT1 regulation

In parallel with DNA methylation patterns in cancer cells, it has also been observed that DNMT1 expression is increased in many tumors (Issa, Vertino et al. 1993). In fact, increased DNMT1 expression has become an accepted mechanism of cellular transformation. Overexpression of DNMT1 in mouse fibroblasts result in cellular transformation (Wu, Issa et al. 1993; Bakin and Curran 1999) and in a time-dependent increase in methylation of several CpG islands (Vertino, Yen et al. 1996). To further implicate DNMT1 in carcinogenesis, reduction of DNMT1 protein levels results in demethylation and reexpression of tumor suppressor genes (Fournel, Sapieha et al. 1999), induction of cellular growth arrest (Knox, Araujo et al. 2000) and reversal of tumorigenesis *in vivo* and *in vitro* (MacLeod and Szyf 1995; Ramchandani, MacLeod et al. 1997). To understand how DNMT1 induces cellular transformation, we must ascertain

the exact mechanism through which DNMT1 expression is regulated in normal cells and how it is deregulated in cancer.

DNMT1 transcription has been shown to be a regulated event under the control of several cellular pathways. The Ras signaling pathway has been shown to have a direct effect on DNMT1 expression and under conditions of aberrant Ras signaling, a common occurrence in cancer (Bos 1989; Ayllon and Rebollo 2000), DNMT1 transcription is induced(Bigey, Ramchandani et al. 2000). Another cellular pathway that has been shown to regulate DNMT1 expression is the Adenomatous Polyposis Coli (APC) tumour suppressor. Normally the APC gene product inactivates the β-catenin/Tcf transcription factor complex. Homozygous deletion of APC in mice leads to a reduction of DNMT1 expression, as well as an inhibition of colorectal cancer tumorigenesis (Laird, Jackson-Grusby et al. 1995; Eads, Danenberg et al. 1999; Campbell and Szyf 2003). E2F-Rb is another signaling pathway associated with tumorigenesis, and has been shown to regulate DNMT1 expression. Rb inactivation and E2F gene target induction have been shown to be a common occurrence, either through gene mutation (Weinberg 1995) or epigenetic inactivation (Sakai, Toguchida et al. 1991; Ferres-Marco, Gutierrez-Garcia et al. 2006). It has been shown that DNMT1 gene expression is regulated and induced by binding of E2F to the DNMT1 promoter (Kimura, Nakamura et al. 2003; McCabe, Low et al. 2006). This induction of DNMT1, through modulation of the pRB/E2F pathway may be involved in cellular transformation. Putative analysis of the DNMT1 promoter revealed potential binding sites for transcription factors AP-1, E2F and the enhancer c-jun (Bigey, Ramchandani et al. 2000). Studies have shown that upon infection by the human immunodeficiency virus (HIV), the DNMT1 gene is upregulated by the AP-1 pathway (Youngblood and Reich 2008). Differentiation of p19 embryocarcinoma cells has been shown to induce DNMT1 transcription in a c-Jun dependent manner (Slack, Pinard et al. 2001).

While DNMT1 gene transcription is a highly regulated event it has been shown that DNMT1 gene transcription remains constant throughout the cell cycle (Szyf, Bozovic et al. 1991) while DNMT1 mRNA and protein levels vary (Szyf, Kaplan et al. 1985; Robertson, Keyomarsi et al. 2000; Detich, Ramchandani et al. 2001). This implies that DNMT1 can also be regulated post-transcriptionally. It was initially shown that upon arrest at G_1, DNMT1 mRNA levels were at their lowest, while entry into S-Phase and synthesis of DNA lead to the highest levels of DNMT1 (Robertson, Keyomarsi et al. 2000), all while DNMT1 gene transcription rates remained unchanged. The concurrent increase in DNMT1 levels with DNA synthesis has been hypothesized as a mechanism through which appropriate DNMT1 levels are maintained for copying the methylation pattern onto newly synthesized strands of DNA. Loss of this coordination could lead to improper establishment of methylation patterns and tumorigenesis. A study by Detich examined the mechanism through which DNMT1 mRNA was regulated during the cell cycle. It was discovered that an AU-rich motif in the 3' untranslated element (3'UTR) of DNMT1 mRNA served as a mechanism of control and upon deletion of the 3'UTR, DNMT1 mRNA levels remained unaffected by the cell cycle (Detich, Ramchandani et al. 2001). It was further identified that a 40 kDa protein served to negatively regulate DNMT1 mRNA through binding of the 3'UTR. Deregulation of this control led to cellular transformation, further emphasizing the impact of DNMT1 regulation on the overall health of the cell.

In addition to transcriptional and post-transcriptional regulation, the DNMT1 protein has also been shown to be regulated post-translationally. The DNMT1 protein was first shown to be regulated by the proteasome in 2005 (Ghoshal, Datta et al. 2005). The KEN box domain of DNMT1 served as a target motif for the proteasome. The KEN box is a signature sequence (KENxxxN), in which the last amino acid is not absolutely conserved, for the proteasomal degradation of many cell cycle regulatory proteins (Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004). Degradation of DNMT1 protein by

the proteasome was observed to occur through ubiquitination. DNMT1 protein itself has been shown to be stabilized through association with Heat-Shock Protein 90 (Hsp90). Upon disassociation of DNMT1 from Hsp90, DNMT1 is ubiquitinated and degraded by the proteasomal pathway (Zhou, Agoston et al. 2008). In addition to Hsp90, Phosphatidylinositol 3-kinase was shown to stabilize DNMT1 protein. Inhibition of Phosphatidylinositol 3-kinase led to global hypomethylation, indicative of decreased DNMT1 protein levels (Sun, Zhao et al. 2007).

The observation that DNMT1 levels iscontrolled through multiple layers of regulation reveals the importance of its normal function. Disregulation of DNMT1 at any of these levels leads to alterations in genomic methylation patterns, gene expression and cellular transformation. For this reason it is important to understand the exact mechanisms through which DNMT1 is regulated and how that regulation can impact cancer therapy.

2.8. Epigenetic Targeting

While maintenance methylation copies DNA methylation patterns using a pre-existing template of the template strand, *de novo* methylation has no template strand to copy. However establishment of *de novo* methylation patterns occur naturally in development (Okano, Bell et al. 1999) as well as in tumorigenesis. For this to occur a relationship must exist between the DNMTs and complexes that can recruit them to specific regions that are required to be methylated. As with DNA methylation, active DNA demethylation requires the recruitment of targeting complexes to specifically demethylate regions of DNA. Without such a targeting mechanism DNA methylation would occur randomly. This cannot be the case with DNA methylation patterns since it

has been established that CpG methylation occurs in a non-random manner (Saxonov, Berg et al. 2006).

2.8.1. Targeting DNA methylation

Along with the *de novo* methyltransferases DNMT3a and 3b, DNMT1 has also been shown to have *de novo* methyltransferase activity in addition to its role as the maintenance DNMT. Two mechanisms exist that recruit DNMTs to specific targets in the genome.

The first mechanism involves recruitment of DNMTs to specifically targeted areas of the genome through interactions with DNA binding proteins. For example, the transcription factor Myc was found to associate with DNMT3a (Brenner, Deplus et al. 2005). This recruitment of DNMT3a facilitates silencing methylation of myc binding sites in the promoter of p21/Cip1 (Brenner, Deplus et al. 2005). PML-RAR is a fusion protein of the myeloid gene PML with retinoic acid receptor alpha (Kakizuka, Miller et al. 1991) and it acts as negative transcriptional regulator of retinoic acid target genes (Benedetti, Grignani et al. 1996). PML-RAR was also shown to recruit DNMT3a and 3b to specific promoters. Association between PML-RAR and DNMT3a and 3b allows specific targeting and methylation of RARα sites in the promoter of RARβ2 (Di Croce, Raker et al. 2002). Complexing of DNMT1 with the transcription factor E2F1 served as a transcriptional repressor complex of E2F1 target sites (Robertson, Ait-Si-Ali et al. 2000).

The second mechanism through which DNA methylation can be targeted to specific sites occurs via association of DNMTs with proteins that recognize specific chromatin modifications, typically those associated with a repressed state. DNMT3L

serves as a recruitment partner for DNMT3a and 3b (Bourc'his, Xu et al. 2001) where it can recognize and bind unmethylated H3K4 and induce *de novo* methylation of DNA flanking the recognized region of chromatin (Ooi, Qiu et al. 2007). DNMT1 has previously been shown to interact with HP-1, known to interact with methylated H3K9 and H4 (Bannister, Zegerman et al. 2001; Polioudaki, Kourmouli et al. 2001) in heterochromatin. This recruitment results in DNA methylation of heterochromatinized DNA and disruption of this complex leads to DNA demethylation and gene activation (Smallwood, Esteve et al. 2007).

Recently a new mechanism was described that involves recognition of a specific histone modification rather than a histone modifying protein by a DNMT. An arginine histone methylation H4R3me2 was found to serve as a direct binding site for DNMT3A (Zhao, Rank et al. 2009).The association of the histone methyltransferase EZH2 and DNMT1 draws attention to the relationship between DNA methylation and histone modifications in site-specific repression. EZH2 (see section 2.6.1) is a histone methyltransferase that interacts with DNMT1 and can methylate H3K27 residues, which serves as a repressive mark (Laible, Wolf et al. 1997). EZH2 has been shown to interact DNMT1 and to recruit it to specific sites through binding of histones (Vire, Brenner et al. 2006). EZH2 serves as a link between DNA methylation and chromatin repression. Interestingly it was discovered that a group of CpG islands that were not methylated in normal tissue but underwent *de novo* methylation in lung cancer were already bound by EZH2 in normal tissue (Rauch, Wang et al. 2007). Thus,EZH2 mediated H3K27 methylation serves as a mark for *de novo* methylation. H3K27 methylation was established on unmethylated CpG island genes early in development and maintained in differentiated cell types by the EZH2-containing Polycomb complex (Bracken, Dietrich et al. 2006; Lee, Jenner et al. 2006). During tumorigenesis, the EZH2 complex recruits DNMTs leading to *de novo* methylation of these regions (Schlesinger, Straussman et al. 2007). EZH2 is known to bind and methylate the promoters of tumor suppressors p16 (Bracken, Kleine-Kohlbrecher et al. 2007) and RUNX3 as well as E-cadherin and RUNX-3 (Fujii, Ito et al. 2008; Fujii and Ochiai 2008). It is not yet known how EZH2 recruits

DNMT1 to specifically methylated histones and not others. These results reveal that upon cellular transformation CpG islands are targeted for *de novo* methylation by DNMT1 through the action of EZH2. This highlights the biological relevance of targeting DNMT1 to specific promoters. However the critical question is why DNMT1 is targeted to EZH2 in tumor cells and not in normal cells. The fifth chapter in this thesis addresses this question.

2.8.2. Targeting DNA demethylation

While there is no direct evidence that implicates certain proteins in targeting site specific demethylation, there are indications that DNA demethylases require targeting to demethylate particular sequences. Site-specific demethylation occurs during development and cellular differentiation. The transcription factor NGFI-A was shown to interact with MBD2 in targeting specific sequences in the Glucocorticoid Receptor promoter for demethylation(Weaver, D'Alessio et al. 2007). Other examples of gene-specific demethylation mediated by transcription factors include demethylation of the *Kappa Immunoglobulin* gene enhancer in B cells requires the function of the intronic kappa chain enhancer and the transcription factor NF-κB (Lichtenstein, Keini et al. 1994). The transcriptional activator TnpA was shown to mediate the demethylation of the maize Suppressor-mutator transposon(Bruniquel and Schwartz 2003).

Inhibition of HDAC and increased acetylation were shown to induce active demethylation (Cervoni, Bhattacharya et al. 1999; Cervoni and Szyf 2001; Cervoni, Detich et al. 2002). Histone acetylation could be triggered by binding of transcription factors and recruitment of HATs to specific sequences. This could also be a mechanism for induction of site specific demethylation. Interestingly, demethylation induced by

Trichostatin A (TSA) occurred in a RNA polymerase II (Pol II) dependent manner, where Pol II was required to bind and initiate transcription before DNA demethylation occured. This suggests that the transcriptional machinery may serve as a targeting complex for DNA demethylation. Inhibition of HDACs with TSA was observed to induce promoter-specific DNA demethylation of E-cadherin and RAR-β2, without altering expression of other genes (Ou, Torrisani et al. 2007). TSA-mediated promoter demethylation occurs in a specific manner and may activate pathways that are normally involved in targeting genes for demethylation.

2.9. Cancer therapeutics targeting DNA methylation

Treatment of cancer by targeting the DNA methylation machinery is based on the notion that DNA methylation patterns are severely disrupted in cancer. As previously mentioned (see section 2.3.1. on gene silencing), tumor suppressor genes are commonly methylated and silenced in cancer. The hypothesis behind DNA demethylation therapy was to re-activate tumor suppressors and their growth inhibitory function by demethylation of their promoters. Therefore small molecule inhibitors were developed that would inhibit DNMT function and facilitate demethylation of silenced genes (MacLeod and Szyf 1995). However, since it is clear that DNMTs can also promote cancer by mechanisms that do not involve DNA methylation through their protein-protein interactions (Knox, Araujo et al. 2000; Milutinovic, Brown et al. 2004), targeting the DNMTs generally has become a viable method of treating cancer.

Blocking global hypomethylation through targeting the function of DNA demethylases has been proposed as an approach to block the expression of cancer and metastasis promoting genes activated by demethylation (Slack, Bovenzi et al. 2002).

2.9.1 DNMT inhibitors

The most extensively studied DNMT inhibitors are 5-azacytidine (Azacytidine/Vidaza) and 5-aza-2′-deoxycytidine (Decitabine/Dacogen). These inhibitors are chemical analogues of cytidine, which function in mimicking 5-methyl cytosine (Figure 2.4).

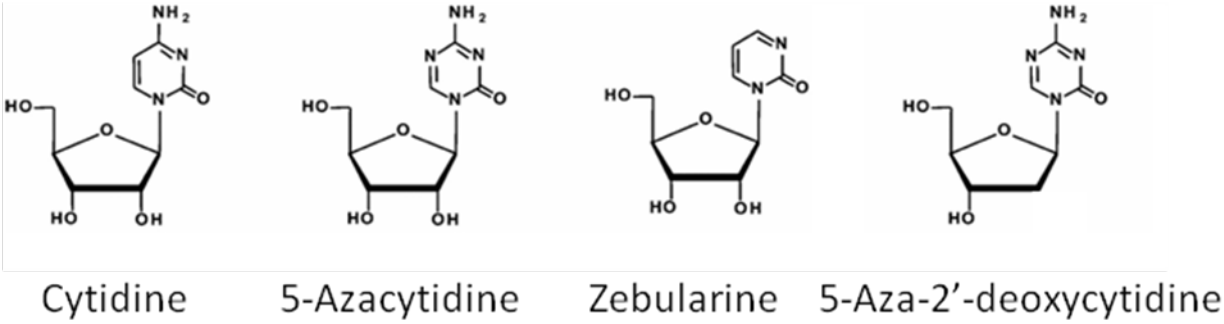

Figure 2.4. Chemical structure of cytidine and cytidine analogues 5-azacytidine, Zebularin and 5-Aza-2′-deoxycytidine.

The analogues are first phosphorylated into a triphosphate nucleotide and incorporated into DNA during DNA synthesis in S-phase. DNMTs form a covalent bond with the carbon position 6 of the cytosine analogue. Normally the DNMT transfers the methyl group from SAM to the 5′ carbon position of the ring. However, the cytosine analogues do not allow methyl group transfers to take place and the DNMT remains covalently bound to DNA(Jones and Taylor 1980). Once the DNMTs are immobilized, they are unavailable to methylate the nascent strand of DNA resulting in passive loss of the methylation after several rounds of cellular division.

The difference between 5-azacytidine and 5-aza-2′-deoxycytidine is that 5-azacytidine is partially incorporated into RNA, thereby interfering with protein

translation, while 5-aza-2′-deoxycytidine is incorporated only into DNA, causing more specific and efficient inhibition of DNMTs. Both analogues have been tested in clinical trials. These inhibitors have shown positive results in myelodysplastic syndrome. 50-70% of treated patients showed increased quality of life and survival rates (Issa, Garcia-Manero et al. 2004; Kantarjian, Oki et al. 2007). However, there was no significant success reported in solid tumors (Weiss, Metter et al. 1977). Several studies have shown that the greatest therapeutic potential of these agents lie in administering them in combination with other chemotherapeutic agents (Schrump, Fischette et al. 2006), in particular with HDAC inhibitors (Bhalla 2005). Analogues of these drugs have also been developed. These analogues include 5,6-dihydro-5-azacytidine and 5-fluoro-2′-deoxycytidine (Villar-Garea and Esteller 2003), which have been shown to be much more stable but with decreased therapeutic effect (Curt, Kelley et al. 1985; Boothman, Briggle et al. 1989). Zebularine (Figure 2.4), another cytidine-analogue, has been shown to have increased DNA demethylation activity, selectivity for tumor cells but reduced effect on decreasing tumour growth (Cheng, Matsen et al. 2003; Cheng, Yoo et al. 2004).

To avoid the toxic effects of nucleoside DNMT inhibitors, non-nucleoside DNMT inhibitors have been investigated. Procainamide and procaine inhibit DNMTs by perturbing interactions between the protein and its target sites (Villar-Garea and Esteller 2003). DNA demethylating activity of the antihypertensive compound hydralazine has been observed and is explained by the interaction of hydralazine and the DNMT active site (Segura-Pacheco, Trejo-Becerril et al. 2003). Epigallocathechin-3-gallate, a natural product derived from green tea, inhibits DNMT activity by blocking its active site (Fang, Wang et al. 2003; Lee, Shim et al. 2005). Another non-nucleoside analogue DNMT inhibitor is an oligonucleotide hairpin inhibitor(Bigey, Knox et al. 1999). This oligonucleotide contains complementary sequences of which only one is methylated, so that upon self-hybridization a hemimethylated hairpin is formed. This hairpin resembles a hemimethylated substrate for DNMT1. Due to a chemically modified backbone, DNMT1 binds this hairpin preferentially and prevents available DNMT1 protein molecules from complexing with chromatin. Hairpin inhibitors have

been shown to cause rapid growth arrest (Knox, Araujo et al. 2000) and induce p21 tumor suppressor re-expression through a methylation-independent mechanism (Milutinovic, Knox et al. 2000). Possible mechanisms by which hairpin inhibitors arrest DNA replication have been suggested. Elimination of available DNMT1 could disrupt the stability of the components of the PCNA replication complex (Araujo, Croteau et al. 2001), leading to the formation of an unstable replication complex. This instability could cause the formation of abnormal replication foci leading to the activation of a replication arrest (section 2.9.2).

The effectiveness of the nucleoside analogues is demonstrated by their abilities to demethylate and re-activate silenced genes including tumor suppressors p15 (Herman, Jen et al. 1996), p16 (Merlo, Herman et al. 1995; Otterson, Khleif et al. 1995), RAR β (Cote and Momparler 1997), RASSF1A (Liu, Yoon et al. 2002) as well as metastasis suppressor proteins E-cadherin (Graff, Herman et al. 1995) and TIMP-3 (Bachman, Herman et al. 1999), and DNA repair enzymes MLH1 (Herman, Umar et al. 1998) and BRCA1 (Dobrovic and Simpfendorfer 1997). Epigallocathechin-3-gallate was shown to induce demethylation and re-expression of p16, RAR β and hMLH1 (Fang, Wang et al. 2003).

Despite the effectiveness of DNMT inhibitors in reactivating silenced genes, there have also been reports of re-activation of silenced pro-metastatic genes. Studies have shown that lung cancer cells treated with 5-azaC showed a significant increase in metastasis and a 40 times increase in the number of tumor nodules in nude mice (Bennett, Dexter et al. 1986; Ormerod, Everett et al. 1986). Breast and prostate cancer cell line models have shown that 5-azacytidine treatment resulted in the reactivation of the metastatic gene urokinase-type plasminogen activator (uPa)(Xing and Rabbani 1999). 5-azaC has also been shown to activate the MAGE family of genes, known to be involved in promoting metastasis (Milutinovic, Zhuang et al. 2003). While targeting DNA methylation results in DNA demethylation and re-expression of silenced genes, this may

also lead to re-expression of genes that can promote metastasis, one of the most morbid aspects of cancer.

2.9.2. DNMT1 depletion

Preventing the unwanted effects of re-expressing metastatic genes silenced by DNA methylation could possibly be addressed by targeting specific DNMT isoforms. The previously mentioned DNMT inhibitors display no specificity for any particular DNMT, therefore the toxic effects of these drugs may be linked to inhibition of other isoforms. Upregulation of DNMT1 is a common occurrence in cancer (el-Deiry, Nelkin et al. 1991) and plays a role in cellular transformation (Detich, Ramchandani et al. 2001). Targeting DNMT1 specifically could potentially have therapeutic value in cancer therapy. We have previously shown that depletion of DNMT1, as opposed totreatment with 5-aza-CdR, does not cause global hypomethylation (Milutinovic, Zhuang et al. 2003). It is therefore possible that inhibiting DNMT1 would avoid the unwanted effects caused by global hypomethylation. Targeting of DNMT1 has been made possible through initial development of antisense RNA corresponding to specific sequences of DNMT1 mRNA. Antisense RNA binds its complimentary RNA and prevents translation leading to an overall depletion of DNMT1 protein levels following turnover of the remaining proteins (Dias and Stein 2002). Initial treatment of adrenocortical tumor cell line Y1 with antisense DNMT1 RNA revealed a decreased ability to grow in an anchorage-independent manner, decreased tumorigenicity in syngeneic mice and re-expression of silenced genes (MacLeod and Szyf 1995; Ramchandani, MacLeod et al. 1997). Further studies revealed that depletion of DNMT1 lead to decreased DNA replication, with decreased firing of replication complexes from origins of replication, and an arrest in S-phase (Knox, Araujo et al. 2000). Clinical trials with antisense to DNMT1 are underway and have met with mixed results. Phase I trials have provided varied results indicating some efficacy of treatment (Davis, Gelmon et al. 2003; Stewart, Donehower et al. 2003). Phase II trials have met with disappointing results suggesting that effectiveness of the

drug may be due to inefficient cellular uptake of the antisense molecule (Winquist, Knox et al. 2006; Klisovic, Stock et al. 2008).

Additional investigations into the effects of DNMT1 depletion on replication and cell cycle yielded many new discoveries regarding DNMT1 function. Depletion of DNMT1 led to re-expression of the epigenetically silenced tumor suppressor gene p21 (Milutinovic, Brown et al. 2004). However this re-expression occurred independent of DNA methylation and histone demethylation, suggesting that depletion of DNMT1 induced novel cellular responses never before associated with the DNMTs. A comparison of effects of DNMT inhibition by 5-azacytidine and DNMT1 depletion on gene expression and cell-cycle revealed a difference in effects between the two drugs (Milutinovic, Zhuang et al. 2003). 5-azacytidine induced a number of genes, including the MAGE family of proteins, pro-apoptotic BIK and SSX2. DNMT1 depletion induced a series of genotoxic stress response genes associated with the DNA damage checkpoint, including ARF-3, JunB and GADD45β. The overall differences in expression patterns reveal that elimination of the DNMT1 protein induces a distinct cellular response from global inhibition of DNA methylation by 5azaC.

The response to DNMT1 depletion elicited a known DNA damage checkpoint. This type of response is also seen when replication forks are stalled during cell division. Cells respond to the appearance of single-stranded DNA (Zou and Elledge 2003) that arise from stalled replication forks during DNA replication or DNA damage (by ATM and ATR effector kinases) (Bao, Tibbetts et al. 2001) by intiating a signaling pathway (Falck, Petrini et al. 2002). This pathway involves activation of the checkpoint kinases (ChK) leading to phosphorylation and degradation of cell division control protein 25a (CDC25a). As a consequence, the downstream effect is the decreased capacity to load CDC45 onto replication origins and therefore leading to impaired recruitment of replication complexes and DNA replication arrest. Further effects of the checkpoint are to induce cell death (Callegari and Kelly 2007), which could explain the anti-growth properties of DNMT1 antisense DNA. A potential explanation to link DNMT1 to the

induction of this DNA damage response pathway could be that the absence of DNMT1 induces unstable DNA replication structures read by the cell as damaged DNA. The results of these studies indicate that targeting DNMT1 specifically may prove to be more successful in targeting the growth properties of cancer cells without inducing demethylation of metastatic genes.

2.9.3. DNMT3 depletion

Most of the focus on epigenetic anti-cancer therapy has centered on the inhibition of DNMTs and specific targeting of DNMT1. However this does not preclude the potential role of DNMT3a and 3b as targets for chemotherapeutics. The role of DNMT3b in cancer was illustrated by the discovery that DNMT3b aids DNMT1 in silencing genes in human cancer cells (Rhee, Bachman et al. 2002) and contributes to promoter hypermethylation (Roll, Rivenbark et al. 2008). Interestingly, knockdown of DNMT3b was shown to induce apoptosis of human cancer cells but not normal cells (Beaulieu, Morin et al. 2002). Further studies have shown that DNMT3a and 3b mediate the anti-cancer effects of 5-azacytidine treatment (Oka, Meacham et al. 2005). Studies have shown that targeting of DNMT3b in conjunction with DNMT1, with mRNA-specific small interfering RNA (siRNA), provided a viable mechanism of re-establishing expression of silenced genes in cancer, specifically inducing CXCL12 expression in breast cancer and pancreatic carcinoma cell lines (Sowinska and Jagodzinski 2007), and suppressing the growth of human cholangiocarcinoma cell line (Zuo, Luo et al. 2008). In fact, the effectiveness of co-depletion of DNMT3b with DNMT1 may depend on the proposition that DNMT1 and DNMT3B interact for inactivation of tumor suppressor genes (Zuo, Luo et al. 2008). While studies have yet to show an important role of DNMT3a in anti-cancer therapy, targeting of DNMT3b in conjunction with DNMT1 may yield a potential combinatorial therapy.

2.9.4. Demethylase inhibitors

MBD2 has been proposed as an ideal cancer target as it was shown to be dispensable for normal cells, yet critical for the development of tumors. Depletion of MBD2 in cancer cells, via specific antisense treatment, was shown to decrease anchorage-independent growth of cancer cells and growth of xenograft tumors in nude mice (Slack, Bovenzi et al. 2002). These initial investigations into MBD2 as a target for anti-cancer therapy were later confirmed by crossing the MBD2 knockout mouse with the Apc $^{min/+}$mouse (Sansom, Berger et al. 2003), a model for colorectal and intestinal cancer (Aoki, Tamai et al. 2003). The mbd2-/- min-/- showed dramatically reduced intestinal adenomas. This decreased propensity to form adenomas was associated with altered methylation patterns. Intravenous injection of MBD2 antisense was shown to decrease the volume and growth of tumors (Campbell, Bovenzi et al. 2004) providing a viable therapeutic agent targeting MBD2. Depletion of MBD2 was shown to induce silencing of the metastatic genes uPa and MMP2, leading to an overall decrease in metastasis (Pakneshan, Szyf et al. 2004; Shukeir, Pakneshan et al. 2006). Due to the limited work and knowledge regarding the molecular identity of DNA demethylase, anti-cancer therapy using demethylases as a target remains in its infancy.

2.10. Summary

This review of the literature provides evidence that the proper epigenetic regulation of gene expression is essential for healthy cellular function. When this epigenetic regulation is altered we witness a dramatic reworking of gene expression patterns that favor uncontrolled growth and proliferation of cells as well as metastasis. Those states are commonly known as cancer and metastatic cancer, respectively. In cancer, we frequently observe silencing of tumor suppressor genes as a consequence of the upregulation of DNMTs. Depletion of DNMT1 in cancer cells leads to an inhibition of growth and a reversal of tumorigenesis. However, more data is required to understand

the exact mechanisms through which the regulation of the epigenome becomes impaired in cancer and the consequences of treating cancer cells through targeting DNMT1.

Examined in this thesis are two aspects of cellular function: cell cycle regulation of DNMTs and replication arrest checkpoints. This thesis will determine how these functions play a role in a relationship with the epigenome. By examining how DNMT1 is regulated through the cell cycle, we can determine the exact mechanisms that may occur during the initial stages of cancer that lead to elevated levels of DNMT1 as well as altered DNA methylation profiles. Through investigation of the consequences of DNMT1 depletion in cancer we can also determine exactly how the cell responds to the elimination of this oncogenic signal and can further elucidate better methods of treating this disease.

Chapter 3

Objectives

I hypothesize that DNMT1 mRNA levels are regulated by a protein that controls DNMT1 levels to control the oncogenic propensity of elevated DNMT1 protein levels. This regulation over DNMT1 is a tightly controlled event and upon its disruption, DNMT1 protein levels increase and the genome becomes hypermethylated. I believe that genomic hypermethylation is a programmed event and DNMT1 methylates specific regions by commandeering promoter specific targeting pathways. I also hypothesize that depletion of DNMT1 in cancer cells induces a specific replication arrest checkpoint and that overexpression of DNMT1 in cancer acts as a fundamental inhibitor of this pathway. The specific objectives are the following:

- To identify the protein that destabilizes DNMT1 mRNA in the G_1/G_0 cell cycle phase and the exact mechanism through which this protein destabilizes DNMT1 mRNA.
- To determine how the regulation of DNMT1 mRNA levels is controlled throughout the cell cycle.
- To establish the effect of eliminating cell cycle regulation over DNMT1 mRNA levels and the impact on DNA methylation.
- To determine the mechanism through which DNMT1 depletion causes S-phase arrest.

Chapter 4

AUF1 cell cycle variations define genomic DNA methylation by regulation of *DNMT1* mRNA stability

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DNA methylation is a major determinant of epigenetic inheritance. The DNA methyltransferase 1 (*DNMT1*) is the enzyme responsible for the maintenance of DNA methylation patterns during cell division, deregulated expression of which leads to cellular transformation. We show herein that AUF1/hnRNP D interacts with an AU-rich conserved element in the 3'-UTR of the *DNMT1* mRNA and targets it for destabilization by the exosome. AUF1 protein levels are regulated with the cell cycle by the proteasome resulting in cell cycle specific destabilization of *DNMT1* mRNA. AUF1 knock down leads to increased *DNMT1* expression and modifications of cell cycle kinetics, increased DNA methyltransferase activity and genome hypermethylation. Concurrent AUF1 and DNMT1 knock down abolishes this effect suggesting that the effects of AUF1 knock down on the cell cycle are mediated at least in part by DNMT1. In this study, we demonstrate a link between AUF1, the RNA degradation machinery and maintenance of the epigenetic integrity of the cell.

INTRODUCTION

DNA methylation patterns are a critical component of the epigenome, controlling gene expression in vertebrates (Razin and Szyf 1984; Razin 1998). The enzyme DNA methyltransferase 1, *DNMT1* is responsible for maintenance and propagation of DNA methylation patterns. These patterns are altered in tumorigenesis (Herman 1999; Baylin and Herman 2000). The overexpression of *DNMT1* in NIH-3T3 mouse fibroblasts cells causes cell transformation (Wu, Issa et al. 1993) while *DNMT1* overexpression in human fibroblasts results in aberrant methylation of endogenous CpG islands (Vertino, Yen et al. 1996). In parallel, the down regulation of *DNMT1* inhibits cancer growth in animal models (Laird, Jackson-Grusby et al. 1995; MacLeod and Szyf 1995; Ramchandani, MacLeod et al. 1997). Based on these reports, *DNMT1* was therefore proposed as a target for anticancer therapy (Szyf 1994; Szyf 2003).

As was expected from its critical role in maintaining epigenomic integrity, *DNMT1* expression was shown to be controlled with cell growth (Szyf, Kaplan et al. 1985; Szyf, Bozovic et al. 1991; Robertson, Keyomarsi et al. 2000). Multiple mechanisms such as transcriptional (MacLeod, Rouleau et al. 1995; Rouleau, MacLeod et al. 1995; Bigey, Ramchandani et al. 2000; Kimura, Nakamura et al. 2003; McCabe, Davis et al. 2005), post-transcriptional (Detich, Ramchandani et al. 2001) and post translational (Ding and Chaillet 2002; Agoston, Argani et al. 2005) ensure a tight regulation of its expression during the cell cycle. It was suggested that deregulated expression of *DNMT1* during the cell cycle might be critical for cell growth control (Robertson, Keyomarsi et al. 2000; Szyf, Knox et al. 2000) and DNA replication (Knox, Araujo et al. 2000; Milutinovic, Zhuang et al. 2003). Deregulated cell cycle control of *DNMT1* was observed in breast cancer and colorectal cancers (De Marzo, Marchi et al. 1999; Nass, Ferguson et al. 1999).

Our previous study showed that *DNMT1* 3'-untranslated region (3'-UTR) contains a highly conserved non canonical AU-rich region, which is responsible for regulating its expression level during the cell cycle (Detich, Ramchandani et al. 2001). Deletion of this conserved region resulted in cellular transformation. We also observed a binding of a protein with an apparent size of ~40 kDa on this region, which triggered the destabilization of *DNMT1* transcript in G_0/G_1 phase.

Using affinity capture with the 3'UTR of *DNMT1* mRNA and MALDI-TOF-MS/MS analysis we identified ARE/Poly-(U)-Binding/Degradation Factor (AUF1), which is also called Heterogenous Nuclear Ribonucleoprotein D (hnRNP D) (Brewer 1991; Zhang, Wagner et al. 1993) and determined its role in posttranscriptional regulation of *DNMT1* mRNA through the exosome. AUF1 is expressed as four isoforms (p37, p40, p42 and p45) arising through alternative splicing of a common pre-mRNA (Wagner, DeMaria et al. 1998; Wilson and Brewer 1999). While differences in the activities of the various AUF1 isoforms have been documented, all isoforms enhance target mRNA decay (Laroia, Cuesta et al. 1999; Loflin, Chen et al. 1999). AUF1 was previously shown to influence the stability of many transcripts encoding proteins involved in mitogenic stimulation, immune response such as Interleukin 10 (Brewer, Saccani et al. 2003), stress response and, cell cycle such as *p16* (Wang, Martindale et al. 2005) and *p21* (Lal, Mazan-Mamczarz et al. 2004). In particular, Cyclin D1 is present at low abundance in quiescent cells but rapidly accumulates after stimulation with serum or mitogens. It is suggested that its rapid cell cycle regulation requires AUF1 binding to the 3'-UTR of this mRNA (Lin, Wang et al. 2000; Lal, Mazan-Mamczarz et al. 2004). We describe here a cell cycle dependent regulation of AUF1 by the proteasome. We further show that cell cycle regulation of AUF1 can post-transcriptionally control *DNMT1* mRNA, and is critical for maintaining the integrity of genomic methylation levels.

MATERIALS AND METHODS

Materials and antibodies

Serum starved Hela cell pellets were purchased from Cilbiotech. Recombinant AUF1 protein was obtained from Upstate Biotechnology. The following antibodies were used: *AUF1* (Upstate Biotechnology), β-actin (Sigma) and *DNMT1* (New England Biolabs). The hRrp 40p, hRrp41p, hRrp46p, generously given by Dr Schilders and Pruijn; hRrp4 by Dr Tollervey; PM-Scl 75 by Dr. van Venrooij. Cycloheximide was purchased from Sigma, MG-132 and Streptavidin agarose suspension from Calbiochem.

Cell culture and transfections

HEK-293, BALB-c and Hela cells were maintained in DMEM/ 10% FCS, antibiotics (Invitrogen). MCF7 and T24 cells were maintained in MEM and Mc Coy medium, respectively. Human fibroblasts GM01887, provided by the Coriell Cell Repositories (Camden, New Jersey), were grown in MEM/10% FCS. HEK-293 cells were transfected using calcium phosphate while Lipofectamine 2000 (Invitrogen) was used for transfection of Hela cells, and Lipofectin (Invitrogen) for MCF7 cells, human fibroblasts GM01887 and T24 cells. For serum starvation studies, cells were grown for 2-14 days in their medium/0.5% FCS and then released from cell-cycle arrest by addition of serum (10%) for 6 to 48 hours.

Plasmids and siRNA oligonucleotides

Construction of pBluescript SK 3'-UTR *DNMT1*-polyA vector: the pSK Δ5'259 vector, containing the h*DNMT1* 3'-UTR conserved region 5349-5405 (accession number NM001379.1) (Detich, Ramchandani et al. 2001) was annealed with a poly-A primer. PCR

was performed using a 3'-poly-T primer and 5'-primer containing a h*DNMT1* 3'-UTR complementary region flanked by a T3 promoter sequence. The PCR product was cloned into pcDNA3.1 V5-HIS-TOPO/A (Invitrogen). The pSP64 Poly-(A) vector was purchased from Promega.

pFLAG-CMV2-AUF1 isoforms vectors were kindly provided by Dr. R. Schneider (Sarkar, Xi et al. 2003), pSilencer 2.0-U6 and pSilencer AUF15 by Dr. M. Gorospe (Wang, Martindale et al. 2005). pSuper AUF1 and pSuper CT was generated by inserting the following sequence into *Hind* III/*Bgl* II pSR-Neo sites (Oligoengine), respectively: 5'-AGCTTTTCCAAAAAGATCCTATCACAGGGCGATTCTCTTGAAATCGCCCTGTATAGGATC-3' and 5'-GATCCCCGACGACGACGACGACGATGTTTTCAAGAGAAAC ATCGTCGTCGTCGTCGTCTTTTTGGAAA-3'. 3'-UTR *DNMT1* deletion constructs were generated by PCR and inserted in frame into pcDNA3.1 His B (Invitrogen). Oligonucleotide antisense for *DNMT*1 was previously described (Knox, Araujo et al. 2000). CT siRNA, 5'-UGGAGAGCACCGUUCUCC-3', hRrp40 P4 ,PM-Scl 75 siRNA (Stoecklin, Mayo et al. 2006) and AUF1 exon 3 siRNA (Raineri, Wegmueller et al. 2004) were purchased from Dharmacon.

3'-UTR *DNMT1*-polyA vector contained the h*DNMT1* 3'-UTR conserved region 5349-5405 (accession number NM001379.1) (Detich, Ramchandani et al. 2001). pcDNA3-hRrp4-TAP vector provided by Dr Chen was described (Chen, Gherzi et al. 2001). pFLAG-CMV2-AUF1 isoforms vectors were kindly provided by Dr. R. Schneider (Sarkar, Xi et al. 2003), pSilencer 2.0-U6 and pSilencer AUF15 by Dr. M. Gorospe (Wang, Martindale et al. 2005). pSuper AUF1 and pSuper CT was generated by inserting the following sequence into *Hind* III/*Bgl* II pSR-Neo sites (Oligoengine), respectively : 5'-AGCTTTTCCAAAAAGATCCTATC ACAGGGCGATTCTCTTGAAATCGCCCTGTATAGGATC-3' and 5'-GATCCCCGACGA CGACGACGACGATGTTTTCAAGAGAAACATCGTCGTCGTCGTCTTTTTGGAAA-3'. 3'-UTR *DNMT1* deletion constructs were generated by PCR and inserted in frame into pcDNA3.1 His B (Invitrogen). Oligonucleotide antisense for *DNMT*1 was previously described (Knox, Araujo et al. 2000). CT siRNA, 5'-UGGAGAGCACCGUUCUCC-3', hRrp40 P4 ,PM-Scl 75 siRNA

(Stoecklin, Mayo et al. 2006), AUF1 exon 3 siRNA (Raineri, Wegmueller et al. 2004) and *p21* siRNA were purchased from Dharmacon.

In vitro RNA transcription

pSP64 Poly(A) and pBluescript SK 3'-UTR *DNMT1*-polyA vectors were *in vitro* transcribed with either T3 or SP6 polymerase using the *in vitro* transcription kit (Ambion). For affinity chromatography column, larger quantities of RNA were synthesized using the MEGAscript kit (Ambion).

RNA Affinity chromatography

500 µg of *in vitro* transcribed RNA (*DNMT1* 3'-UTR or Control) were hybridized in incubation buffer (Hepes pH 8.0, 10 mM; $MgCl_2$ 3 mM; KCl 40 mM; Glycerol 20%; DTT 1 mM; complete protease inhibitors® (Roche Diagnostics)) with 500 mg of oligo-(dT)-cellulose beads (Sigma). Whole cell protein extracts (1mg) from serum starved Hela cells were incubated with RNA probe/oligo-(dT) beads in incubation buffer supplemented with tRNA. Proteins/RNA-beads complexes were pelleted and unbound proteins were eliminated by two 50 ml-washes in the incubation buffer and 5 times with 50 ml-washing buffer (Hepes pH 8.0, 10 mM; $MgCl_2$ 3 mM; KCl 40 mM; glycerol 20%; DTT 1 mM; NaCl 500 mM; protease inhibitors®). Bound proteins were eluted by a step-wise gradient (0.8 M to 4.3 M NaCl). The fractions from each NaCl concentration were desalted and concentrated using Amicon Ultra-4 centrifugal filters (Millipore). Five µl of the concentrated fraction were loaded onto a 15% acrylamide gel and

silver stained using the BioRad Silver Staining Kit (BioRad). For mass spectrometry analysis, samples were stained by mass spectrometry-compatible Coomassie blue.

Mass spectrometry Maldi-TOF-tandem MS

DNMT1 3' UTR specific binding proteins were identified by comparing the *DNMT1* 3' UTR and the control lanes. Gel slices were excised and digested with porcine trypsin on a MassPrep robotic workstation (Micromass). Tryptic peptides were analysed on a QTrap 4000 ion trap mass spectrometer (Applied Biosystems). The tryptic peptides were applied Pico Frit column containing BioBasic C18 packing. Eluted peptides were electrosprayed as they exited the column and doubly, triply or quadruply charged ions were selected for passage into a collision cell. MS/MS data were analyzed by BioAnalyst 1.4 software (Applied Biosystems) and submitted to Mascot (Matrix Science) for identification by analysis against the NCBI non-redundant database. MS/MS analyses were performed by the Genome Quebec Proteomic Facility (Montreal, Quebec).

RNA-protein UV cross-linking

Twenty µg of cell extract or 1-2 µg of AUF1 purified protein, were subjected to RNA UV cross linking using the indicated RNA probes as previously described (Detich, Ramchandani et al. 2001).

Protein and RNA immunoprecipitation

HEK-293 cells were transfected with FLAG-tagged AUF1 or hRrp4p-TAP vectors. T24 cells or transfected HEK-293 cells were lysed and protein precipitation of the cytoplasmic fraction was performed with hRrp4p, M2 anti-flag antibody (Sigma) or pull down using TAP

purification system. hRrp4p was immunoprecipitated from T24 cells. For AUF1 immunoprecipitation, antibodies were cross-linked to protein G agarose beads (Roche Diagnostics) using dimethyl pimelimidate (DMP). RNA was prepared from the supernatants (SPNT) and pellets following immunoprecipitation and subjected to RT-PCR as described below.

Reverse Transcription-PCR and quantitative-Real Time-PCR

Total RNA was extracted using RNAeasy kit (Qiagen). cDNA was synthesized and PCR were performed as previously described (Detich, Ramchandani et al. 2001), using the following primers: *DNMT1* and β-Actin (Detich, Ramchandani et al. 2001); *DNMT3A* forward: 5'-ACCCTCCAAAGGTTTACCCACCTG-3'; *DNMT3A* reverse: 5'-CA TACCGGGAAGGTTACCCCAGAA-3'; *DNMT3B* forward: 5'-GACTGGACCGTGCGCCTGCAGGCC-3'; *DNMT3B* reverse: 5'-GAAGCGACGT ACTTTCCTACCTTT-3'; *p*16 (Kondo, Shen et al. 2003); AUF1 (Yasuda, Wada et al. 2004) and *p21* (Milutinovic, Knox et al. 2000) The numbers of cycles were selected so that the PCR amplification remained in the linear range after a series of amplification at different numbers of cycles. Each PCR was performed in triplicate; the intensity of signal obtained for each messenger was determined by densitometry (NIH Image and normalized to the intensity of the signal obtained for β-actin. For some PCR, nucleic acids were transferred by Southern blot to nylon membrane. An oligonucleotide specific for the *DNMT1* mRNA sequence, 5'-CCTCGAGGCCTAGAAACAAA GGGAAGGGCAAG-3' was synthesized, radiolabeled and then hybridized to the membranes, which were exposed to PhosphorImager screens. The screens were scanned by PhosphorImager (Molecular Dynamics). ROD readings were determined using a computer-assisted densitometry program (Molecular Dynamics). Real-time PCR analysis was performed using the Roche Light Cycler. PCR was performed in 25-µl reactions with Syber Green (SuperArray). All the primer sets used produced no signal in control reactions lacking template. Dissociation-curve analysis showed that single products with the expected T_m values were generated by each primer set. Standard curves were

determined for each primer set by dilution of the input DNA. The amount of each cDNA was calculated from the cycle threshold (C_T) for each primer set using the standard curves. The relative units recovered for each primer set were determined by dividing the calculated amount of cDNA by the amount β-Actin cDNA. The following primer sequences were used: *DNMT1* forward: 5'-TTTGTATGTTGGCCAAAGCCCGAG-3'; *DNMT1* reverse: 5'-TTCATGTCAGCCA AGGCCACAAAC-3'; *DNMT3A* forward: 5'-GACTCCATCACGGTGGGCATGG-3'; *DNMT3A* reverse: 5'-TGTCCCTCTTGTCAC TAACGCC-3'; *DNMT3B* forward: 5'-GA GTCCATTGCTGTTGGAACCG-3'; *DNMT3B* reverse: 5'-ATGTCCCTCTTGTCGCC AACCT-3'(Jinawath, Miyake et al. 2005); β-Actin forward: 5'-AGATGTGGATCAGCAAGCAGGAGT-3'; β-Actin reverse: 5'-GCAATCAAAGTCC TCGGCC ACATT-3'.

In vitro RNA degradation assay

RNA decay rates were assessed as previously described (Buzby, Brewer et al. 1999). Following autoradiography, the relative signal strength of the [^{32}P]-labeled RNA was quantified by two-dimensional densitometric scanning with NIH imaging system. Quantitative decay of the RNA was calculated as the percentage of signal remaining as compared to signal at time 0.

Adenoviral infection

Adenoviral vectors encoding h*DNMT1* and h*DNMT1* lacking its 3'-UTR as well as the adenoviral particle production and infection were previously described (Detich, Ramchandani et al. 2001).

Northern blot and *DNMT1* mRNA half-life measurement

DNMT1, *GFP,* and neomycin mRNA levels were analyzed by Northern blot analysis (Detich, Ramchandani et al. 2001). After transfection with a combination of pSilencer AUF15 and pSuperAUF1 or the corresponding CT siRNA vector, HEK-293 cells were treated with actinomycin D (5μg/ml) for the indicated time. *DNMT1* and neomycin mRNA levels were estimated by Northern blot. Quantitative decay of the RNA was calculated as the percentage of signal remaining as compared to signal at time 0. The following probes were used: h*DNMT1* (Detich, Ramchandani et al. 2001), GFP (pEGFP C2 (Clontech)), X-press (pcDNA3.1 His B (Invitrogen)) and neomycin (pcDNA3.1 His B). In T24 cells, detection of *DNMT1* mRNA after actinomycin treatment was performed by RT-PCR followed by oligonucleotide hybridization described in (Detich, Ramchandani et al. 2001) and quantified by q-RT-PCR.

Flow cytometry analysis

Cells were stained with propidium iodide and the DNA content was measured by flow cytometry. Data were analyzed using WinMDI v2.8 software.

CAT reporter activity assays

T24 cells were transfected with the previously described pMet-P1□HXCAT plasmid (Bigey, Ramchandani et al. 2000). As a control, a plasmid with the same fragment in the opposite orientation was used. CAT assays were performed as described previously (Rouleau, Tanigawa et al. 1992).

[^{3}H]-**Thymidine incorporation DNA synthesis assay**

Cells were incubated for 4 h with 1 μCi/ml of [^{3}H]-thymidine (Perkin Elmer). Cells were fixed in 10% trichloroacetic acid and then lysed with NaOH 1N/SDS 1%. Lysates were collected and applied onto a liquid scintillation cocktail. [^{3}H]-thymidine incorporation was measured using a liquid scintillation counter (1211Rackbeta-LKB Wallac).

Assay of DNA methyltransferase activity

DNA methyltransferase activity in nuclear extracts from human fibroblasts was assayed as described previously (Szyf, Bozovic et al. 1991).

5-Methylcytosine quantification by nearest neighbor analysis

5-methylcytosine level was quantified by nearest-neighbor analysis as described in (Ramsahoye 2002; Jackson, Krassowska et al. 2004). The intensities of 5 methylcytosine and cytosine mononucleotide spots were measured using a phosphorimager screen and Image Quant quantification.

Statistical analysis

Experiments were performed in triplicate. Averages and the standard deviations were calculated. A Student's t-test was performed and critical values for statistical significance are expressed as *p<0.05 and **p<0.01.

RESULTS

Isolation and identification of AUF1/hnRNP D as a 3'-UTR *DNMT1* mRNA binding protein.

Our previous studies suggested that the depletion of *DNMT1* mRNA during the G_0/G_1 phase of the cell cycle was mediated by a ~40 kDa protein binding to a conserved *DNMT1* 3'-UTR (Detich, Ramchandani et al. 2001). As expected, a binding protein at ~40 kDa, which interacts specifically with the *DNMT1* 3'-UTR (Figure 1A) was detected by UV cross-linking (Figure 1B) in cytoplasmic extracts from serum starved HeLa cells. Cell cycle regulation of *DNMT1* was verified in this cell line (Supplementary figure S1). Using RNA affinity chromatography with either the 3'UTR sequence or the control RNA sequence as bait (Figure 1A) we partially purified this protein from the extracts (Supplementary figure S2). In the 3.2M NaCl fraction, a ~40 kDa protein was found to interact specifically with the 3'-UTR *DNMT1* RNA. This fraction was analyzed by UV cross-linking to confirm the presence of a ~40 kDa *DNMT1* 3'-UTR specific-binding protein (Supplementary figure S3). Two bands from the 3.2 M fractions were excised together and analyzed by MALDI-TOF-MS/MS (Figure 1C). Eight sequenced peptides corresponding to the AUF1/hnRNP D protein were identified which were common to all of the four known AUF1 isoforms (Figure 1D and Table 1). The presence of AUF1 protein in the fractions eluted from *DNMT1* 3'-UTR-RNA matrix was confirmed by Western-blot analysis (Figure 1E).

Binding of AUF1 to *DNMT1* 3'-UTR results in a decrease of *DNMT1* expression levels by destabilizing its mRNA.

We confirmed that AUF1 binds to *DNMT1* 3'-UTR RNA, by UV cross-linking purified AUF1 with a labeled *DNMT1* 3'-UTR-RNA (Figure 2A). Whereas no signal was observed with the probe alone (Figure 2A, lane 1), AUF1 exhibited an enhanced interaction with the *DNMT1* 3'-UTR probe (3'-UTR) as compared to the non specific probe (CT) (Figure 2A, lane 2-

3 and 6-7). In addition, the binding of AUF1 to *DNMT1* 3'-UTR probe was weakly competed out by an excess of unlabeled control probe (Figure 2A, lane 4) whereas the binding was much more effectively competed out by an excess of unlabeled *DNMT1* 3'-UTR probe (Figure 2A, lane 5).

To determine whether the different AUF1 isoforms could interact with *DNMT1* mRNA in living cells we resorted to transient transfection in human embryonic kidney HEK-293 cells which can be transfected by exogenous expression vectors at high efficiency. An UV cross-linking assay revealed that all four AUF1 isoforms bind the *DNMT1* 3'-UTR probe in HEK-293 cells (Figure 2B). RNA immunoprecipitation followed by RT-PCR using AUF1 antibody revealed that AUF1 is endogenously associated with *DNMT1* mRNA (Figure 2C) as well as *p21* mRNA which is a known AUF1 target but not with β-actin which is not known to interact with AUF1. The same non quantitative approach using anti-Flag antibody confirmed that endogenous *DNMT1* mRNA can physically associate with all known AUF1 isoforms in living cells. Indeed, while both *DNMT1* and β-actin mRNAs were detected in all the supernatant fractions (Figure 2D, upper panel), only *DNMT1* mRNA was present in all tagged-AUF1 immunoprecipitates (Figure 2D, bottom panel). We further observed that the AUF1 overexpression led to a reduction of *DNMT1* mRNA level (Figure 2D, upper panel).

We examined whether AUF1 altered the stability of *DNMT1* mRNA. Using *in vitro* RNA degradation assays, we showed that extracts derived from HEK-293 cells ectopically expressing the AUF1 isoforms (p37, p40, p42 and p45) degraded *DNMT1* 3'-UTR RNA at an accelerated rate ($t_{1/2}$: 10.5, 8.9, 12.0 and 9.0 min, respectively) relative to control vector transfected cell extracts that contained markedly lower levels of endogenous of AUF1 ($t_{1/2}$: 27.3 min) (Figure 2E). Moreover, Western blot analysis revealed that the overexpression of AUF1 isoforms in HEK-293 led to a reduction of *DNMT1* protein levels (Figure 2F) which are in agreement with the mRNA levels (Figure 2D, upper panel).

Interestingly, overexpression of AUF1 isoforms which decreased *DNMT1* levels also led to a decreased HEK-293 cell numbers (Figure 2G). This is consistent with previous data

which showed that *DNMT1* was part of the DNA replication complex (Rountree, Bachman et al. 2000) and that inhibition of *DNMT1* inhibited DNA replication and cell growth (Knox, Araujo et al. 2000).

Down regulation of AUF1 induces *DNMT1* expression by stabilizing its mRNA.

To ascertain the cellular role of the 3'-UTR in mediating destabilization of *DNMT*1 mRNA by AUF1, different X-press tagged *DNMT1* 3'-UTR deletion constructs were transiently transfected into HEK-293 cells (Figure 3A). Northern blot analysis revealed that the deletion of the 54 nt AUF1 binding sequence flanked by an additional 64nt upstream sequence (construct *DNMT1*-Δ5285) increased the steady state mRNA levels (Figure 3B). The strict deletion of the AUF1 binding site (construct *DNMT1*-□5347) also resulted in a similar increase that strongly suggests that the AUF1 binding region in the *DNMT1* 3'-UTR confers instability to the *DNMT1* mRNA. We also determined the effect of AUF1 depletion mediated by AUF1 siRNA on the half-life of *DNMT1* mRNA. Down regulation of AUF1 protein level, as visualized by Western blot (data not shown) extended the half-life of *DNMT1-FL* mRNA ($t_{1/2}$: 29.0 min) as compared to the half-life in control cells ($t_{1/2}$: 17.7 min) (Figure 3C). AUF1 knock down also increased the levels of endogenous *DNMT1* protein levels (Figure 3D).

We further confirmed that AUF1 down regulates *DNMT1* mRNA using a different human cell line, bladder carcinoma T24 cell line, in which *DNMT1* was shown to be cell-cycle regulated (Robertson, Keyomarsi et al. 2000).. We verified by RNA immunoprecipitation that AUF1 is also associated with endogenous *DNMT1* mRNA in this cell line (data not shown). siRNA knock down of AUF1 (Figure 4A, middle panel) resulted in an increase in endogenous *DNMT1* protein levels (Figure 4A top panel). We then examined whether the effect of AUF1 required the 3'-UTR. T24 cells were infected with adenoviral vectors expressing *DNMT1* mRNA that either contain the entire 3'-UTR (pAD-DNMT1) or lack this region (pAD-DNMT1-ΔUTR). An adenoviral vector expressing DNMT1 mRNA with an UTR lacking the AUF1 binding site (pAD-DNMT1-Δ3'56) was also used (Detich, Ramchandani et al. 2001) (Figure 4B).

Northern blot analysis indicated that *DNMT1* transcripts that lacked the 3'-UTR or the AUF1 binding site were more abundant than those containing the entire 3'-UTR (Figure 4C, lane 1, 3 and 5). Moreover, AUF1 depletion increased the level of *DNMT1* mRNA containing the 3'-UTR (Figure 4C, lane 1 and 2) but had no significant effect on the level of *DNMT1* mRNA which does not contain the 3'-UTR (Figure 4C, lane 3 and 4) or the AUF1 binding site (Figure 4C, lane 5 and 6). These data show that AUF1 modulation of *DNMT1* mRNA expression in T24 cells requires the 3'-UTR. In addition, an *in vitro* degradation assay revealed an increased half-life of *DNMT1* 3'-UTR RNA probe incubated with a cytosolic extract from AUF1 depleted T24 cells from 5.9 to 12.7 min (Figure 4D) confirming that, in T24 cells, AUF1 is involved in destabilization of the *DNMT1* transcripts.

In order to exclude the possibility that AUF1 could also act at the transcriptional level by indirectly modulating *DNMT1* promoter activity, we tested whether AUF1 depletion would increase h*DNMT1* promoter driven CAT activity (Figure 4E) (Bigey, Ramchandani et al. 2000). There was no observed change in *DNMT1* promoter activity.

To test whether the regulation of *DNMT1* by AUF1 is specific to cancer cells or whether it also applies to non-transformed cells, we investigated the effects of a knock down of AUF1 protein on *DNMT1* expression in non-transformed human skin fibroblast line, GM01887. We first established that *DNMT1* expression was also regulated with the cell cycle in human fibroblasts. Indeed, culture in serum starved conditions for 14 days led to an arrest of a large population of cells in G_o/G_1 phase measured by FACS analysis whereas a subsequent serum supplementation for 48h triggered an entry into S/G_2M phase (Figure 4F). mRNA and protein *DNMT1* expression was measured in these two populations and demonstrated that *DNMT1* was also regulated in a cell cycle-dependent manner in this human fibroblast cell line (Figure 4G and H). AUF1 down regulation triggered a marked increase in *DNMT1* protein as determined by Western blot (Figure 4I, top panel) indicating that AUF1 also controls *DNMT1* level in non-transformed cells.

AUF1 destabilizes specifically *DNMT1* mRNA in T24 cells and human fibroblasts and not other *DNMT*s.

It was previously shown that *DNMT3A* and *DNMT3B* as well as *DNMT1* expression are regulated during the cell cycle in T24 cells (Robertson, Keyomarsi et al. 2000). We found no significant changes in *DNMT3A* and *DNMT3B* levels in either in either T24 (Figure 4J) or human fibroblast cells (Figure 4K) upon AUF1 knock down. This indicates that AUF1 selectively targets *DNMT1* mRNA. The cell cycle regulation of *DNMT3A* and *3B* must entail other cell regulating mechanisms.

Biological consequences of AUF1 depletion on DNA replication are partially mediated by *DNMT1* in T24 cells

We previously showed that an acute knock down of *DNMT1* leads to arrest of firing of origin of replication (Knox, Araujo et al. 2000) and results in an intra-S-phase arrest of DNA replication (Milutinovic, Zhuang et al. 2003). We therefore tested whether AUF1 knock down would affect cell cycle kinetics (Figure 5A). AUF1 depletion in T24 cells resulted in an increase in the fraction of cells in S and G_2/M phase as compared to control siRNA transfected cells (siCT), (S: 19.5%; G_2/M: 32.6% and S: 13.1%, G_2/M: 18.5%, respectively) and a decrease in the number of cells in G_0/G_1 (47.9% versus 68.4%). AUF1 regulates a number of transcripts important for cell cycle regulation such as *p16* (Wang, Martindale et al. 2005) and *p21* (Lal, Mazan-Mamczarz et al. 2004). We determined whether *DNMT1* was a downstream effector of AUF1 action on DNA replication by concurrent knock down of AUF1 and *DNMT1.* AUF1 knock down stimulated DNA synthesis as predicted from the FACS analysis and this increase was diminished by concurrent *DNMT1* knock down with a *DNMT1* antisense oligonucleotide (Knox, Araujo et al. 2000) (Figure 5B). The fact that double knock down of AUF1 and *DNMT1* did not result in an increase in DNA replication suggests that AUF1 knock down effects on DNA replication require the presence of *DNMT1*. In the absence of *DNMT1*, AUF1 does not induce DNA replication. However, this does not rule out the possibility that

the double-knock down effect could be a combination of two independent effects as well. Simultaneous knock down of *p21* and AUF1 led on the other hand to stimulation of DNA replication and increased the fraction of cells in the S phase of the cell cycle (Supplementary figure S4). Thus, the effects of AUF1 knockdown on the cell cycle are dependent on different effectors. Some of the AUF1 targets enhance entry into S such as *DNMT1* while others repress cell cycle progression such as *p16* and *p21.*

AUF1 depletion induces *DNMT1* expression in human fibroblasts and increases global DNA methylation.

Since AUF1 depletion in T24 cells modified the cell cycle, we next asked whether a similar effect would be observed in non transformed cells. Interestingly, up-regulation of *DNMT1* generated by an AUF1 knock down observed in Figure 4I did not result in a distinct change in cell cycle kinetics (Figure 5C) or cell proliferation (Figure 5D) in non-transformed human fibroblasts.

However, ectopic expression of *DNMT1* was previously shown to transform mouse immortalized fibroblast cells (Wu, Issa et al. 1993). Since an AUF1 knock down did not result in cellular transformation of human fibroblasts (data not shown), we hypothesized that a knock down of AUF1 would unleash other control mechanisms that would override *DNMT1* action on cell cycle kinetics. One possibility is that AUF1 knock down activates tumor suppressor genes that counteract the impact of increased *DNMT*1. It was previously shown that AUF1 depletion triggers *p16* gene expression in human fibroblasts (Wang, Martindale et al. 2005). We validated that AUF1 knock down induced tumor suppressor gene *p16* mRNA expression in these particular human fibroblasts (Figure 5E).

Next, we tested whether the knock-down of AUF1 would result in an overall increase in the DNA methylation of genomic DNA since *DNMT1* expression is elevated in the absence of concomitant increase in DNA synthesis. We first showed that DNA methyltransferase

activity on hemimethylated DNA substrate in nuclear extract prepared from AUF1 siRNA treated cells was elevated in comparison with extracts from control siRNA treated cells (Figure 5F). This increase in DNA methyltransferase activity was associated with a significant global increase in CpG methylation level as measured by nearest neighbor analysis (Figure 5G). These data suggest that AUF1 depletion leads to an increased methylation capacity of normal human fibroblasts and increased genomic methylation by up regulating *DNMT1* mRNA. Our earlier findings that *DNMT3A* and *3B* are not regulated by AUF1 (Figure 4J and 4K) are consistent with the hypothesis that the increased DNA methyltransferase activity and global methylation observed are primarily caused by increased *DNMT1* mRNA and protein.

AUF1 expression is inversely correlated with *DNMT1* expression levels during the cell cycle.

Our *in vitro* and in cell culture experiments showed that AUF1 interacted with *DNMT1* mRNA to negatively regulate its expression. We tested the hypothesis that AUF1 was regulated in a cell cycle dependent manner which regulated, in turn, *DNMT1* mRNA expression in T24 cells. T24 cells were arrested by serum starvation and entry into the cell cycle was induced by serum supplementation. The fractions of cells at the different stages of the cell cycle were determined by FACS analysis (Figure 6A). AUF1 and *DNMT1* protein levels during the progression of the cell cycle were measured were analyzed by Western blot analysis (Figure 6B) and RT-PCR (Figure 6C). As seen in Figure 6A, serum deprivation arrested a large fraction of the cells at G_0/G_1. Serum supplementation induced an entry into the S phase of the cell cycle. As expected, an increase in *DNMT1* protein levels was observed 18h post serum stimulation (Figure 6B) to reach a maximum after 24h. In contrast, a simultaneous decrease of AUF1 protein level was observed upon entry into the cell cycle. After 48h, levels of *DNMT1* and AUF1 protein levels returned to basal levels. In contrast to AUF1 protein levels, no changes were observed in AUF1 mRNA concentration during the cell cycle (Figure 6C) indicating that AUF1 regulation in the course of the cell cycle is most likely

post-translational. The graph in Figure 6D represents the levels of *DNMT1* mRNA and AUF1 in cell populations at different stage of the cell cycle. This cell cycle regulation of AUF1 was also observed in other normal and cancer cell lines from human and mouse origin (Supplementary figure S5) suggesting that this mechanism of regulation of AUF1 is not an idiosyncrasy of T24 cells. SiRNA knock down of AUF1 in serum starved cells increased *DNMT1* levels suggesting that the higher levels of AUF1 observed in G_0/G_1 cells are, at least in part, responsible for the down regulation of *DNMT1* during G_0/G_1 (Figure 6E). Our data are therefore consistent with the hypothesis that the changes in *DNMT1* during the cell cycle could be partially caused by inverse cell-cycle regulation of AUF1 levels.

The proteasome machinery is responsible for an increased AUF1 degradation rate during S phase

Since we observed a decrease in AUF1 protein levels during S phase, which was not explained by reduced transcription of AUF1 mRNA, we determined whether differences in protein stability were responsible. We therefore compared AUF1 protein stability, subsequent to a cycloheximide block of *de novo* protein synthesis, in serum starved (mostly in Go/G_1 phase) and in an 18h-serum released T24 cell population (mostly in S phase). This analysis revealed a shorter AUF1 half-life in the S phase cell population (Figure 6F, upper panel) than in the serum-starved cells. Several studies have previously demonstrated that AUF1 isoforms were subjected to ubiquitination mediated degradation by the proteasome machinery (Laroia, Cuesta et al. 1999; Laroia, Sarkar et al. 2002; Laroia and Schneider 2002). We observed that AUF1 degradation rate in S phase cell population was clearly diminished by the addition of the proteasome inhibitor MG-132 (Figure 6F, bottom panel) suggesting the participation of the proteasome in this cell cycle dependent regulation. Furthermore, we wanted to evaluate the consequences of a MG-132 treatment on *DNMT1* mRNA in T24 cells. Unfortunately, upon further investigation it was determined that MG-132 treatment itself significantly modified the cell cycle which would bias the measurement of cell cycle

regulated mRNA such as *DNMT1* (Supplementary figure S6). Nevertheless, these MG-132 effects were not observed in the T24 cell populations in which protein translation was chemically blocked by cycloheximide (Supplementary figure S7).

The exosome participates in the AUF1 triggered-degradation of *DNMT1* mRNA

AUF1 was shown to interact with exosome and to be responsible for the targeting of (ARE)-containing mRNA to this RNA degradation machinery (Chen, Gherzi et al. 2001). Immunoprecipitation experiments confirmed a physical interaction between the four AUF1 isoforms and components of the exosome complex such as hRrp4p, hRrp41 (Figure 7A). In parallel, immunoprecipitation of hRrp4p in T24 cells showed the presence of *DNMT1* mRNA in the precipitated complex (Figure 7B). As anticipated if AUF1 mediates the interaction between the 3'-UTR and the exosome, an AUF1 knockdown led to a reduced amount of *DNMT1* mRNA detected in the hRrp4p immunoprecipitate (Figure 7B). These data demonstrate for the first time that AUF1 links *DNMT1* mRNA and the exosome complex and provides a mechanism for how AUF1 affects *DNMT1* mRNA stability.

To confirm the role of the exosome in *DNMT1* mRNA degradation, we decided to disrupt exosome function using a siRNA strategy. In brief, we targeted PM-Scl 75 protein, a core exonuclease of the exosome (Stoecklin, Mayo et al. 2006) and hRrp40, another component of the exosome (Figure 7C). The consequences of this exosome knock down in T24 cells were a slowing of *DNMT1* mRNA degradation (Figure 7D) resulting subsequently in an increase in protein (Figure 7C) and mRNA expression (Figure 7E). In addition, combined knockdown of the two *DNMT1* mRNA destabilizing agents, AUF1 and the exosome, potentiated *DNMT1* mRNA expression (Figure 7F). Inversely, hRrp4 overexpression of the exosome protein hRrp4p led to a decrease of *DNMT1* protein (Figure 7G) and mRNA expression (Figure 7H) in HEK-293 cells. Pull down of the hRrp4p protein revealed the presence of *DNMT1* mRNA indicating that *DNMT1* mRNA is physically associated with this component of the exosome in (Figure 7 I).

Since we showed that AUF1 protein level varied during the cell cycle, we also measured the expression level of different components of the exosome. Western blot analysis did not show dramatic changes in terms of protein levels during the cell cycle (Figure 7J). We therefore hypothesized that the AUF1 protein variations during the cell cycle may control, at least in part, the relative amount of *DNMT1* mRNA/protein during the cell cycle.

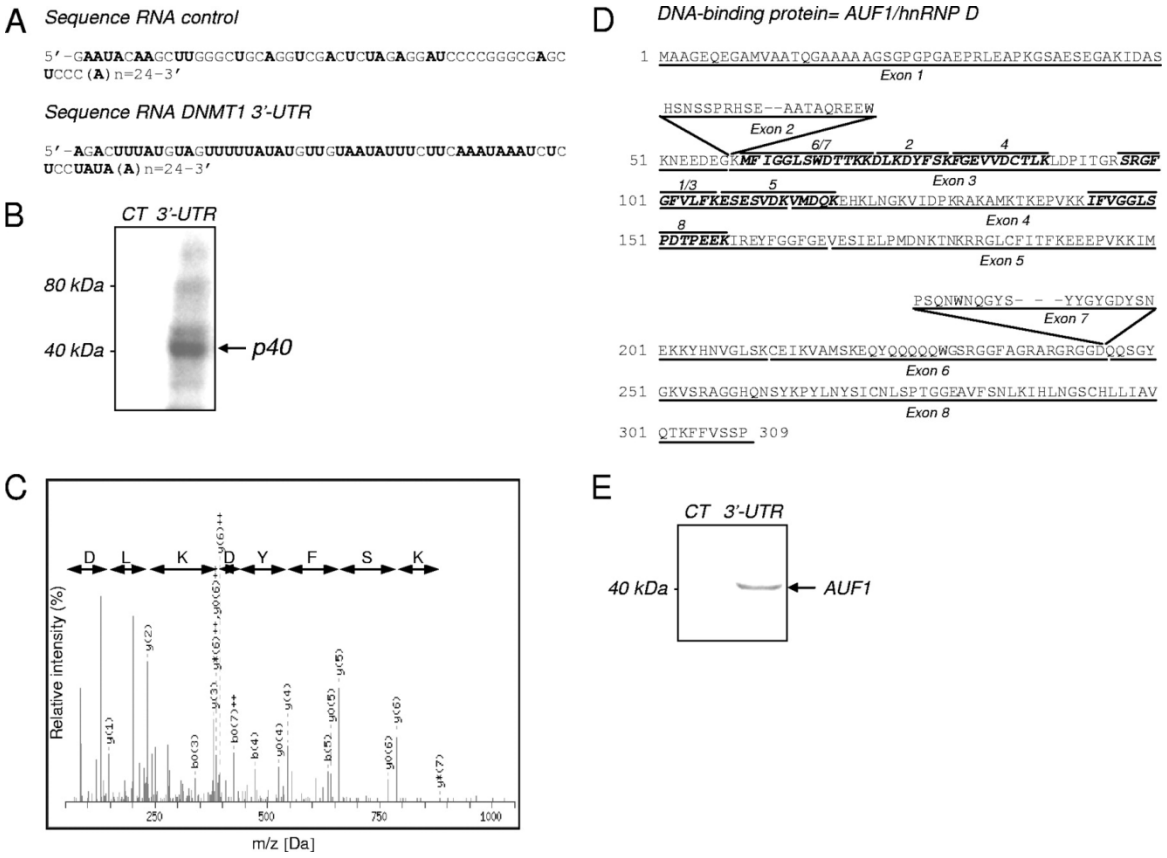


Figure 1. Identification of AUF1/hnRNPD as a 3'-UTR *DNMT1* mRNA binding protein. (A) *In vitro* transcribed RNA sequences encoding the AU-rich conserved element of *DNMT1* 3'-UTR (81 nt) and a control sequence (77 nt) extended by a 24 nucleotides-poly-A tail. Adenine and uracile nucleotides are shown in bold. **(B)** Cytoplasmic extracts of serum starved Hela cells were incubated and UV cross linked with a [^{32}P]-labeled *DNMT1*-UTR (3'-UTR) or control RNA probe (CT). The arrow indicates a RNA/protein complex with an apparent size of ~40 kDa. **(C)** MS/MS spectrum of an identified peptide corresponding to AUF1/hnRNPD sequence. **(D)** Peptide sequence of hnRNP D/AUF1 isoforms. Boldface/italic type represents peptides sequence identified by Maldi-TOF-MS/MS. The positions of exon 2 and 7 are indicated above the line. **(E)** Western-blot analysis performed on the 3.2 M eluted fractions.

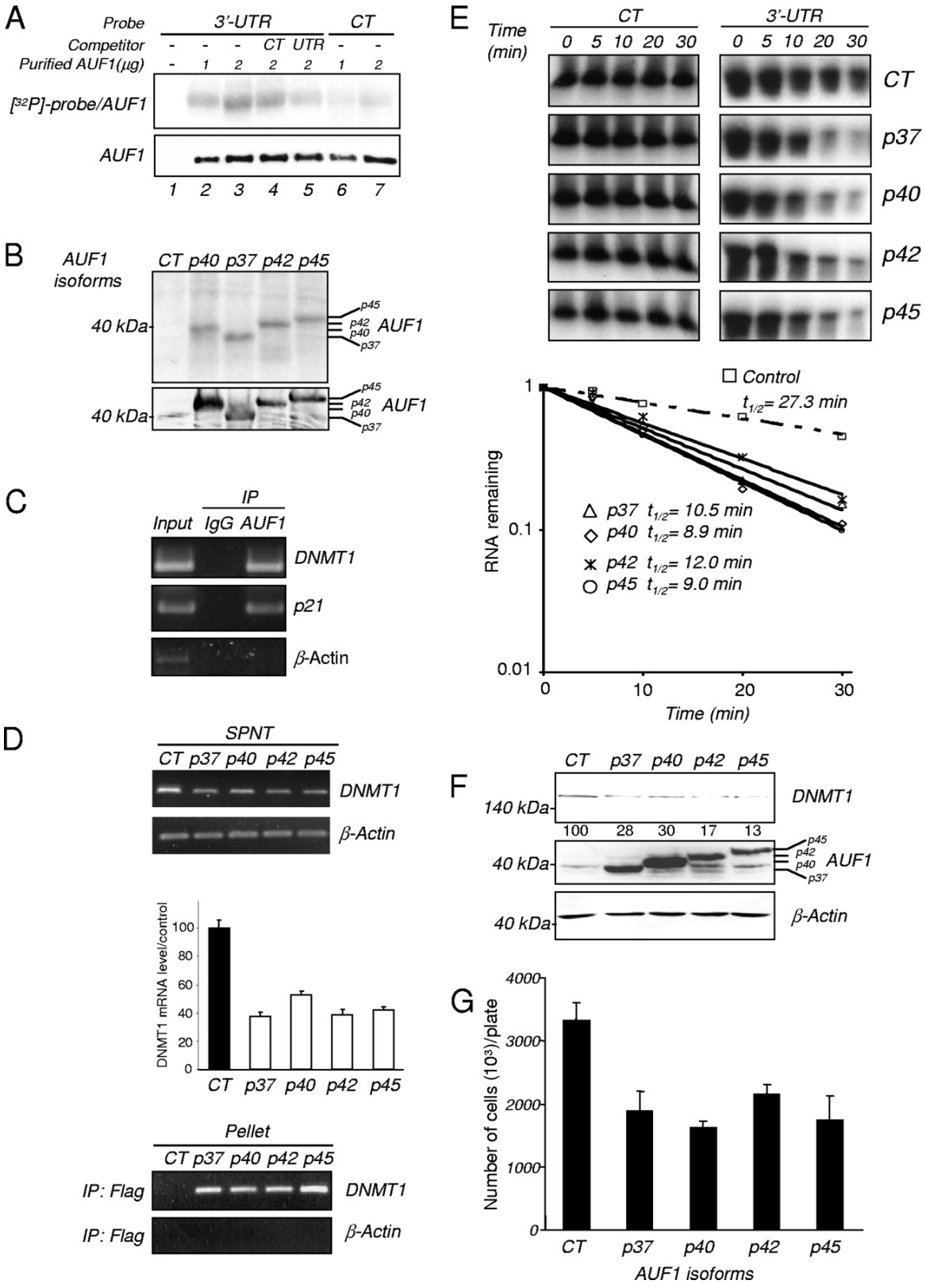

A
Probe
3'-UTR
CT
Competitor
CT UTR
Purified AUF1(μg)
[32P]-probe/AUF1
AUF1
1 2 3 4 5 6 7
B
AUF1 isoforms
CT p40 p37 p42 p45
40 kDa
p45
p42
p40
p37
AUF1
C
IP
Input IgG AUF1
DNMT1
p21
β-Actin
D
SPNT
CT p37 p40 p42 p45
DNMT1
β-Actin
DNMT1 mRNA level/control
100
80
60
40
20
0
CT p37 p40 p42 p45
Pellet
IP: Flag
DNMT1
β-Actin
E
Time (min)
CT
3'-UTR
0 5 10 20 30
CT
p37
p40
p42
p45
Control
t1/2= 27.3 min
RNA remaining
1
0.1
0.01
p37 t1/2= 10.5 min
p40 t1/2= 8.9 min
p42 t1/2= 12.0 min
p45 t1/2= 9.0 min
0 10 20 30
Time (min)
F
CT p37 p40 p42 p45
140 kDa
DNMT1
100 28 30 17 13
40 kDa
AUF1
β-Actin
G
Number of cells (10³)/plate
4000
3000
2000
1000
0
CT p37 p40 p42 p45
AUF1 isoforms

Figure 2. AUF1 binding to *DNMT1* 3'-UTR leads to a decrease in *DNMT1* levels. (A) UV cross linking assay. One (lanes 2 and 6) or 2 μg (lanes 3-5 and 7) of purified AUF1 were incubated with a [^{32}P]-labeled 3'-UTR *DNMT1* (3'-UTR) (lanes 2-5) or control RNA (CT) probe (lanes 6 and 7) and UV cross-linked. *DNMT1*-3'UTR probe alone was used as a control (lane 1). RNA-protein complexes were separated on a 7.5% SDS-PAGE gel and visualized by autoradiography (top panel). Purified AUF1 was incubated with the [^{32}P]-labeled 3'-UTR *DNMT1* probe in the presence of the 10 fold molar excess of the unlabelled control (lane 4) or 3'-UTR *DNMT1* RNA probe (lanes 5). The amounts of purified AUF1 per lane were controlled by Western blot analysis (bottom panel). **(B)** Cytoplasmic extracts of HEK-293 transfected with the different AUF1 isoforms cDNA or an empty vector (CT) were incubated in presence of a [^{32}P]-labeled 3'-UTR *DNMT1* (3'-UTR) and UV cross linked. RNA-protein complexes were resolved by SDS-PAGE and visualized by autoradiography (top panel). The overexpression of the AUF1 isoforms was verified by Western blot (bottom panel). **(C)** RNA immunoprecipitation assay. AUF1 protein was immunoprecipitated from HEK-293 extracts using anti-AUF1 antibody (AUF1). Immunoprecipitation using rabbit IgG was performed as a negative control (IgG). Immunoprecipitated RNA were extracted and subjected to RT-PCR as well as RNA present in an aliquot of the initial extracts (Input). Detection of *DNMT1* and p21 mRNA was performed by RT-PCR. β-actin amplification was used as negative control. **(D)** RNA immunoprecipitation assay. HEK-293 cells were transfected with the four different AUF1 isoforms cDNA or an empty vector (CT). Flag antibody-precipitated RNAs were extracted from the supernatants (*SPNT*) (top panel) and the pellets (bottom panel). *DNMT1* mRNAs were visualized by RT-PCR and quantified in *SPNT* samples by q-RT-PCR. β-actin amplification was used as control. The graph represents the average percentage of *DNMT1* mRNA expression in the *SPNT* samples relative to the control level. **(E)** *In vitro* degradation assay. Cytoplasmic extracts from transfected HEK-293 (CT or AUF1 cDNAs) cells were incubated with radiolabeled *DNMT1* 3'-UTR RNA transcript or control RNA probe for various lengths of time and electrophoresed. The signals were detected by autoradiography and quantified by densitometry. The half-lives were obtained by determining the time point at which 50% of the RNA had been degraded. The graph only illustrates the half lives of *DNMT1* 3'-UTR RNA probe in the presence of HEK-293 extracts. **(F)** HEK 293 cells were transfected with either the four different AUF1 isoforms or an empty vector (CT). *DNMT1* (top panel) and AUF1 (middle panel) protein levels were estimated by Western blot analysis. β-actin antibody was used as a loading control. Numbers indicate the percentage of *DNMT1* protein expression relative to the control level. **(G)** Transfected HEK-293 cells (CT or AUF1 cDNAs) were counted in the presence of trypan blue. The values presented are the average of 3 measurements from three different plates.

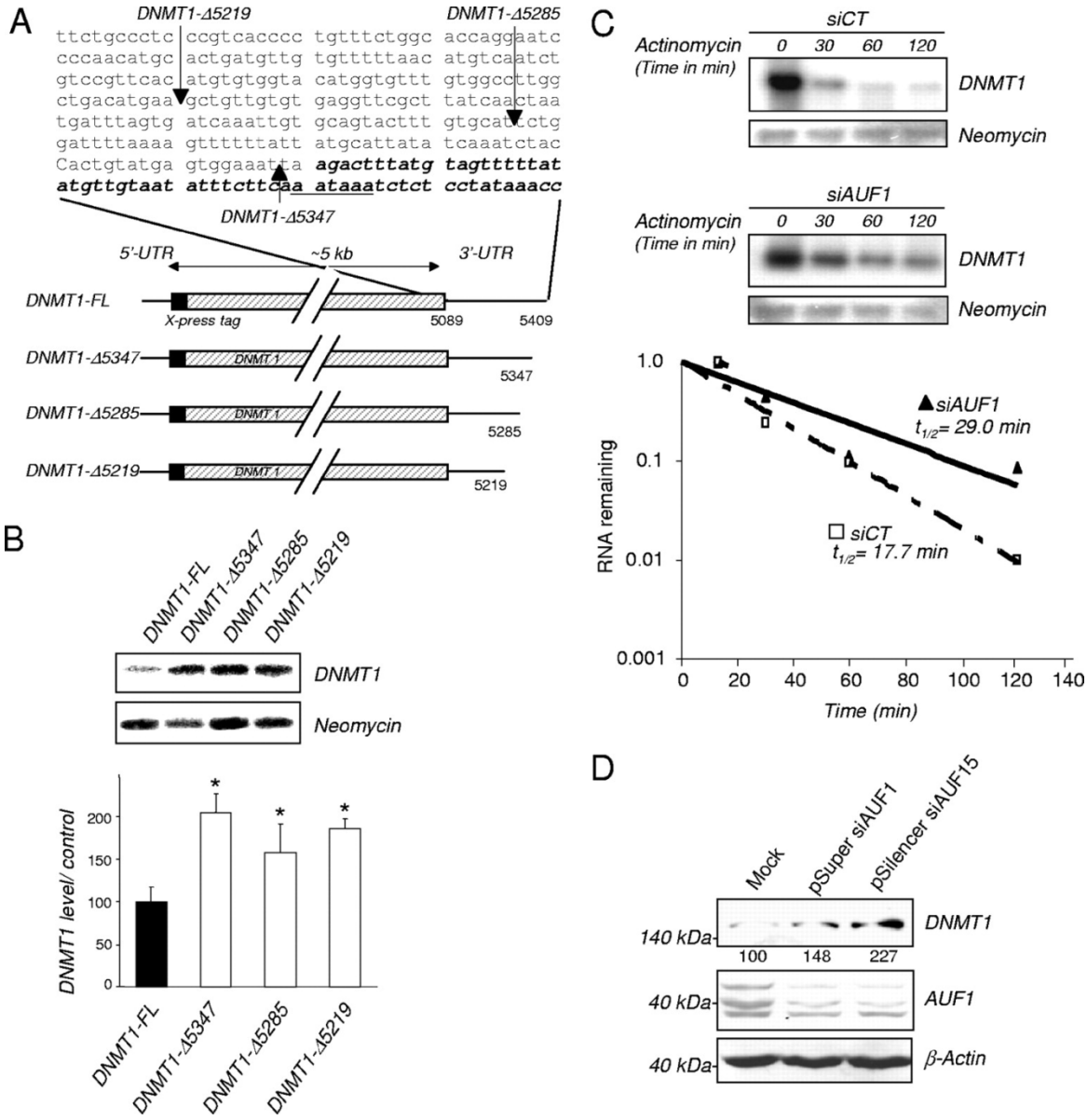
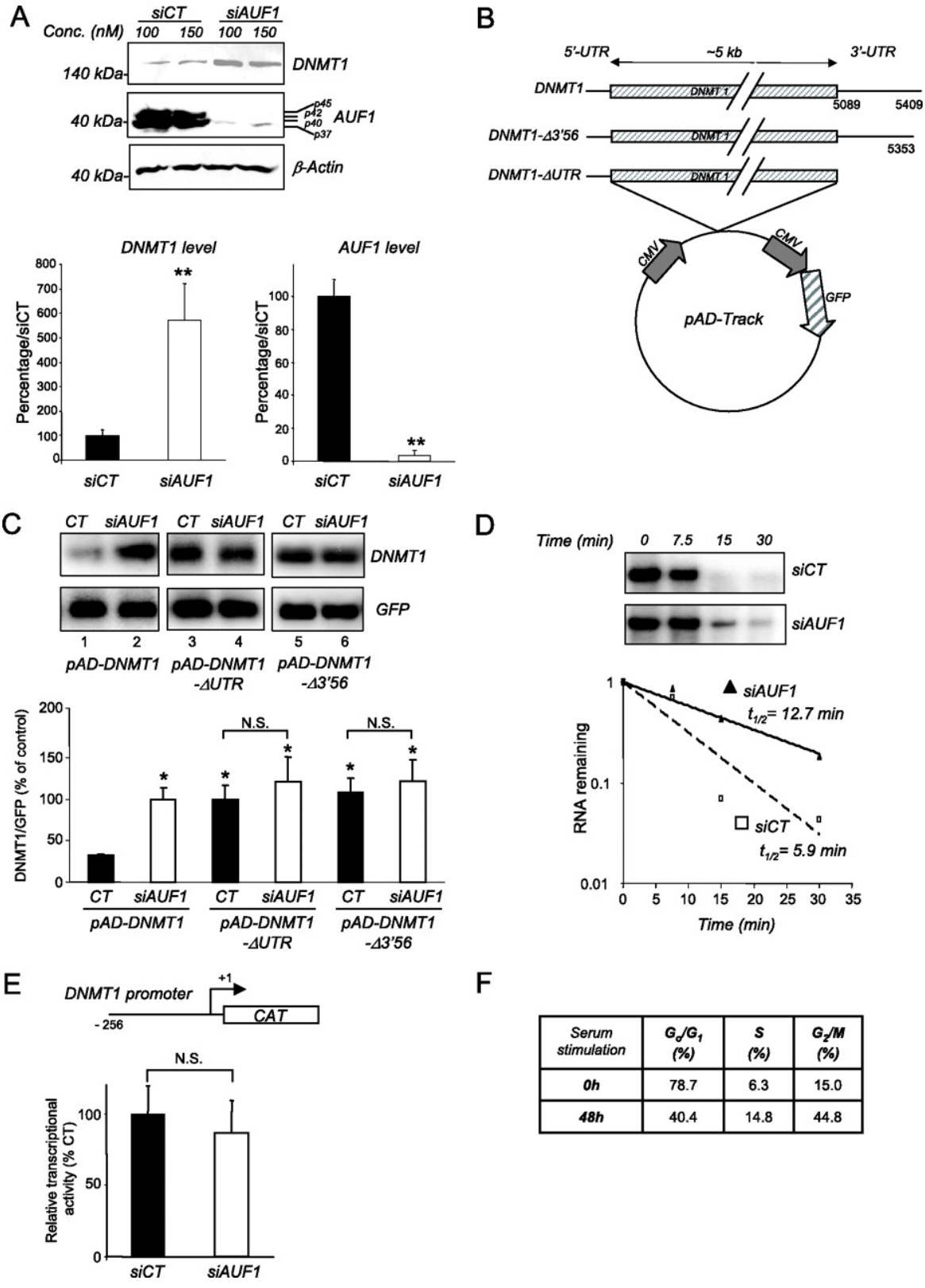


Figure 3. AUF1 depletion stabilizes *DNMT1* mRNA. (A) Schematic representation of *DNMT1* 3'-UTR deletion constructs and *DNMT1* 3'-UTR sequence (GenBank accession number NM001379.1). Boldface/italic type represents the AU rich highly conserved region of *DNMT1* 3'-UTR. **(B)** Levels of expression of the different *DNMT1* 3'-UTR deletion construct mRNAs were estimated by Northern blot using an X-press tag probe normalized by hybridization with neomycin as loading control probe. The graph indicates the percentage of normalized *DNMT1* mRNA expression of the deletion constructs relative to the normalized expression of the full length construct *DNMT1* mRNA(100%). A star indicates statistical significance as tested by a Student t test $p<0.05$. **(C)** *DNMT1* mRNA half life measurements. *DNMT1* FL construct was co-transfected into HEK-293 cells with AUF1 siRNA encoding plasmids. Cells were treated with actinomycin D for the indicated period of time. X-press tagged *DNMT1* and neomycin mRNA levels were determined by Northern blot analysis and the normalized levels of *DNMT1-FL* were calculated. The autoradiogram is representative of three different assays.

(D) HEK-293 cells were transfected with pSuper siAUF1, pSilencer siAUF15 or mock vector. Proteins were extracted and the levels of *DNMT1* and AUF1 were determined by Western blot analysis. Measurement of β-actin levels was used as a loading control. The values indicate the percentage of normalized *DNMT1* (to β-actin) relative to the control levels.



Serum stimulation	G_0/G_1 (%)	S (%)	G_2/M (%)
0h	78.7	6.3	15.0
48h	40.4	14.8	44.8

Figure 4

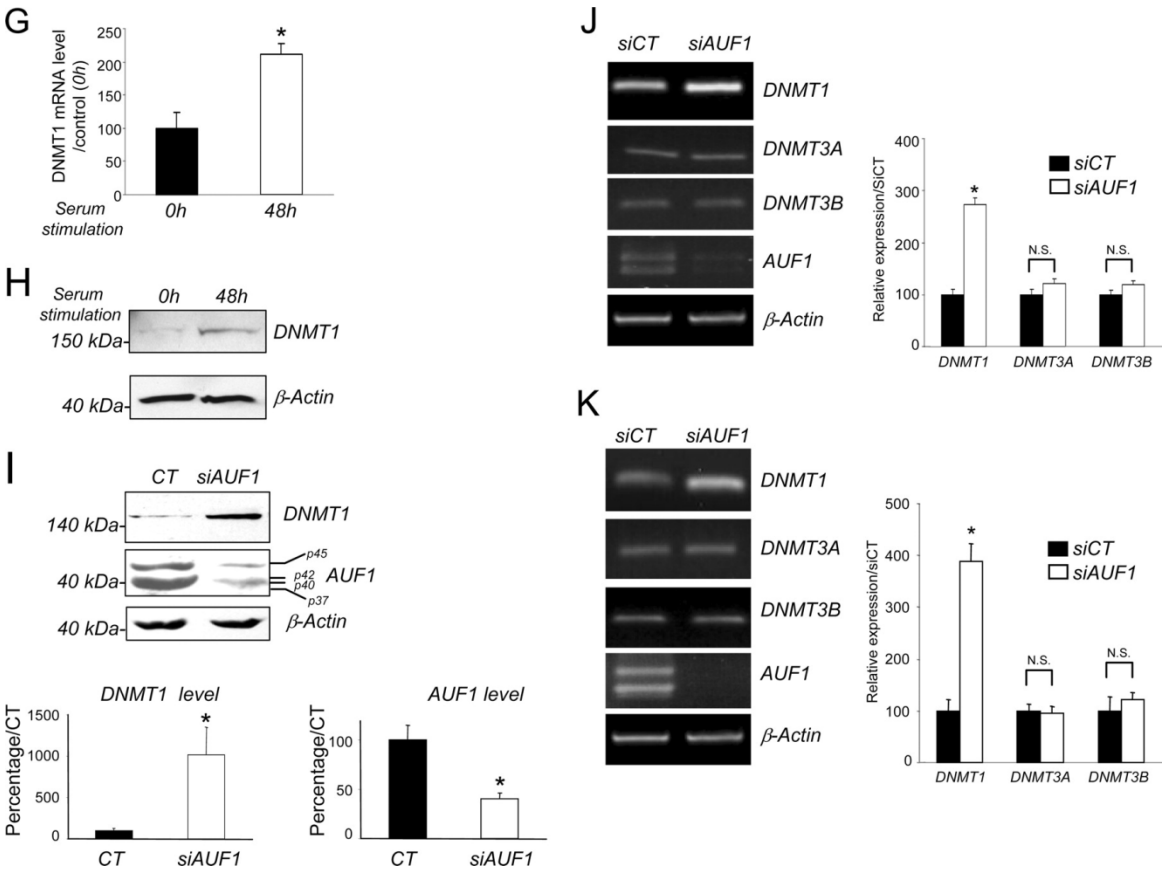


Figure 4 (continued). AUF1 depletion leads to an increased expression and increased stability of *DNMT1* mRNA but not other DNMTs. (A) T24 cells were transfected with AUF1 siRNA (siAUF1) or control siRNA (siCT). Levels of *DNMT1* and AUF1 proteins were determined by Western blot analysis. The chart represent an average of 3 independent determinations +/- SEM. Statistical significance was tested by a Student t test $p<0.05$. **(B)** Schematic representation of the adenoviral constructs pAd-*DNMT1*, pAd-*DNMT1-Δ3'56* and pAD-*DNMT1*-ΔUTR. **(C)** AUF1 depleted or wild type T24 cells were infected with the indicated adenoviral constructs. *DNMT1* and GFP mRNA levels were measured by Northern blot analysis. *DNMT1* mRNA levels were normalized to GFP mRNA as a control for infection efficiency. The graph represents an average of three different adenoviral infections relative to the control *pAD-DNMT1 siAUF1* (100%). Statistical significance was tested by a Student t test $p<0.05$. **(D)** *In vitro* degradation assay. Radiolabeled *DNMT1* 3'-UTR RNA transcript was incubated with cytoplasmic extracts from AUF1 siRNA transfected T24 cells (siAUF1) or control siRNA transfected cells (siCT) for indicated lengths of time. The signals were detected by autoradiography and quantified by phosphorimaging densitometry. **(E)** Analysis of *DNMT1* promoter activity. pMet-P1ΔHX-CAT construct was transfected into AUF1 depleted T24 cells (siAUF1) or si RNA control cells (siCT). CAT activity was measured as described in Materials and Methods. Each value represents the mean of three independent transfections. A Student t test confirmed no statistical difference between the treatment groups. **(F)** Percentages of human fibroblasts in the different stages of the cell cycle after serum starvation (14 days) and serum released (48h) were determined by flow

cytometry. **(G)** Human fibroblasts were serum starved (14 days) and released to enter into the cell cycle for the indicated period of time. *DNMT1* mRNA level was determined by q-RT-PCR. *β*-Actin was used as control. Statistical significance was tested by a Student t test. The difference for *DNMT1* was significant $p<0.05$. **(H)** *DNMT1* protein level in serum starved or serum released fibroblasts was determined by Western blot. *β*-Actin was used as loading control. **(I)** Human fibroblasts were transfected with AUF1 siRNA (siAUF1) or control siRNA (siCT). Levels of *DNMT1* and AUF1 were determined by Western blot analysis. **(J / K)** Measurement of *DNMT1*, *3A* and *3B* mRNA levels in AUF1 depleted T24 cells (J) and human fibroblasts (K) following AUF1 depletion by reverse transcription and q-RT-PCR analysis. Graphs represent the average of three different amplifications. Statistical significance was tested by a Student t test. The difference for *DNMT1* was significant $p<0.05$. No statistical significance was observed for the other *DNMT*s $p>0.5$.

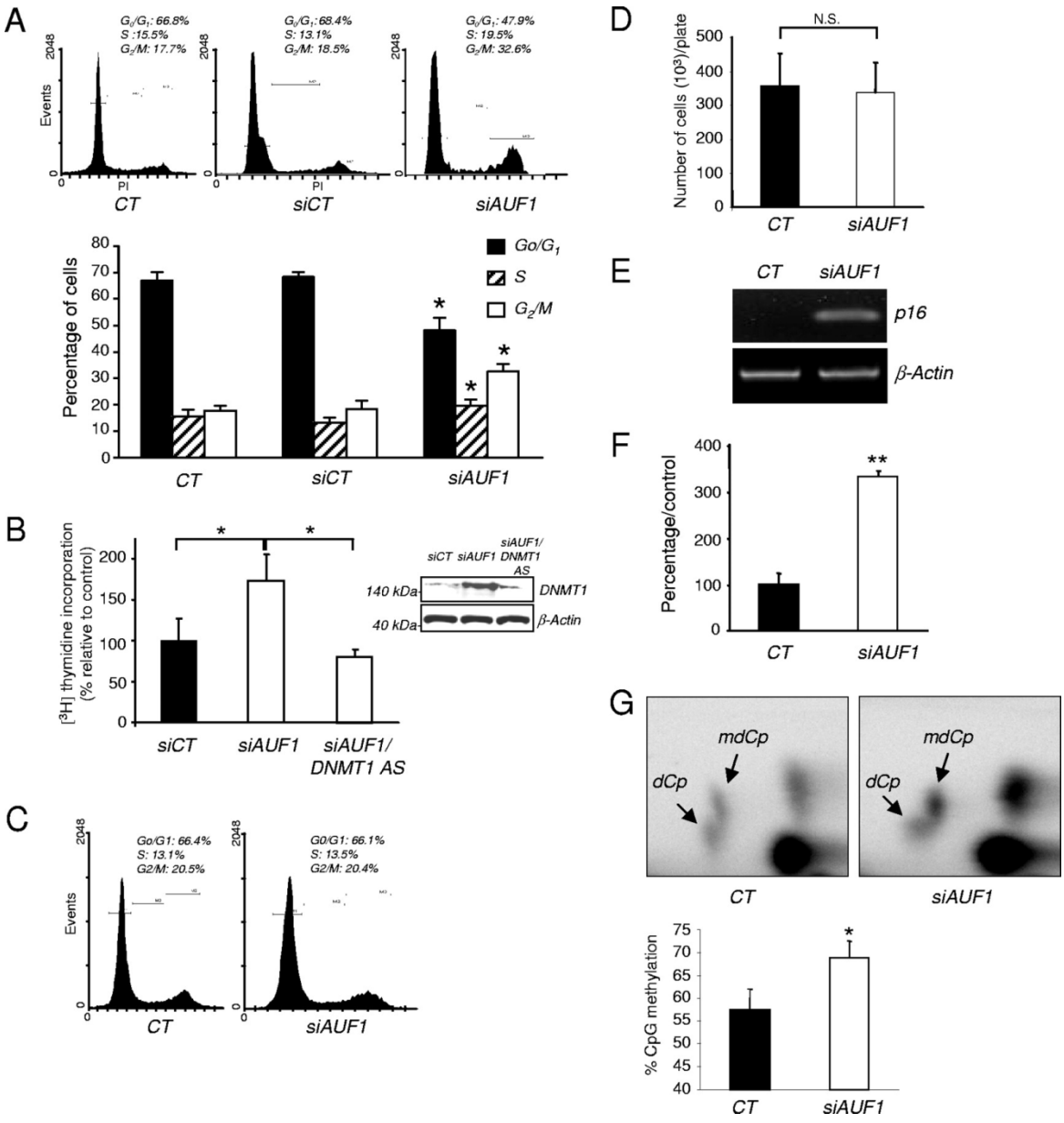


Figure 5. Biological consequences of AUF1 depletion in T24 cells and human fibroblasts.

(A) The fraction of untransfected (CT) cells or transfected with a control siRNA (siCT) or AUF1 siRNA (siAUF1) cells found at the different stages of the cell cycle was determined by flow cytometry. The graph represents the average of three different experiments. A star indicates statistical significance as tested by a Student t test $p<0.05$. **(B)** T24 cells were transfected with CT siRNA, AUF1 siRNA or a combination of AUF1 siRNA/*DNMT1* antisense oligonucleotide. [^{3}H] thymidine uptake was measured as described in Materials and Methods. The results are an average of three independent experiments. A star indicates statistical significance as tested by a Student t test $p<0.05$. The level of *DNMT1* after the different treatments was measured by Western blot analysis. **(C)** Percentage of AUF1 siRNA (siAUF1) or CT siRNA (siCT) transfected human fibroblasts at the different stages of the

cell cycle was determined by flow cytometry. **(D)** AUF1 siRNA (siAUF1) or CT siRNA (siCT) transfected human fibroblasts were counted 3 days post-transfection. The number of cells represents the average of three measurements from three different plates. A Student t test showed no statistically significant difference between the treatment groups $p>0.5$. **(E)** *p16* mRNA expression in AUF1 siRNA (siAUF1) or CT siRNA (siCT) transfected human fibroblasts was evaluated by semi-quantitative RT-PCR. This amplification is representative of three different experiments. **(F)** DNA methyltransferase assay. DNA methyltransferase activity in AUF1 siRNA (siAUF1) or CT siRNA (siCT) transfected human fibroblasts was measured as described in Experimental procedures. The graph represents the average of three different estimations. A star indicates statistical significance as tested by a Student t test $p<0.05$. **(G)** The level of CpG methylation in AUF1 siRNA (siAUF1) or CT siRNA (siCT) transfected human fibroblasts was measured by nearest-neighbor analysis. The graph represents the average of three different determinations. A star indicates statistical significance as tested by a Student t test $p<0.05$.

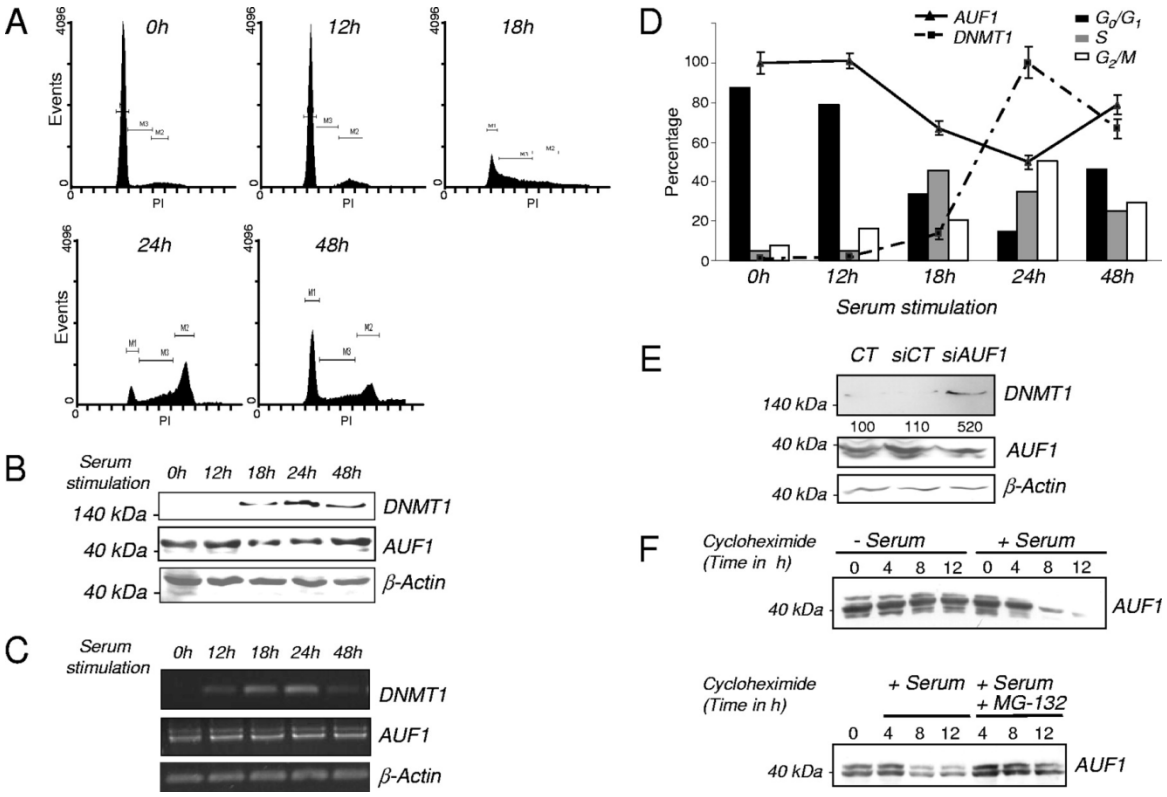


Figure 6. AUF1 expression is inversely correlated with *DNMT1* level during the cell cycle and controlled by the proteasome. (A) Percentage of T24 cells in the different stages of the cell cycle was determined by flow cytometry. **(B)** T24 cells were serum starved and released to enter the cell cycle for the indicated lengths of time. *DNMT1* and AUF1 protein levels were measured by Western blot analysis. β-Actin was used as a loading control. **(C)** T24 cells were serum starved and released to enter the cell cycle for the indicated period of time. *DNMT1* and AUF1 mRNA levels were visualized by semi-quantitative RT-PCR. **(D)** Graphical representation of *DNMT1*, AUF1 protein levels and the percentage of T24 cells at the different stages of the cell cycle at different time points after serum release. *DNMT1* mRNA level was measured by q-RT-PCR. **(E)** Serum starved T24 cells (CT) were transfected with CT siRNA (siCT) or AUF1 siRNA (siAUF1). *DNMT1* and AUF1 protein levels were analyzed by Western blot. Numbers indicate the levels of *DNMT1* protein as percentage over control. **(F)** *In vivo* degradation assay of AUF1 protein at G_0/G_1 and S phases of the cell cycle. Serum starved or 18h serum released T24 cells were treated with cycloheximide for the indicated period of time. Levels of AUF1 were then determined by Western blot (upper panel). 18h-serum released cells treated with cycloheximide were also treated or with MG-132 a proteasome inhibitor (bottom panel). AUF1 levels were determined by Western blot.

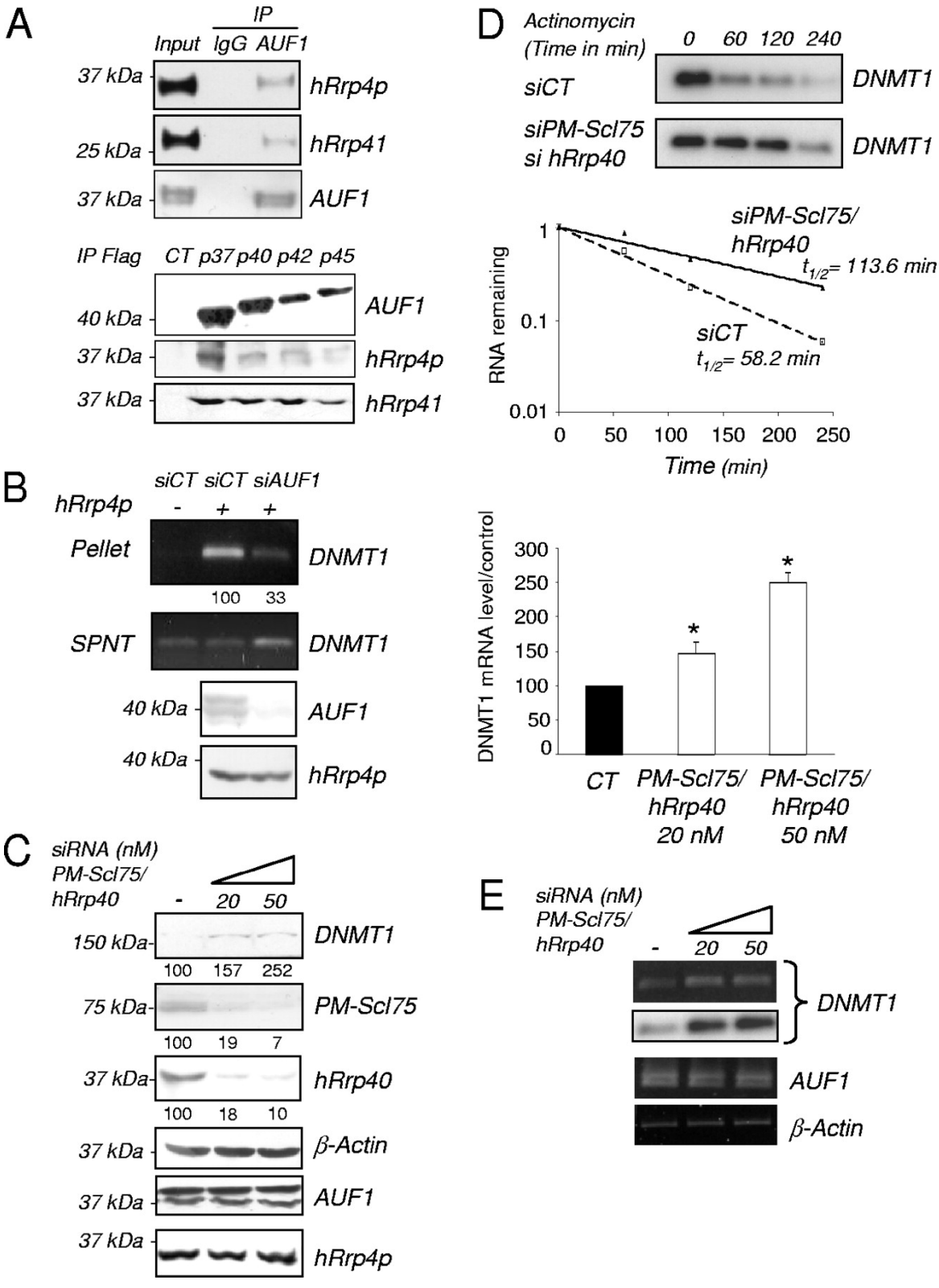

A
IP
Input IgG AUF1
37 kDa
hRrp4p
25 kDa
hRrp41
37 kDa
AUF1
IP Flag CT p37 p40 p42 p45
40 kDa
AUF1
37 kDa
hRrp4p
37 kDa
hRrp41
B
siCT siCT siAUF1
hRrp4p - + +
Pellet
DNMT1
100 33
SPNT
DNMT1
40 kDa
AUF1
40 kDa
hRrp4p
C
siRNA (nM)
PM-Scl75/
hRrp40
- 20 50
150 kDa
DNMT1
100 157 252
75 kDa
PM-Scl75
100 19 7
37 kDa
hRrp40
100 18 10
37 kDa
β-Actin
37 kDa
AUF1
37 kDa
hRrp4p
D
Actinomycin
(Time in min)
0 60 120 240
siCT
DNMT1
siPM-Scl75
si hRrp40
DNMT1
siPM-Scl75/
hRrp40
t1/2= 113.6 min
siCT
t1/2= 58.2 min
RNA remaining
1
0.1
0.01
0 50 100 150 200 250
Time (min)
DNMT1 mRNA level/control
300
250
200
150
100
50
0
*
*
CT
PM-Scl75/
hRrp40
20 nM
PM-Scl75/
hRrp40
50 nM
E
siRNA (nM)
PM-Scl75/
hRrp40
- 20 50
DNMT1
AUF1
β-Actin

Figure 7

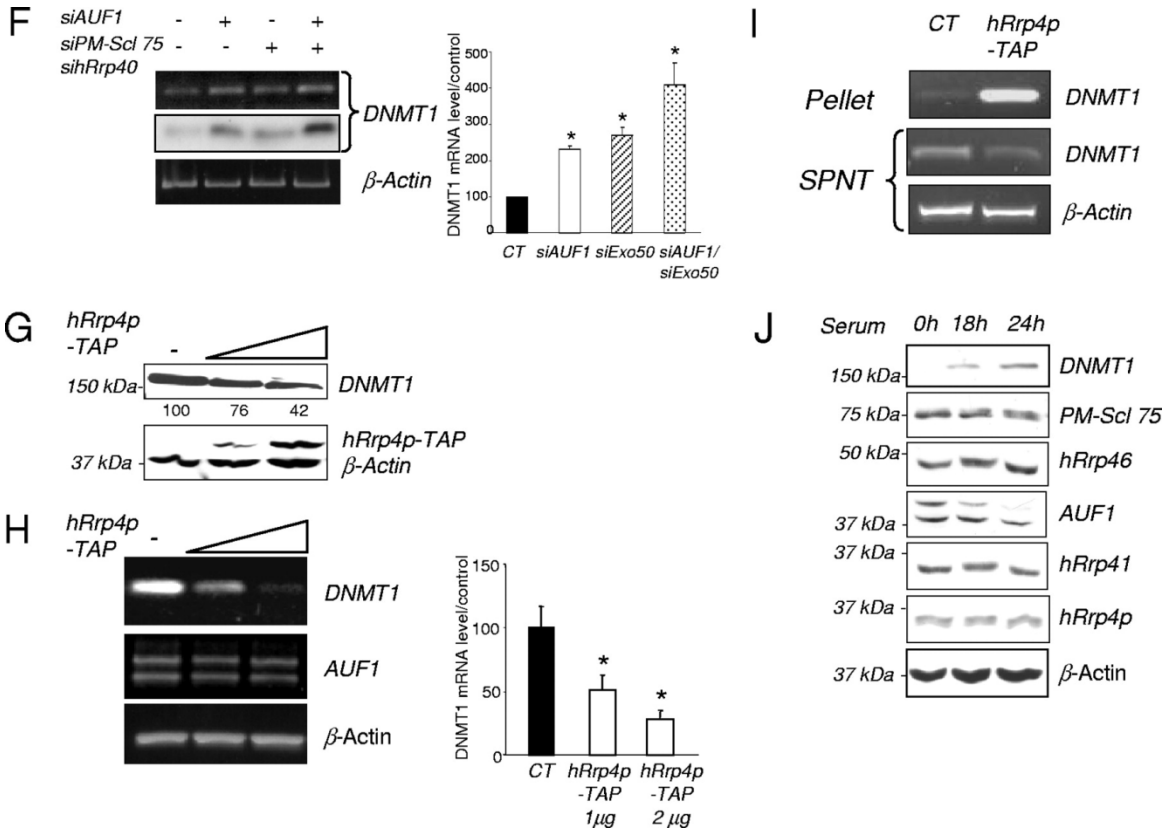


Figure 7 (continued). The exosome participates in the AUF1 triggered-degradation of *DNMT1* mRNA. (A) AUF1 proteins were immunoprecipitated from HEK-293 whole cell extracts. The presence of hRrp4p and hRrp41 in the immunoprecipitates was determined by Western blot analysis (top panel). A pre-immune antibody (IgG) was used as control for the immunoprecipitation. HEK-293 cells were transfected with the different tagged-AUF1 cDNA encoding vectors. AUF1 isoforms were immunoprecipitated using flag antibody (bottom panel). The presence of hRrp4p and hRrp41 in the immunocomplexes was determined by Western blot. **(B)** hRrp4p was immunoprecipitated from T24 cells treated with either control (SiCT) or AUF1 SiRNA (SiAUF1). Precipitated RNAs were extracted from the supernatants (*SPNT*) and the pellets. *DNMT1* mRNA was detected by RT-PCR and quantified by q-RT-PCR. The numbers indicate the level of *DNMT1* mRNA expression relative to the level measured in *siCT* (%) The levels of AUF1 and hRrp4p following AUF1 siRNA treatment were determined by a Western blot. **(C)** T24 cells were simultaneously transfected with PM-Scl 75/hRrp40 siRNAs at the indicated concentrations. Levels of the indicated proteins were determined by Western blot. Numbers indicate the level in percentage of *DNMT1,* PM-Scl75and hRrp40 protein expression relative to control. **(D)** RNA degradation assay. Control or PM-Scl 75/hRrp40 siRNAs transfected T24 cells were treated with actinomycin D for the indicated period of time. *DNMT1* mRNA levels were visualized by RT-PCR followed by specific oligonucleotide hybridization and quantified using q-RT-PCR. The graph represents the quantification from three different experiments. **(E)** *DNMT1* and AUF1 mRNA levels corresponding to the experiment in (C) were visualized by RT-PCR analysis and quantified by real time PCR. The graph represents the level in percentage of *DNMT1* mRNA relative

to the control level. A star indicates statistical significance as tested by a Student t test $p<0.05$. **(F)** T24 cells were separately and simultaneously transfected with PM-Scl 75/hRrp40 or AUF1 siRNAs. The level of *DNMT1* mRNA was visualized by RT-PCR (upper panel) followed by specific oligonucleotide hybridization (middle panel) and quantified using q-RT-PCR. The graph represents the quantification by real time PCR of *DNMT1* mRNA levels relative to the control. A star indicates statistical significance as tested by a Student t test $p<0.05$. **(G)** HEK-293 cells were transiently transfected with hRr4p-TAP vector at different concentrations. *DNMT1* and hRrp4p protein levels were determined by Western blot. **(H)** *DNMT1* and AUF1 mRNA levels corresponding to the experiment in (G) were visualized by RT-PCR analysis. The graph represents the quantification of *DNMT1* mRNA level by q-RT-PCR relative to control (100%). A star indicates statistical significance as tested by a Student t test $p<0.05$. **(I)** Pull-down of hRrp4p-TAP protein from hRrp4p transfected HEK-293 cells. Precipitated RNAs were extracted from the supernatants (*SPNT*) and the pellets. Detection of *DNMT1* mRNA was performed by RT-PCR. *β*-actin amplification was used as control. **(J)** T24 cells were serum-starved for 6 days (0h) and supplemented with serum for 18h and 24h. Levels of the indicated protein were determined by Western blot.

	m/z submitted	*MH+ matched*	*dPPM*	*start*	*end*	*Peptide Sequence*
1.	913.44	913.51	-0.006	118	125	GFGFVLFK
2.	1014.44	1014.50	-0.006	91	98	DLKDYFSK
3.	1156.57	1156.64	-0.007	116	125	SRGFGFVLFK
4.	1166.49	1166.56	-0.007	99	108	FGEVVDCTLK
5.	1393.56	1393.64	-0.008	126	137	ESESVDKVMDQK
6.	1354.59	1354.66	-0.007	79	89	MFIGGLSWDTTK
7.	1482.67	1482.75	-0.009	78	90	MFIGGLSWDTTKK
8.	1487.62	1487.75	-0.015	163	176	IFVGGLSPDTPEEK

Table 1 Peptides sequenced by MS/MS from the excised gel (Supplementary figure S2)

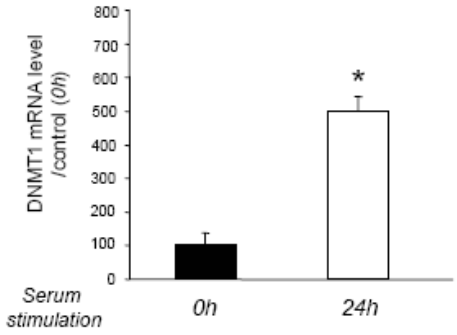
Supplementary figures

S1

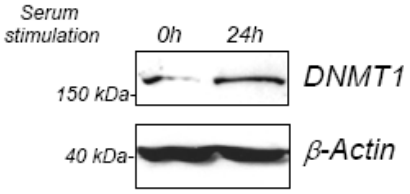
A

Serum stimulation	G_0/G_1 (%)	S (%)	G_2/M (%)
0h	65.3	14.6	20.7
24h	45.0	26.1	28.9

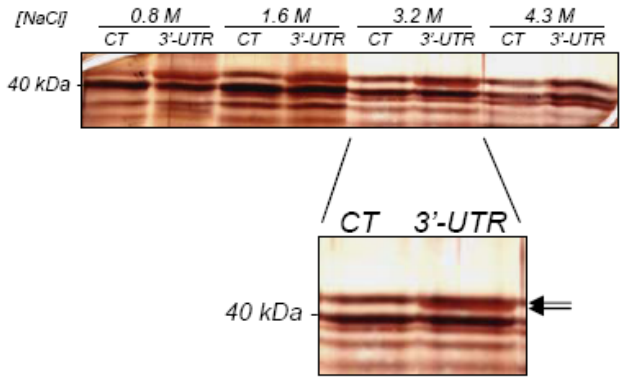
B



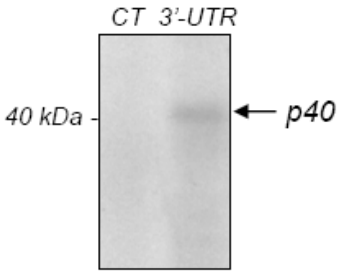
C



S2

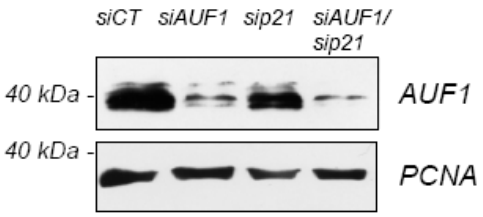


S3

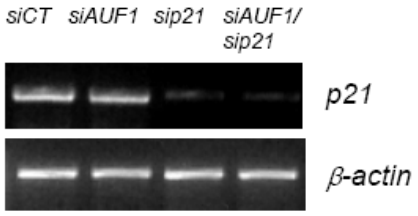


S4

A



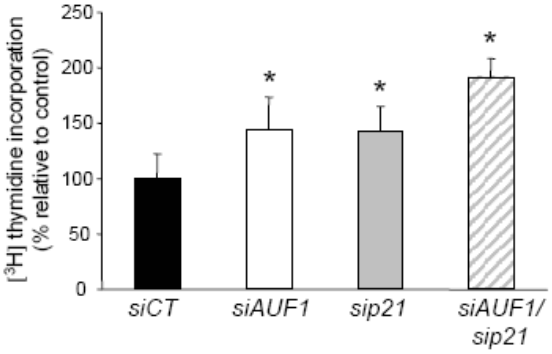
B



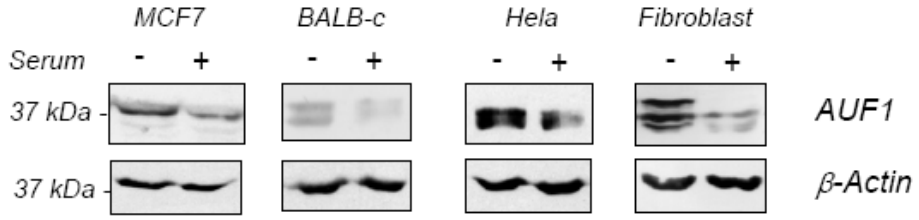
C

FACS Analysis	G_0/G_1 (%)	S (%)	G_2/M (%)
siCT	58.7	23.8	17.5
siAUF1	48.6	29.5	21.9
sip21	50.9	26.8	22.3
siAUF1/sip21	43.6	32.5	23.9

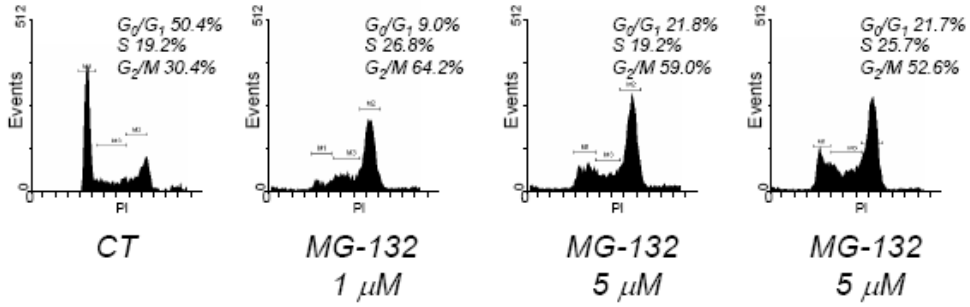
D



S5


MCF7
BALB-c
Hela
Fibroblast
Serum
- + - + - + - +
37 kDa
37 kDa
AUF1
β-Actin

S6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S 19.2%
G2/M 30.4%
G0/G1 9.0%
S 26.8%
G2/M 64.2%
G0/G1 21.8%
S 19.2%
G2/M 59.0%
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S 25.7%
G2/M 52.6%
CT
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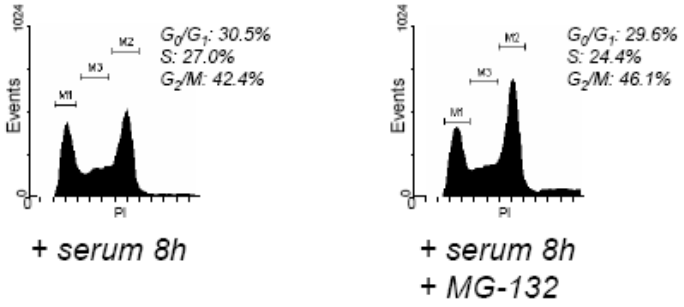
MG-132 5 μM
MG-132 5 μM

S7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PI
G0/G1: 30.5%
S: 27.0%
G2/M: 42.4%
G0/G1: 29.6%
S: 24.4%
G2/M: 46.1%
+ serum 8h
+ serum 8h
+ MG-132

Supplementary figures

(S1) (A) Percentages of Hela cells in the different stages of the cell cycle after serum starvation and serum released (24h) were determined by flow cytometry. **(B)** T24 cells were serum starved and released to enter the cell cycle for the indicated period of time. *DNMT1* mRNA level was determined by reverse transcription followed by q-RT-PCR. β-Actin was used as control. Statistical significance was tested by a Student t test. The difference for *DNMT1* was significant $p<0.05$. **(C)** *DNMT1* protein level in serum starved or serum released-T24 cells was determined by Western blot. β-Actin was used as loading control.

(S2) Protein fractions eluted at different NaCl concentrations from the RNA affinity chromatography columns were fractionated on a 15% SDS PAGE gel and silver stained. The 3.2 M fractions lanes were enlarged in order to visualize a ~40 kDa specific protein (bottom arrow) in the 3'-UTR eluted fraction. The upper arrow indicates a non specific protein.

(S3) Protein fractions eluted at 3.2 M NaCl were incubated with a [^{32}P]-labeled 3'-UTR RNA and UV cross linked. RNA-proteins complexes were separated by SDS-PAGE and visualized by autoradiography. The arrow indicates a 3'-UTR specific complex with an apparent size of ~40 kDa.

(S4) (A) AUF1 protein expression in T24 cells upon AUF1 and *p21* siRNA transfection alone or in combination was measured by Western blot analysis. PCNA was used as loading control. **(B)** *p21* mRNA expression upon AUF1 and *p21* siRNA transfection alone or in combination in T24 cells was estimated by semi quantitative RT-PCR. β-actin was used as loading control. **(C)** The percentages of T24 cells in the different phases of the cell cycle after AUF1 and *p21* siRNA transfection alone or in combination were evaluated by propidium iodide incorporation and FACS analysis. (**D)** T24 cells were transfected with AUF1 and *p21* siRNAs alone or in combination. After 24h, [^{3}H]-thymidine incorporation was measured as described in Materials and Methods. A star indicates statistical significance as tested by a Student t test $p<0.05$.

(S5) AUF1 protein levels in different cell lines serum-deprived (-) or supplemented with serum (+) for 18 to 48 hours: human breast cancer cells, MCF-7; murine embryonic cells, BALB/c, human cervical epithelial carcinoma cells, Hela and human fibroblast were determined by Western blot analysis.

(S6) Percentage of T24 cells in the different stages of the cell cycle after treatment with increasing concentrations of MG-132 for 24 hours was determined by flow cytometry.

(S7) Percentage of 18h-serum released T24 cells in the different stages of the cell cycle after treatment with cycloheximide and MG-132 for 8 hours was determined by flow cytometry.

DISCUSSION

AUF1 was first characterized based on its property of binding AU rich elements and was shown to affect mRNA stability in a number of genes involved in growth control. The observation that *DNMT1* is regulated by a factor, AUF1, which also regulates a number of other growth control genes such as *p16* (Wang, Martindale et al. 2005), *p21* (Lal, Mazan-Mamczarz et al. 2004), provides a mechanism of coordination of *DNMT1* levels with other cell cycle events. Since *DNMT1* has a critical function in copying the epigenetic information during cell division, it is crucial that its expression is tightly coordinated with the state of cell division.

The manner by which AUF1 maintains a dynamic balance of growth control by coordinating the expression of genes with opposite functions is illustrated by the AUF1 siRNA knock down experiments. AUF1 knockdown in non-transformed human fibroblasts results in an induction of *DNMT1* mRNA and protein levels (Figure 4K and 4I), which has been shown to have a growth promoting effect in other human cell lines. However, the AUF1 knock down also results in an induction of the tumor suppressor *p16* gene which may counter the growth promoting effects of *DNMT1* (Figure 5A and E). Therefore, by coordinately destabilizing the expression of both tumor suppressor genes (*p16* (Wang, Martindale et al. 2005), *p21* (Lal, Mazan-Mamczarz et al. 2004)) and growth promoting genes such as *DNMT1*, AUF1 may maintain balanced growth. In other words, AUF1 effects on the cell cycle may result from a simultaneous destabilization of target mRNA encoding proteins with important function in the cell cycle regulation. It is therefore tempting to speculate that the ability of *DNMT1* to transform cells requires the elimination of the counteracting action of tumor suppressor genes by regional hypermethylation, which was shown to be a late consequence of *DNMT1* overexpression (Vertino, Yen et al. 1996) or by mutation.

In this study, we demonstrated for the first time that AUF1 expression itself is regulated during the cell cycle in T24 (Figure 6B) and other cell lines (Supplementary figure

S5) at a post translational level. An *in vivo* degradation assay revealed greater degradation of AUF1 in S phase cells which is dependent on proteasome activity. This observation complements previous findings for the role of the proteasome machinery in AUF1 degradation (Laroia, Cuesta et al. 1999; Laroia, Sarkar et al. 2002; Laroia and Schneider 2002). However, although all four AUF1 isoforms are regulated during the cell cycle, an interesting question raised by our data is why all AUF1 controlled-(ARE)-mRNAs are not inversely regulated during the cell cycle. An explanation might be the dual role of AUF1, in either stabilizing or destabilizing mRNAs depending on the (ARE)-mRNA sequence context (Dean, Sully et al. 2004). Another possible explanation is that other proteins are involved in regulating AUF1 to particular AU containing mRNAs. One recent example is the Pin1 protein, a *cis-trans* isomerase which was recently shown to regulate the association of AUF1 isoforms with GM-CSF mRNA, accelerating or slowing mRNA decay (Shen, Esnault et al. 2005). Moreover, we do not exclude the possibility that other ARE-binding proteins could participate in *DNMT1* mRNA regulation such as HuR.

The exosome functions in several processes involving the 3'-to-5' processing or degradation of RNA. Among them are the maturation of 5.8 S rRNA, the processing of many small nuclear and nucleolar RNAs and the turnover of different type of mRNAs, especially ARE-mRNA (Chen, Gherzi et al. 2001); (Mukherjee, Gao et al. 2002). We show that a similar mechanism is involved in *DNMT1* regulation by AUF1. Since the levels of different exosome elements do not vary during the cell cycle, we suggest that modulation of AUF1 expression is mostly responsible for the cell cycle specific targeting of *DNMT1* mRNA to the exosome for degradation. The regulation of *DNMT1* mRNA stability described here is to our knowledge, the first example of a cell cycle dependent regulation of an mRNA involving the exosome machinery.

Finally, we demonstrate that depletion of AUF1 protein in non transformed human fibroblasts leads to increased levels of *DNMT1* protein and global genomic methylation (Figure 4I and 5G). *DNMT1* protein overexpression and tumor suppressor gene hypermethylation characterize a number of different tumors (Herman 1999; Baylin and

Herman 2000) and levels are believed to contribute directly to tumorigenicity. Changes in AUF1 protein expression have been observed in tumor progression in neoplastic lung tissue (Blaxall, Dwyer-Nield et al. 2000), which could have potential implications on *DNMT1* mRNA regulation. Moreover, Morello's group published that **"**AUF1 p37 transgene induces tumors in mice" (Gouble, Grazide et al. 2002). These interesting findings suggest that in certain cell types, the AUF1 p37 isoform negatively controls more tumor suppressor gene mRNAs than growth promoting gene mRNAs. The overproduction of the AUF1 p37 in these mice might create an increased destabilization of these cell cycle repressor genes mRNA resulting in an aberrant cell growth.

Although many different mechanisms are believed to contribute to the enhanced *DNMT1* expression, we show for the first time that alteration of a post-transcriptional regulation and the exosome function could play an important role in the maintenance of the genome DNA methylation level and therefore in tumorigenesis.

ACKNOWLEGDMENTS

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Connecting Text

In the previous chapter we have established that the protein involved in destabilizing DNMT1 mRNA is AUF1. We demonstrated that AUF1 binds the DNMT1 3' UTR, decreasing the half-life of its mRNA. This occurs through recruitment of the exosome. Degradation of DNMT1 mRNA was mediated by the exosome through recruitment by AUF1. Upon depletion of AUF1 DNMT1 mRNA levels stabilized and DNMT1 protein increased, which lead to an increase in genomic methylation levels. We determined that cell cycle dependent change, in DNMT1 mRNA levels coincided with inverse changes in AUF1 protein levels.

To determine what the impact would be on cellular function if AUF1 regulation over DNMT1 mRNA were disrupted, we next examined how AUF1 is regulated and what the consequences of this disregulation would be on DNMT1 and the epigenome. The AUF1 protein has previously been shown to be regulated at the post-translational level through the function of heat shock proteins(Laroia, Cuesta et al. 1999) and degradation by the ubiquitin-proteasome pathway (Laroia, Sarkar et al. 2002). As AUF1 protein levels were observed to decrease as the cell cycle entered into S-phase, it is possible that the regulation of AUF1 may be tied into the regulatory pathways at that point in the cell cycle. Furthermore the observed increase in genomic methylation levels due to AUF1 inactivation implies potential methylation of specific genes. We next examined the mechanism through which AUF1 protein is regulated and the effects on gene methylation when this mechanism is disrupted.

Chapter 5

Gene specific methylation is defined by Rb regulation over AUF1 and DNMT1.

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DNA methylation serves to coordinate gene regulation of the entire mammalian genome. The main enzyme responsible for maintaining methylation patterns is DNA methyltransferase 1 (DNMT1). DNMT1 activity is required in S-phase for methylation of nascent DNA strands but is not required in other phases of the cell cycle. Previously we have shown that the protein AUF1 binds DNMT1 mRNA and destabilizes it. Here we show that the AUF1 protein is regulated by the cell cycle protein Rb in a proteosome-dependent manner. Elimination of Rb leads to a decrease in AUF1 protein levels and a consequent increase in DNMT1 levels. This increase in DNMT1 is coupled with genomic hypermethylation and increased association with the histone methyltransferase EZH2. Known EZH2 binding sites, including tumour suppressor p16/INK4a were observed to show increased methylation which was both DNMT1 and EZH2 dependent. Promoters that were methylated by DNMT1 and EZH2 were observed to have decreased in expression. This study demonstrates a link between the cell cycle and DNMT1 regulation. When this regulation is impaired, site-specific methylation occurs, mimicking similar events in cancer.

Introduction

DNA methylation patterns are a fundamental constituent of the epigenome, which regulates gene expression. DNA methylation occurs at cytosine bases residing next to guanosines (Gruenbaum, Stein et al. 1981). DNA methylation acts to silence gene expression by either blocking binding of *trans*-acting factors (Comb and Goodman 1990), or indirectly through binding of methylated DNA binding proteins which attract repressor complexes to the gene resulting in chromatin reconfiguration (Nan, Cross et al. 1998). However, not all methylated CGs necessarily signal gene silencing. For example in plants, methylation of the body of moderately transcribed genes correlates with gene activity (Zilberman, Gehring et al. 2007). DNA methylation patterns are laid down by a family of enzymes known as the DNA methyltransferases (DNMTs). It was believed that the maintenance DNMT1 was mainly responsible for automatically copying DNA methylation patterns during cell division while *de novo* methyltransferases DNMT3A and DNMT3B were responsible for introducing specific new methylation patterns during early development (Razin and Riggs 1980). Recent experiments suggest that the difference between maintenance and *de novo* methylation might be blurred (Rhee, Jair et al. 2000; Liang, Chan et al. 2002; Rhee, Bachman et al. 2002; Ting, Jair et al. 2004; Ting, Jair et al. 2004).

DNA methylation patterns are altered in tumorigenesis, where an overall alteration in genomic methylation is observed, combined with regional hypermethylation of gene promoters (Baylin, Esteller et al. 2001). Promoter hypermethylation has been shown to block normal growth control mechanisms, specifically the tumor suppressors *p16 (Merlo, Herman et al. 1995; Suzuki, Yamada et al. 2006)*, repair enzymes MLH1 and MSH2 (Murata, Khattar et al. 2002).The silencing of these genes has been shown to contribute to the transformed phenotype. Furthermore,

alterations in levels of DNMT1 have been observed in human cancers (Robertson, Uzvolgyi et al. 1999; Cervoni and Szyf 2001). In fact, overexpression of DNMT1 has been observed to induce cellular transformation of mouse fibroblasts (Wu, Issa et al. 1993; Detich, Ramchandani et al. 2001), while inhibition of DNMT1 serves to inhibit cancer growth (Laird, Jackson-Grusby et al. 1995; MacLeod and Szyf 1995; Ramchandani, MacLeod et al. 1997).This evidence serves to implicate DNMT1 as an essential part of tumorigenesis and cellular transformation.

Evidence also suggests that DNA methylation can be a targeted process. The maintenance methyltransferase function of DNMT1 has recently been shown to require targeting by UHRF1 and Np95 (Bostick, Kim et al. 2007; Sharif, Muto et al. 2007; Arita, Ariyoshi et al. 2008; Hashimoto, Horton et al. 2008). However recent studies (Vire, Brenner et al. 2006) have proposed a relationship between DNMT1 and EZH2, leading to a direct control over DNA methylation patterns in a gene specific manner. Enhance of Zeste Homolog 2 (EZH2) is a histone methyltransferase associated with the methylation of histone H3 at lysine 27 (H3K27), which serves as an anchor point for Polycomb group Proteins (PcG), which are repressors(Cao, Wang et al. 2002; Kuzmichev, Nishioka et al. 2002). A physical interaction between EZH2 and DNMT1 mediates recruitment of DNMT1 to EZH2-specific promoters and their subsequent methylation (Vire, Brenner et al. 2006). Furthermore, loci associated with H3-histone methylated at the K27 residue are poised to be methylated in cancer (Schlesinger, Straussman et al. 2007).

Previous studies have determined that levels of DNMT1 mRNA vary at different phases of the cell cycle (Robertson, Keyomarsi et al. 2000; Cervoni, Detich et al. 2002), with levels at their highest in S phase and lowest in G_0/G_1. We have recently demonstrated that DNMT1 mRNA stability is determined by the AU-rich element (ARE)/poly(U)-binding/degradation factor (AUF1) (Torrisani, Unterberger et al. 2007). AUF1 regulation of *DNMT1* mRNA occurred in a cell-cycle dependent manner, in which it was observed that AUF1 protein, but not mRNA, levels were inversely correlated with

DNMT1 mRNA levels. Previous studies have observed regulation of AUF1 at the protein level, which was mediated by Heat Shock Protein 70 (Hsp70) (Laroia, Cuesta et al. 1999) or by ubiquitination (Laroia and Schneider 2002). However, the exact mechanism through which AUF1 is regulated has never been studied nor its implications for the cell when this regulation is impaired.

In this study we present findings suggesting that AUF1 is regulated at the protein level by Rb. Depletion of Rb, most common in cancer through mutation, deletion(Weinberg 1995) or epigenetic silencing (Ferres-Marco, Gutierrez-Garcia et al. 2006), results in proteasome-dependent downregulation of AUF1, upregulation of DNMT1 and aberrant hypermethylation. Hypermethylation was observed to occur in an EZH2-mediated manner, suggesting a level of targeting. We propose that an increase in DNMT1 levels, through loss of regulation over AUF1, led to subversion of the natural targeting function of EZH2, resulting in unnatural hypermethylation of tumor suppressor genes and the subsequent repression of their expression, a necessary first step in cancer progression.

Materials and Methods

Cell Culture, Transfections and drug treatments

Normal untransformed human fibroblasts were obtained from the Coriell Cell Repository (GM01887). Human embryonic kidney HEK-293 cells were obtained from the ATCC. Human untransformed hepatocytes were obtained from Celprogen (Cat# 33003-02). Human fibroblasts were grown in minimum essential medium. Human hepatocytes were grown in Human Hepatocyte Cell Culture Complete Growth Media (Celprogen; M33003-025). HEK-293 cells were grown in DMEM medium. The cells were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Eighteen to 24 h prior to small interfering RNA (siRNA) treatment, cells were plated at a concentration of 4×10^5 cells in a 100-mm tissue culture dish. The following siRNAs, obtained from Dharmacon, were used in this study: human AUF1 siRNA (siAUF1; (Raineri, Wegmueller et al. 2004)), human DNMT1 siRNA (siDNMT1; antisense strand, 5′-GCAUGAGCACCGUUCUCCdTdT-3′), human Rb SMARTpool siRNA (siRb), human Rb specific siRNA (siRb2; antisense strand, 5′-UGGUUCACCUCGAACACCC-3′), human EZH2 SMARTpool siRNA (siEZH2), human Hsp70 SMARTpool siRNA (siEZH2). siRNA oligonucleotides were transfected using Lipofectin (Invitrogen) in serum-free Opti-MEM. The oligonucleotide-containing Opti-MEM was removed from the cells and replaced with regular growth medium after 4 h and recovered for 20h. Human fibroblasts were harvested after two transfections, a total of 48hrs. Human hepatocytes were harvested after three transfections, a total of 72hrs. HEK-293 cells were transfected with10µg of CMV-Flag AUF1 p45 plasmids (Torrisani, Unterberger et al. 2007) or empty vector DNA (Rouleau, MacLeod et al. 1995) by calcium phosphate precipitation. For Actinomycin D (Sigma; A1410-5MG) treatment, cells were treated with 5µg/ml for the described time. For Cycloheximide (Sigma; 01810-5G) treatment, cells were treated with 20µg/ml for 12 hours (Torrisani, Unterberger et al. 2007). For MG-132

treatment (Sigma; C2211-5MG), cells were treated with a concentration of 10μM for 12 hours.

Large scale purification of AUF1 p45

AUF1 p45 or control transfected HEK-293 cells were lysed in the following lysis buffer: Tris-HCl (pH 7.6) 50 mM; KCl 100 mM; Glycerol 10%; EDTA 1mM; Triton X-100 1% and Complete® protease inhibitor (Roche Diagnostics) for 20 minutes on ice followed by a round of freezing and thawing. After the centrifugation step, the supernatant protein contents were quantified using Bradford reaction (Bio-Rad). Ten milligrams of protein were incubated overnight at 4^0C in the lysis buffer (final volume 5 ml) with 250 μl of Flag M2-agarose beads. A control immunoprecipitation was performed using normal rabbit IgG with AUF1 p45 transfected HEK-293 cells. Beads were then centrifuged and washed with lysis buffer. Bound proteins were eluted using glycine solution 0.1 M (pH 3.3) as recommended by the manufacturer and were neutralized by a 0.5 M Tris-HCl (pH 7.6); 1.5 M NaCl solution. Eluted fractions were pooled and concentrated using Ultra 4 centrifugal filter devices (Amicon). The concentrated eluted fractions were subjected to mass spectrometry analysis to identify proteins interacting with AUF1, while a sample of the concentrated eluted fractions were examined by western blot analysis to confirm interactions.

Protein digestion

Following SDS-PAGE and staining with Coomassie blue, gel fragments containing proteins of interest were excised and stored in 1% acetic acid. Before destaining, gel pieces were prewashed with HPLC grade water. After removal of water, gel pieces were destained by incubation in 100 mM ammonium bicarbonate, followed by addition of 100% acetonitrile. Destained and dehydrated gel slices were then sequentially reduced and alkylated by incubating in 50 μl of 10 mM dithiothreitol, followed by addition of 50 μl of 55 mM iodoacetamide and 100 μl of 100% acetonitrile. Liquid was then removed and gel pieces were washed. Gel pieces were dehydrated and then dried for 30 minutes

at 37°C. Proteins were in-gel digested by adding trypsin (sequencing grade modified trypsin (Promega). To extract peptides, 30 µl of a mixture containing 1% formic acid and 2% acetonitrile were added. Thirty µl of the liquid were then transferred into a new cooled (10°C) plate. Two additional extractions were performed. Each of the extractions involved addition of 12 µl of a mix of 1% formic acid and 2% acetonitrile and 12 µl of 100% acetonitrile to the slice. 15 µl of the extraction were transferred and pooled with the first extraction. The final volume of the peptide extracts was 60 µl at 0.54% formic acid and 15.9% acetonitrile.

LC MS/MS analysis

Tryptic peptides were analyzed on a QTrap 4000 linear ion trap mass spectrometer (Sciex-Applied Biosystems, Concord, Ont). Briefly, the sample was applied to a 10 cm x 75 micron Pico Frit column containing BioBasic C18 packing. Peptides were eluted from the column using a 30 minute gradient of 10-95% acetonitrile (v/v) containing 0.1% formic acid (v/v) at a flow rate of 200nl/min. Eluted peptides were electrosprayed as they exited the column. Precursor ion selection for subsequent tandem ms fragmentation analysis was done using predefined information-dependent acquisition criteria. Briefly, up to three doubly, triply or quadruply charged ions of intensity greater than $2x10e^{6}$ counts per second (cps) from each ms survey scan were selected for passage into a collision cell. Collision-induced dissociation was facilitated by collision with nitrogen gas; fragment ions were trapped in Q3 and scanned. Three ms/ms scans at a speed of 4000 amu/second from 70 to 1700 m/z were averaged for each selected precursor ion.

Data analysis

Raw mass spectra were then passed through a bioinformatics pipeline (Kearney, Bencsath-Makkai et al. 2003) which implemented the following analysis procedures. The raw spectra were first subjected to peak-detection using the Distiller product from Matrix Science (http://www.matrixscience.com/distiller.html) with its parameters set at

SNR=10 and CC=0.7. This reduced noise and produced a list of distinct peptide peaks in which all members of the isotopic clusters were collapsed into an equivalent monoisotopic peak.

The peak-lists were then sent to the Mascot tool from Matrix Science (http://www.matrixscience.com) (Perkins, Pappin et al. 1999) for peptide identification by data base search. Fixed modifications were set for carbamidomethyl on cysteine, variable modifications were set for oxidation of methionine, peptide mass tolerance was set at ± 1.5 Da, fragment mass tolerance was set at ± 0.8 Da and the data base searched was NCBI nr frozen on May 11, 2006 (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) database with taxonomy limited to human (273,951 sequences; 98,047,698 residues).

The resulting list of peptide identifications was then processed to eliminate those likely to have arisen by chance. Thus, only peptide identifications with a probability of occurring by chance of < 0.05 were retained (i.e. only those peptides for which the Mascot Peptide Score was > the Id Score were retained). These peptide identifications were then linked to the proteins containing them and sorted by protein to produce an initial list of protein identifications. However, since 5% of the spectra matched more than one peptide and 40% of the peptides identified occur in more than one protein, the protein lists were then processed by a grouping algorithm (Kearney, Blondeau et al. 2005) to generate a list of proteins defined by distinct sets of proteins. That is, the minimum number of protein sequences needed to explain the peptides observed.

Nearest Neighbour Analysis

Levels of 5-methylcytosine in DNA extracted from cells transfected with either control or AUF1 siRNA were measured by nearest-neighbour analysis as described previously (Ramsahoye 2002). Two micrograms of genomic DNA were incubated with *Mbo*I restriction enzyme (MBI-Fermentas) at 37°C overnight. The reaction was heat-inactivated at 65°C for 20 minutes and the DNA was recovered by ethanol precipitation. The *Mbo*I digested DNA was end-labeled with 10 μCi [α-^{32}P]-dGTP (Amersham), 0.5 μl

Klenow enzyme (Roche Diagnostics) and 1.5 µl of 10X Labeling buffer (Roche Diagnostics) at 15°C for 15 minutes. The reaction was stopped by addition of 2 µl of 0.2 M EDTA. Labeled DNA was purified by a Sephadex G50 spin column (Roche Diagnostics). 8 µl of DNA were digested with 1 µl of Micrococcal Nuclease (Roche Diagnostics), 1 µl of Spleen Phosphodiesterase (Roche Diagnostics) and 1 µl of Micrococcal Nuclease buffer (250 mM Tris-HCl, pH 7.4 and 10 mM $CaCl_2$) at 37 ^{o}C overnight. Digested DNA was separated by Thin-Layer Chromatography (TLC) on Polygram CEL 400 TLC sheets (Macherey-Nagel) in two dimensions, the first dimension solution consisted of 66 volumes isobutyric acid: 18 volumes water: 3 volumes 30% ammonia solution, and the second dimension solution consisted of 80 volumes saturated ammonium sulphate: 18 volumes 1 M acetic acid: 2 volumes isopropanol. The intensity of 5-methylcytosine and cytosine mononucleotide spots was measured using a PhosphorImager screen and the Image Quant image analysis program (GE Healthcare). Levels of unmethylated cytosine content are expressed as a percentage of [cytosine]/[cytosine + methylcytosine].

Luminometric Methylation Assay (LUMA)

The assay was performed as previously described (Karimi, Johansson et al. 2006). Genomic DNA was digested with either *HpaII* and *EcoRI* or *MspI* and *EcoRI*, and then subjected to polymerase extension assay using the pyrosequencing platform, which can distinguish the *HpaII*, *MspI* and *EcoRI* cut sites. The level of cytosine methylation was determined by comparing the ratio of *HpaII*/*EcoRI* to *MspI*/*EcoRI* in the different treated samples.

Immunoprecipitation

Five hundred micrograms of protein extracts were precleared with Protein G Agarose (Roche; 11718416001) for 30 min at 4^{o}C. The pre-cleared extract was incubated overnight with 10µg of EZH2 (AbCam 3748) or rabbit IgG (Santa Cruz; sc-2027) antibody at 4^{o}C. The extract:antibody mixture was incubated with Protein G Agarose beads for 2 hours at 4^{o}C. Following this incubation the beads were washed six times with Tris Lysis

Buffer. The immunoprecipitated protein mixture was assayed for DNMT1 presence by western blot analysis.

Western blot analysis

Fifty micrograms of whole-cell protein extracts were fractionated on a 7.5% to 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Protein extracts were isolated following lysis of the cells with Tris Lysis buffer (10 mM Tris-HCl, pH 7.6: 5 mM $MgCl_2$; 0.3 M NaCl; 0.05% Tween and 10% Glycerol) followed by centrifugation. The following antibodies were used: anti-DNMT1 (New England Biolabs), anti-AUF1 (Upstate Biotechnologies), anti-Rb (Santa Cruz), anti-phospho-Rb (Santa Cruz), anti-β-Actin (Sigma; A5316), anti-PCNA (Santa Cruz; PC10), anti-Hsp70 (Upstate Biotechnologies) and anti-FLAG (Sigma-Aldrich). Membranes were blocked in 5% milk in Tris-buffered saline (TBS) with 0.05% Tween (TBST) for 1 h and then exposed to antibody in the TBST-5% milk solution overnight at 4°C. The membranes were washed with TBST and then exposed to either horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG in TBST-5% milk or TBST, respectively, for 1 h at room temperature. The signal was then developed using an ECL enhanced chemiluminescence kit from Amersham (catalog no. 135,136).

Flow Cytometry Analysis

Treated fibroblast cells were trypsinized and washed with phosphate-buffered saline (PBS), and fixed with chilled 70% ethanol. The fixed cells were then centrifuged and washed with PBS. The cells were then treated with 1 U of DNase-free RNase to the cell suspension (10^6 cells in 1 ml), and incubated for 30 minutes at 37°C. Fifty microliters of 1 mg/ml propidium iodide was added directly to the cell suspension, and flow cytometry analysis was performed on a flow cytometer (FACS Calibur; BD Biosciences, Ontario, Canada). Results were analyzed further using the WinMDI software (version 2.8; Scripps Research Institute, La Jolla, CA).

Cell counting

Cell numbers were determined by trypan blue staining. Treated fibroblasts were trypsinized and washed with phosphate-buffered saline (PBS). Cells were resuspended in Trypan Blue staining solution and counted with a hemocytometer. Cell counting was performed in triplicate.

Chromatin Immunoprecipitation (ChIP) analysis

ChIP assays (Crane-Robinson, Myers et al. 1999) were performed as described previously (Cervoni and Szyf 2001), using an anti-EZH2 antibody or normal rabbit IgG antibody (Santa Cruz). DNA was purified from both the immunoprecipitated and preimmune (input) samples and was subjected to PCR amplification. In all experiments, 1/10 of the input sample was used for PCR amplification.

Methylated DNA Immunoprecipitation (mDIP) analysis

mDIP was performed as previously described (Weber, Davies et al. 2005; Keshet, Schlesinger et al. 2006). Briefly, 2µg of human fibroblast sonicated DNA (between 200-700bp) was denatured by heating and immunoprecipitated with 10µl of 5-methyl Cytosine antibody (Calbiochem #NA81). 5mC bound fractions were purified with Sepharose beads and SpinX columns. Fractions were washed a total of 8 times with FB buffer (10mM Tris pH8, 50mM NaCl, 1mM EDTA) and eluted in TE + 0.5% SDS. Eluted DNA was purified by phenol-chloroform and ethanol precipitation. Samples were used for RT-PCR analysis. In all experiments, 1/10 of the input sample was used for PCR amplification.

Real-Time PCR

Quantitative real-time PCR mixtures (25µl) containing DNA or cDNA, SYBR Green master Mix (Roche Diagnostics) and 0.4µM primer were loaded into LightCycler capillaries (Roche Molecular Biochemicals). Samples were run in the LightCycler 3.5 (Roche Molecular Biochemicals). To determine the C_T ratio, a four-point calibration curve of

decreasing amounts of DNA (1,0.1, 0.01 and 0.001 dilutions) as well as a no-template negative control were performed by using separate tubes for each reaction. The primer sequences for each region analyzed in this study are listed in Table 1. PCRs were performed in triplicate.

Sodium Bisulfite Mapping of DNA Methylation status

Sodium bisulfite treatment was performed as previously described (Clarke, MacKay et al. 1994) with minor modifications. 5µg of bisulfite treated DNA samples were subject to PCR amplification using the sequencing primers listed in table 1. The PCR products were cloned using TA cloning kits (Invitrogen; K2060-01) and the clones were subsequently sequenced using the CEQ 8800 Genetic Analysis System (Beckman Coulter).

Results

Regulation of AUF1 protein occurs in an Rb dependent manner.

Our previous study (Torrisani, Unterberger et al. 2007) suggested that AUF1 protein levels were significantly decreased in relation to the cell cycle, specifically a decrease in the S phase of the cell cycle. We determined the effect of serum starvation, a technique known to arrest cells in G_0/G_1 (Torrisani, Unterberger et al. 2007), on AUF1 and DNMT1 protein levels in untransformed human fibroblasts. We used these cells in our study as they served as a model for normal functioning cells, without the potential confound due to transformation. As the fibroblasts entered into the cell cycle, levels of AUF1 protein decreased (Fig. 1A) while levels of DNMT1 increased. We also observed an expected increase in Rb phosphorylation, typical of cell cycle entry into S phase (Chau and Wang 2003). We hypothesized that Rb, due to it being an important mediator of entry into S-phase (Korenjak and Brehm 2005), played a role in regulating AUF1, and consequently DNMT1. Indeed, siRNA specific for Rb lead to a decrease in AUF1 protein levels (Fig. 1B), as well as an increase in DNMT1 levels. Furthermore, we previously demonstrated that AUF1 mRNA levels were unaltered through the cell cycle (Torrisani, Unterberger et al. 2007), which lead us to examine the effect of Rb siRNA treatment on AUF1 mRNA levels (Fig. 1C). Rb siRNA did not significantly affect AUF1 mRNA levels. To establish if our observed regulation of Rb over AUF1 and DNMT1 was limited to our cell line we performed a knockdown of Rb using Rb-specific siRNA (siRb 2), utilizing a different target sequence to the Rb siRNA used in the rest of this study, in untransformed human hepatocytes (Fig. 1D). We observed a similar decrease in AUF1 protein, as well as an increase in DNMT1 protein levels, suggesting a similar level of regulation as in human fibroblasts. This suggests that Rb could serve as a positive

regulator of AUF1. Upon inactivation of Rb, the positive elements of AUF1 regulation were eliminated and an increase in AUF1 protein turnover occurred.

To determine if the effect of Rb depletion of AUF1 was related to an alteration in the cell cycle, we performed a cell cycle analysis on fibroblasts treated with Rb siRNA. We determined that within the time frame of the decrease of AUF1 protein levels, 48 hours, we saw no significant change in siRb treated cells in S-phase (Fig. 1F). Therefore the effect on AUF1 regulation by Rb depletion was not due to a disruption of entry into S-phase of the cell cycle. Furthermore we examined the effect of Rb depletion on cell numbers (Fig. 1E). Within the time frame of the decrease in AUF1 protein, there was no significant change in cell numbers between control siRNA and Rb siRNA, further establishing the regulation of Rb on AUF1 to be limited a specific mechanism, rather than an overall decrease in cell number.

Rb regulates AUF1 protein in a proteasome-dependent manner.

We previously established that decreases in AUF1 protein levels lead to an increase in DNMT1 mRNA stability (Torrisani, Unterberger et al. 2007). We proposed that a decrease in AUF1 levels due to Rb depletion would lead to an increase in DNMT1 mRNA stability. Indeed we observed an increase in DNMT1 mRNA levels upon Rb siRNA treatment (Fig. 2A). To establish the mechanism through which Rb regulated AUF1 protein, we first determined at what level this regulation occurs. We examined the effect of Rb regulation on transcription through treatment of cells with Actinomycin D, a known transcriptional inhibitor. Inhibition of transcription was observed not to effect Rb siRNA regulation of DNMT1 mRNA (Fig. 2A). We next examined the mechanism of regulation at the translational and proteosomal levels. We observed that treatment with cycloheximide, a translational inhibitor, did not inhibit Rb siRNA mediated depletion of AUF1 (Fig. 2B). In fact, it was seen to have an increased effect on AUF1 protein instability, by the elimination of *de novo* AUF1 protein synthesis. Upon treatment with MG-132, a proteosomal inhibitor, the Rb siRNA mediated decrease in AUF1 was abolished, suggesting that AUF1 was regulated by Rb through the proteasome.

Furthermore, this established that Rb serves as a negative regulator of the proteasome, in terms of AUF1, and upon Rb inactivation the proteosomal complex degrades AUF1.

Hsp70 serves as a regulator of AUF1 protein.

Previous studies have implicated Hsp70 as having a role in regulation of AUF1 protein (Laroia, Cuesta et al. 1999). We examined the role of Hsp70 in the mechanism through which Rb regulates AUF1 by using siRNA specific to Hsp70. We observed that upon treatment of fibroblasts with Hsp70 and Rb siRNA the effect of AUF1 depletion was abolished (Fig. 2C) as compared to cells treated only with Rb siRNA. This data suggests that Hsp70 can control the stability of AUF1, but does so in the context of Rb. Furthermore, depletion of Hsp70 eliminates the effect on the Rb siRNA mediated increase in DNMT1 (Fig. 2D). To further understand the role of Hsp70 in terms of AUF1 regulation, we examined the purported relationship between AUF1 and Hsp70 (Laroia, Cuesta et al. 1999). We performed a large-scale purification of AUF1 interacting partners using a FLAG-tagged AUF1 p45 isoform. The eluted AUF1 binding protein fraction was subject to mass spectrometry to determine the identity of interacting proteins. We observed that Hsp70 did indeed bind and interact with AUF1 (Table 2). We confirmed our mass spectrometry results by observing a positive interaction between Hsp70 and AUF1 by western blot analysis of the eluted immunoprecipitated fractions (Fig. 2E).

Impairment of AUF1 regulation leads to genomic hypermethylation.

In this study we have observed that Rb depletion leads to an increase in DNMT1 mRNA and protein levels (Fig. 1B), which would imply a potential alteration in genomic DNA methylation levels. We determined through nearest neighbor analysis that this increase in DNMT1 levels does indeed lead to genomic hypermethylation (Fig. 3A). Furthermore, this increase in genomic methylation is comparable to the increase in global 5-methyl cytosine seen in AUF1 siRNA treated cells, which are consistent with previous results (Torrisani, Unterberger et al. 2007). This indicates that the increase in DNMT1 and the effect on genomic methylation are a result of the decrease in AUF1

protein levels. These results were further validated by LUMA (Fig. 3B) confirming the effect of Rb depletion on genomic methylation.

Impairment of AUF1 and DNMT1 regulation lead to p16 promoter methylation.

While Rb siRNA treatment induces genomic methylation, we examined the effect of this increase in global 5-methyl cytosine on *p16/INK4a* promoter methylation, a known tumor suppressor that is methylated and silenced in tumors and transformed cells (Merlo, Herman et al. 1995; Suzuki, Yamada et al. 2006). We examined the methylation status of p16/INK4a by methylated DNA immunoprecipitation (mDIP) in four different primer sets of the p16 promoter (p16 region1), intron (p16 region 2 and 3) and exon (p16 region 4) regions (Fig. 3C). We determined that with Rb siRNA treatment, there was a significant increase in methylation of the p16 promoter (Fig. 3E. p16 Region 1), as well as region 3 and region 4, but not in region 2 (Fig. 3F). This experiment determined that the increase in global methylation upon Rb siRNA treatment leads to changes in promoter methylation of tumor suppressors. Additionally, bisulfite analysis of the promoter region covered by the p16 region 1 primer set, revealed a concurrent increase in CG methylation of the p16 promoter (Fig. 3G), confirming the results of the mDIP analysis. In determining the effect on global and promoter methylation by Rb siRNA treatment, we examined the role of DNMT1 in these changes in methylation. Using simultaneous treatment of fibroblasts with siRNA for DNMT1 and Rb, we abolished the increase in global methylation (Fig. 3A) and p16 promoter methylation (Fig. 3E,F), implicating the increase of DNMT1 through AUF1 deregulation by Rb as afactor in these increases in DNA methylation. We observed that DNMT1 siRNA treatment in the 48 hour time frame did not significantly lower genomic methylation compared to controls, which is consistent with previous results (Milutinovic, Zhuang et al. 2003; Unterberger, Andrews et al. 2006). This suggests that it was the effect of the DNMT1 siRNA in abolishing the increase of DNA methylation, as opposed to a passive demethylation effect. Finally, we examined the effect of Rb depletion on p16 expression and we observed a significant decrease in expression of p16 mRNA (Fig. 3H), confirming

that the increased methylation of the p16 promoter leads to decreased gene expression.

EZH2 mediates genomic hypermethylation upon disruption of AUF1 and DNMT1 regulation by Rb.

We observed that the increase in p16 methylation was not consistent throughout the area (Fig. 3F. p16 region 2), suggesting that the increase in DNMT1 levels was inducing hypermethylation of specific areas, as opposed to a non-specific increase in methylation of all CGs in the genome. Furthermore we used primers (Bracken, Kleine-Kohlbrecher et al. 2007) for regions of p16 that are known to be binding sites for EZH2 (Fig. 3D), a known mediator of promoter specific methylation by DNMT1 (Vire, Brenner et al. 2006). We established the binding capacity of EZH2 to known EZH2 binding sites (Bracken, Dietrich et al. 2006; Vire, Brenner et al. 2006), WYNT1, KCNA, CNR1 and the p16/INK4A promoter by chromatin immunoprecipitation (ChIP) (Fig. 3D and 4A). We also examined β-Actin, which served as a negative control with no known EZH2 promoter association. Previous studies (Vire, Brenner et al. 2006) have shown a direct physical interaction between DNMT1 and Rb protein, which we were able to observe in our human fibroblast cell line (Fig. 4B). We observed that depletion of Rb led to increased binding of DNMT1 to EZH2 (Fig. 4B). We next examined if this increased association led to any changes in methylation of the promoters of the EZH2 binding sites by mDIP analysis using the same primer set used to determine EZH2 association. Depletion of Rb lead to a significant increase in promoter methylation of KCNA and CNR1, while there was no significant changes in WYNT1 and β-Actin methylation (Fig. 4D). Additionally, bisulfite analysis of the CNR1 promoter region covered by the DNA primer set revealed a site-specific increase in CG methylation upon Rb depletion (Fig. 4E, F). To fully establish the effect of promoter methylation we observed a subsequent decrease in CNR1 expression in Rb depleted cells (Fig. 4G)

We simultaneously depleted the human fibroblasts of Rb and EZH2 to determine if EZH2 mediated the gene specific changes in methylation (Fig. 3C). The increase in

methylation of KCNA and CNR1 upon Rb depletion was abolished upon treatment with EZH2 siRNA (Fig. 4C, D). Furthermore, we examined if the effect of EZH2 depletion on p16 promoter methylation and we determined that EZH2 depletion in siRb treated cells lead to an abolishment of the increased methylation status (Fig. 3E) and a relief in suppression of CNR1 gene expression (Fig. 4G). This demonstrates that the increase in DNMT1 leads to promoter hypermethylation and decreased gene expression in a gene specific manner mediated by EZH2.

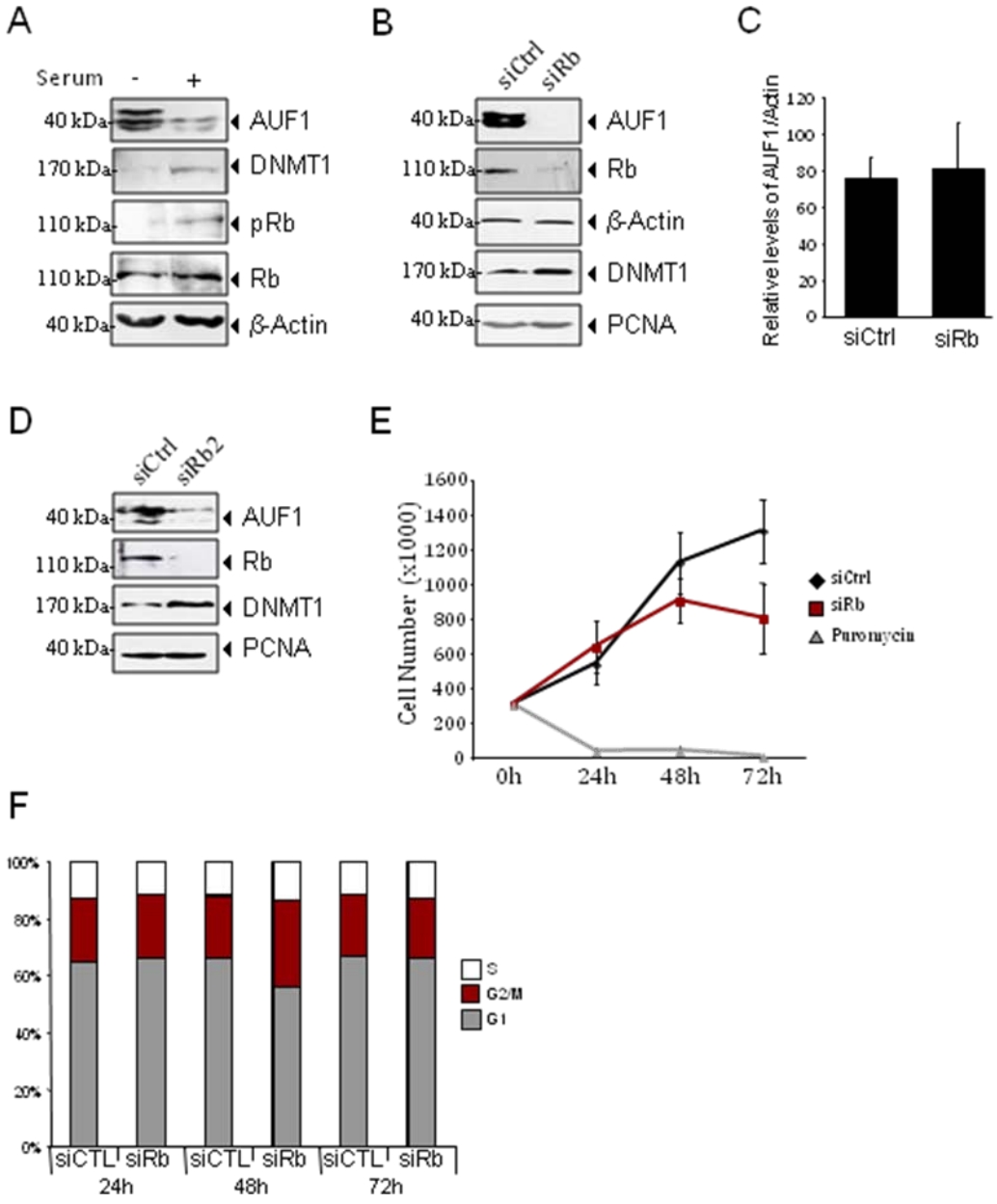


Figure 1 Cell Cycle Dependent Regulation of AUF1. (A) During the cell cycle AUF1 protein levels are inversely correlated with DNMT1 protein levels. Western blot analysis of protein levels of AUF1, DNMT1, Rb, phospho-Rb and β-Actin in human untransformed fibroblasts serum starved for 12 days (Serum -) or serum released for 48 hours (Serum +). **(B)** Rb knockdown by siRNA decreases AUF1 protein levels and increases DNMT1 levels. Western blot analysis of protein levels of AUF1, DNMT1, Rb, PCNA and β-Actin in

whole cell extracts prepared from human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) twice over a period of 48h. **(C)** Rb regulation of AUF1 protein is independent of AUF1 mRNA. Real-time PCR analysis of AUF1 and β-Actin levels of cDNA prepared reverse-transcribed RNA of human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) twice over a period of 48h. Values are means ± standard error (*, $P < 0.05$, Student's *t* test; $n = 3$). **(D)** Rb knockdown in human hepatocytes decreases AUF1 protein levels and increases DNMT1 protein levels. Western blot analysis of protein levels of AUF1, DNMT1, Rb and PCNA in whole cell extracts prepared from human untransformed hepatocytes treated with 150nM control siRNA (siCtrl) or 150nM of Rb siRNA (siRb2) three times over a period of 72h. **(E)** Rb siRNA treatment does not significantly reduce cell numbers in the 48h time frame. Trypan blue staining of human untransformed fibroblasts treated with 100nM control siRNA (siCtrl), 100nM Rb siRNA (siRb) or 2μg/ml of Puromycin over a period of 0, 24, 48 or 72h. Values are means ± standard error ($n = 3$). Puromycin served as a positive control for cell death. **(F)** Rb siRNA does not affect S-phase levels in the 48h time frame. Cell cycle distribution analysis was carried out by FACS in human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) over a period of 24h (transfected once), 48h (transfected twice every 24h) or 72h (transfected three times every 24h).Data were analyzed by WinMDI version 2.8 and plotted.

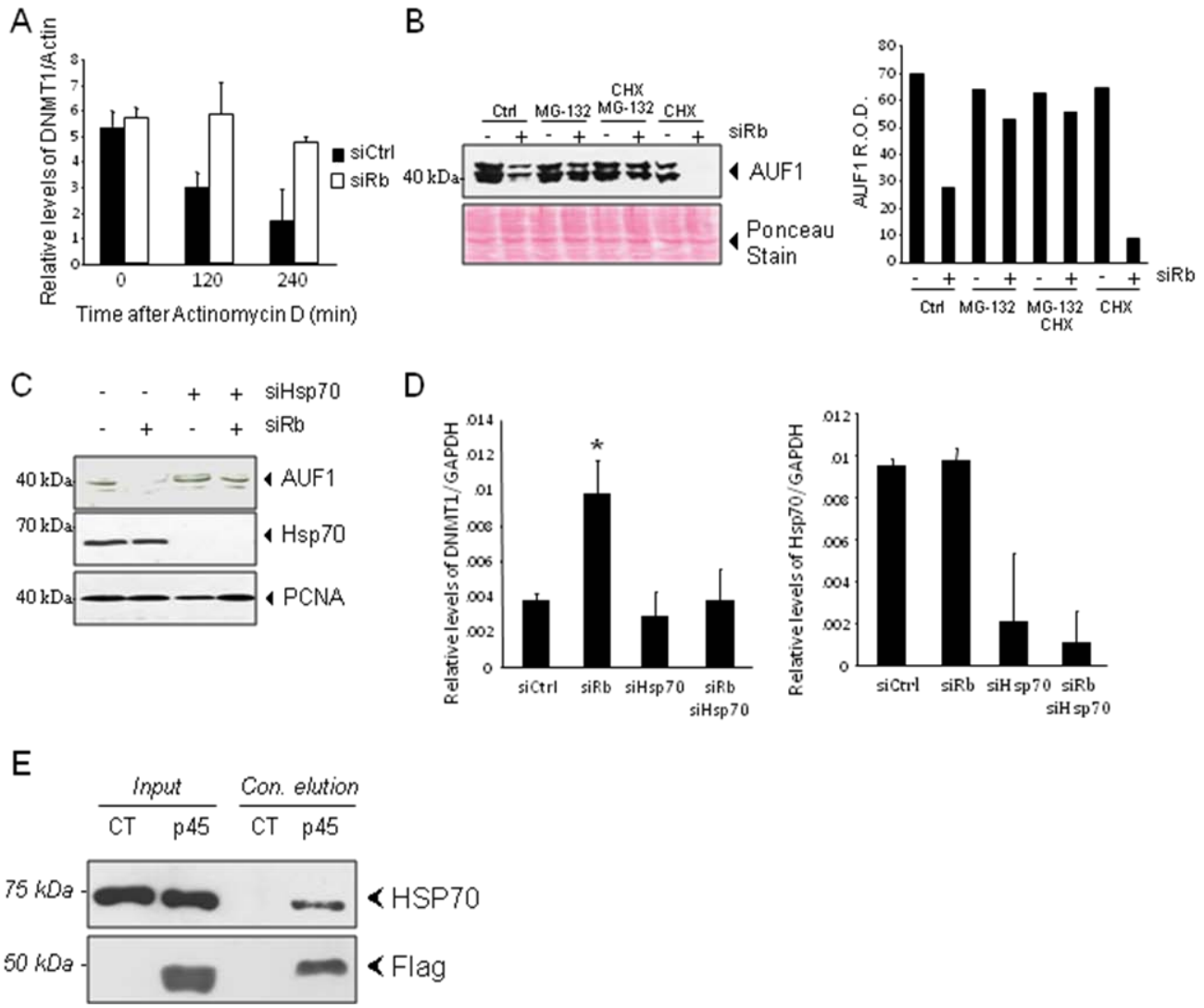
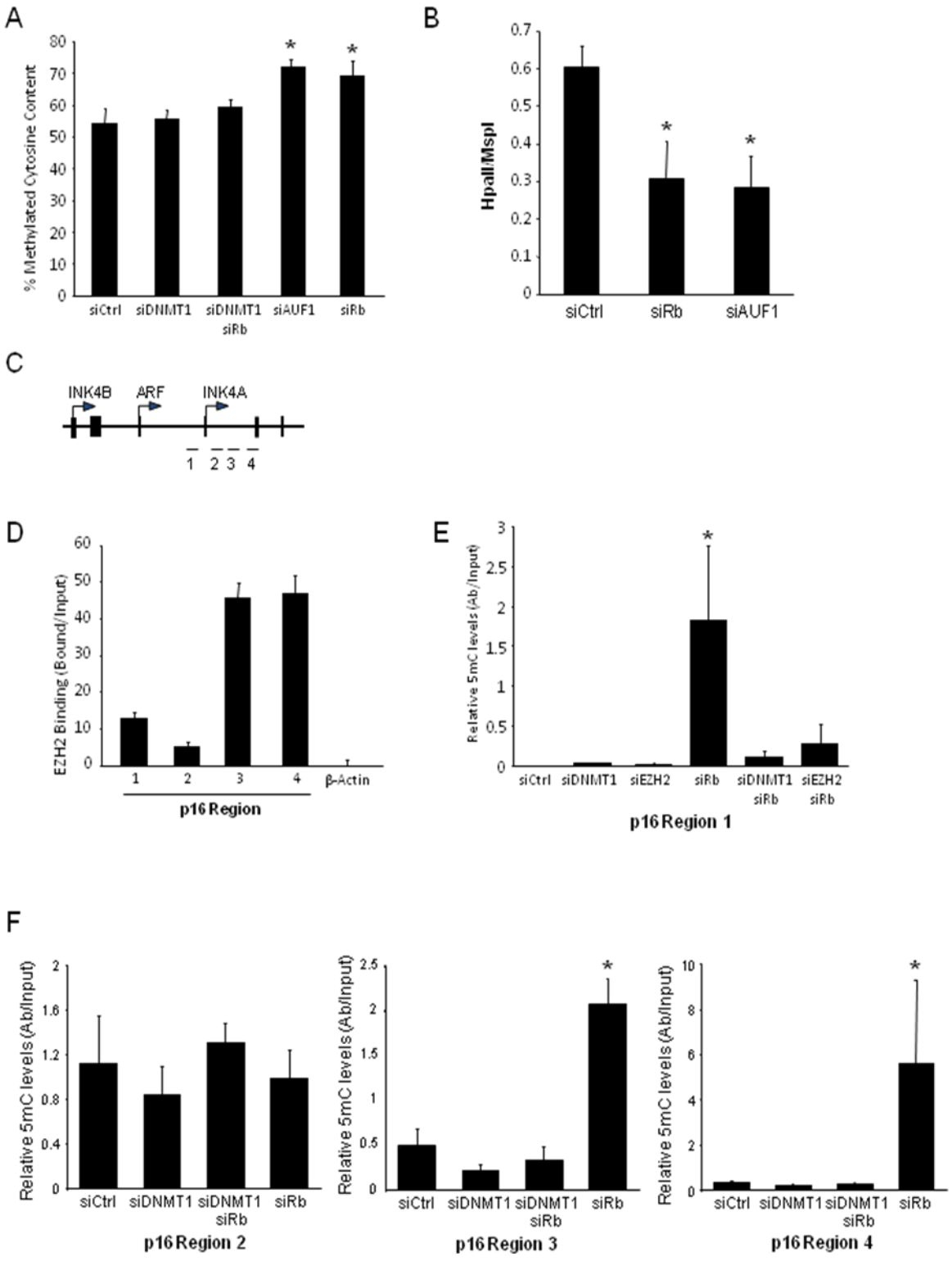


Figure 2 Rb mediates regulation of AUF1. (A) Regulation of DNMT1 by Rb occurs independently of transcription. Real-time PCR analysis of DNMT1 and β-Actin levels of cDNA prepared reverse-transcribed RNA of Actinomycin D treated human untransformed fibroblasts pre-treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) twice over a period of 48h. Values are means ± standard error (*n* = 3). **(B)** Rb regulates AUF1 protein leves in a proteosomal dependent manner. Western blot analysis of protein levels of AUF1 in whole cell extracts of control, MG-132, Cycloheximide or a combination of MG-132/Cycloheximide treated human untransformed fibroblasts, which were pre-treated with 100nM control siRNA (-) or 100nM Rb siRNA (+) twice over a period of 48h. Ponceau staining for total protein levels was performed as a control. Relative Optical Density (R.O.D.) of AUF1 was determined using the signal for the corresponding lane of the Ponceau stain to the AUF1 western blot. **(C)** Hsp70 is required for siRb mediated decrease of AUF1 protein. Western blot

analysis of protein levels of AUF1, Hsp70 and PCNA in whole cell extracts prepared from human untransformed fibroblasts treated with 100nM control siRNA (-/-), 100nm Rb siRNA (siRb), 100nm Hsp70 siRNA (siHsp70) or a combination of Rb and Hsp70 siRNA (100nM) twice over a period of 48h. **(D)** Hsp70 is required for siRb mediated increase in DNMT1 mRNA. Real-time PCR analysis of AUF1, Hsp70 and GAPDH levels of cDNA prepared from reverse-transcribed RNA of human untransformed fibroblasts treated with 100nM control siRNA (siCtrl), 100nm Rb siRNA (siRb), 100nm Hsp70 siRNA (siHsp70) or a combination of Rb and Hsp70 siRNA (100nM) twice over a period of 48h. Values are means ± standard error (*, $P < 0.05$, Student's *t* test; $n = 3$). **(E)** Western blots analysis of Hsp70 and 14-3-3 association with p45-AUF1 after immunoprecipitation with anti-FLAG antibodies in whole cell extracts prepared from HEK-293 cells transfected with AUF1-p45-FLAG. Flag western blot was performed to determine positive precipitation of AUF1-p45.


A
% Methylated Cytosine Content
siCtrl
siDNMT1
siDNMT1 siRb
siAUF1
siRb
B
HpaII/MspI
siCtrl
siRb
siAUF1
C
INK4B
ARF
INK4A
1 2 3 4
D
EZH2 Binding (Bound/Input)
1
2
3
4
β-Actin
p16 Region
E
Relative 5mC levels (Ab/Input)
siCtrl
siDNMT1
siEZH2
siRb
siDNMT1 siRb
siEZH2 siRb
p16 Region 1
F
Relative 5mC levels (Ab/Input)
siCtrl
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siDNMT1 siRb
siRb
p16 Region 2
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p16 Region 4

Figure 3

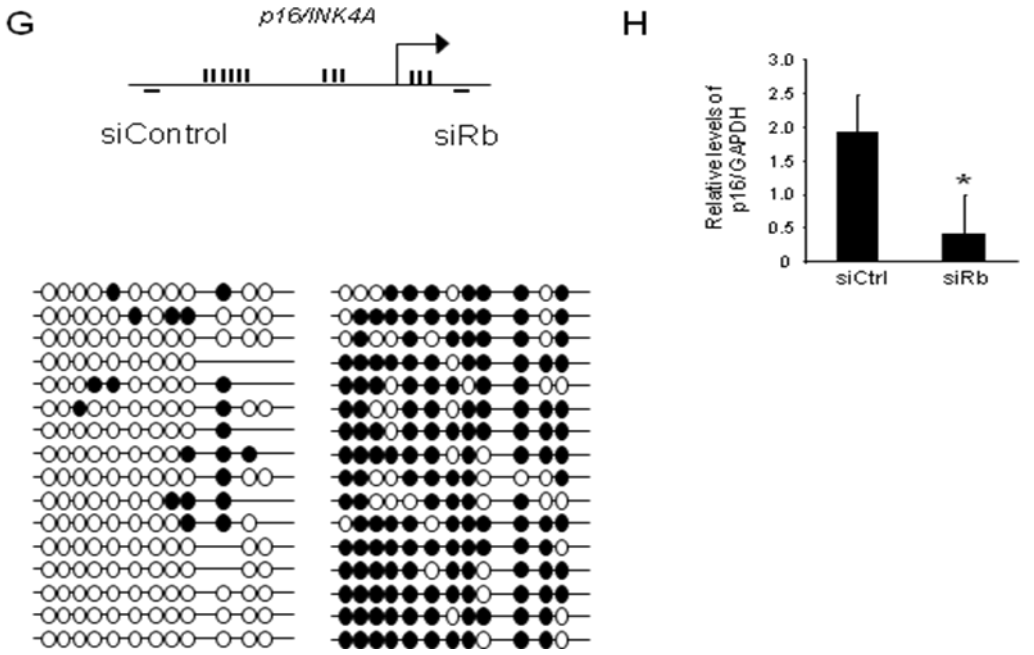


Figure 3 (continued) Effect of AUF1 disruption on DNA methylation. (A) siRb induces global hypermethylation due to the increase in DNMT1. Quantification of changes in global methylation by Nearest Neighbour analysis of genomic DNA extracted from human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), DNMT1 siRNA (siDNMT1), a combination of DNMT1 and Rb siRNA, AUF1 siRNA (siAUF1) or 100nM Rb siRNA (siRb) twice over a period of 48h. (*, $P < 0.05$, Student's *t* test; $n = 3$). **(B)** An induction of genomic methylation levels by Rb siRNA treatment. Quantification of changes in global methylation by Luminometry Methylation Assay of genomic DNA extracted from human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), AUF1 siRNA (siAUF1) or 100nM Rb siRNA (siRb) twice over a period of 48h. (*, $P < 0.05$, Student's *t* test; $n = 3$). **(C)** Representative image of the *p16/Ink4a* promoter and the location of the p16 Region DNA primers. **(D)** Assessment of EZH2 binding capacity to four known EZH2 target sites in the p16/INK4A promoter. Chromatin Immunoprecipitation was performed on cross-linked DNA of human untransformed fibroblasts. Real-time PCR was performed on EZH2 precipitated DNA, as well as Input, using primers for p16 regions 1, 2, 3, 4 and B-Actin. B-Actin served as a negative control for EZH2 binding(n=3). **(E)** Depletion of Rb causes an increase in methylation of region 1 of *p16/Ink4a* promoter in a DNMT1 and EZH2 dependent manner. Relative 5-methyl Cytosine levels were determined by methylated DNA immunoprecipitation of genomic DNA from human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), DNMT1 siRNA (siDNMT1), Rb siRNA (siRb), EZH2 siRNA (siEZH2), a combination of DNMT1 and Rb siRNA, or a combination of EZH2 and Rb siRNA, twice over a period of 48h. (*, $P < 0.05$, Student's *t* test; $n = 3$). Relative 5mC levels were determined by the real-time PCR signal of the 5mC immunoprecipitation and the Input real-time PCR signal using the primers for p16 Region 1. **(F)** Depletion of Rb causes an increase in methylation of the *p16/Ink4a* promoter in a DNMT1 dependent manner. Relative 5-

methyl Cytosine levels were determined by methylated DNA immunoprecipitation of genomic DNA from human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), DNMT1 siRNA (siDNMT1), Rb siRNA (siRb), or a combination of DNMT1 and Rb siRNA, twice over a period of 48h. (*, $P < 0.05$, Student's *t* test; $n = 3$). Relative 5mC levels were determined by the real-time PCR signal of the 5mC immunoprecipitation and the Input real-time PCR signal using the primers for p16 Region 2, 3 and 4. **(G)** Rb siRNA mediates an increase in methylation of the p16/INK4a promoter. Bisulfite mapping of the p16 promoter from genomic DNA of human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl) or Rb siRNA (siRb). Each line represents an individual clone, each circle represents an individual CG. A filled circle represents a methylated CG, an open circle represents an unmethylated CG. Three independent PCRs were performed for each condition. **(H)** Rb depletion leads to suppressed p16 expression. Real-time PCR analysis of p16 and β-Actin levels of cDNA prepared reverse-transcribed RNA of human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) twice over a period of 48h. Values are means ± standard error (*, $P < 0.05$, Student's *t* test; $n = 3$).

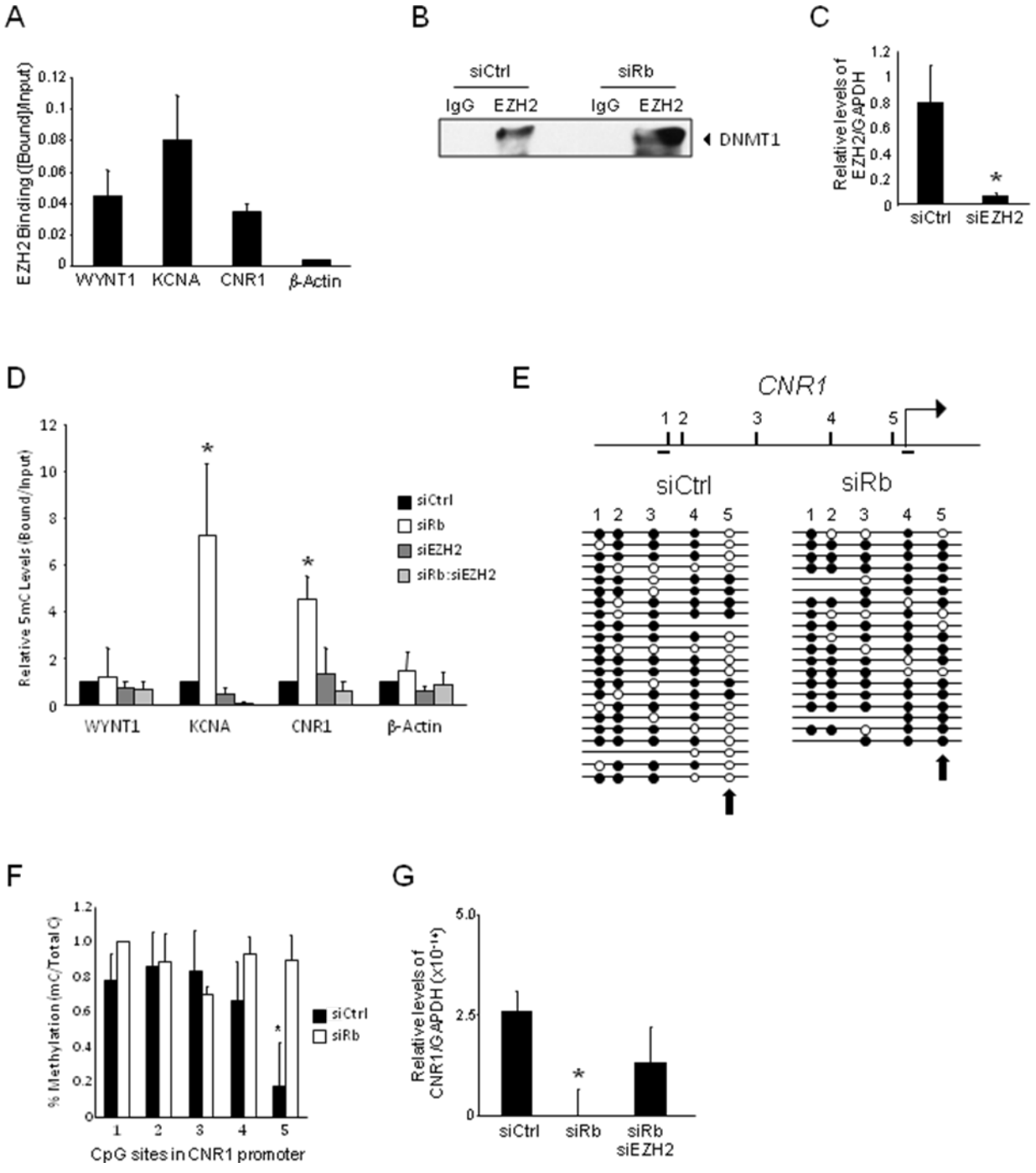


Figure 4. Rb disruption leads to promoter specific hypermethylation, mediated by EZH2. (A) Assessment of EZH2 binding capacity to known EZH2 targets (WYNT1, KCNA and CNR1). Chromatin Immunoprecipitation was performed on cross-linked DNA of human untransformed fibroblasts. Real-time PCR was performed on EZH2 precipitated DNA, as well as Input, using primers for WYNT1, KCNA, CNR1 and *β*-Actin. *β*-Actin served

as a negative control for EZH2 binding (n=3). **(B)** Depletion of Rb by siRNA leads to increased association of EZH2 with DNMT1. Western blot analysis of DNMT1 association with EZH2 was performed on EZH2 immunoprecipitated protein extracts from human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM EZH2 siRNA (siEZH2) twice over a period of 48h. **(C)** Confirmation of EZH2 depletion after treatment with siRNA. Real-time PCR analysis of EZH2 and β-Actin levels of cDNA prepared reverse-transcribed RNA of human untransformed fibroblasts treated with 100nM control siRNA (siCtrl) or 100nM Rb siRNA (siRb) twice over a period of 48h. Values are means ± standard error (*, $P < 0.05$, Student's *t* test; $n = 3$). **(D)** siRb mediates an increase in methylation of promoters of KCNA and CNR1 in an EZH2 dependent manner. Relative 5-methyl Cytosine levels were determined by methylated DNA immunoprecipitation of genomic DNA from human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), Rb siRNA (siRb), EZH2 siRNA (siEZH2) or a combination of EZH2 (siEZH2) and Rb siRNA, twice over a period of 48h. Relative 5mC levels were determined by the real-time PCR signal of the 5mC immunoprecipitation and the Input real-time PCR signal using primers for WYNT1, KCNA, CNR1 and B-Actin. B-Actin served as a negative control due to its lack of EZH2 binding (*, $P < 0.05$, Student's *t* test; $n = 3$). **(E)** Depletion of Rb mediates a site-specific increase in methylation of the *CNR1* promoter. Bisulfite mapping of the *CNR1* promoter from genomic DNA of human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl) or Rb siRNA (siRb). Each line represents an individual clone, each circle represents an individual CG. A filled circle represents a methylated CG, an open circle represents an unmethylated CG. Three independent PCRs were performed for each siRNA condition. **(F)** The 5^{th} CG of the CNR1 promoter was significantly methylated in the siRb treated cells. Quantification of the bisulfite mapping of each CG site of the amplified region of the CNR1 promoter. Three independpent PCRs were performed for each siRNA condition. (*, $P < 0.05$, Student's *t* test; $n = 3$). **(G)** Depletion of Rb reduces CNR1 expression in an EZH2 dependent manner. Real-time PCR analysis of EZH2 and GAPDH levels of cDNA prepared reverse-transcribed RNA of human untransformed fibroblasts treated with 100nM of control siRNA (siCtrl), Rb siRNA (siRb), or a combination of EZH2 (siEZH2) and Rb siRNA, twice over a period of 48h. Values are means ± standard error (*, $P < 0.05$, Student's *t* test; $n = 3$).

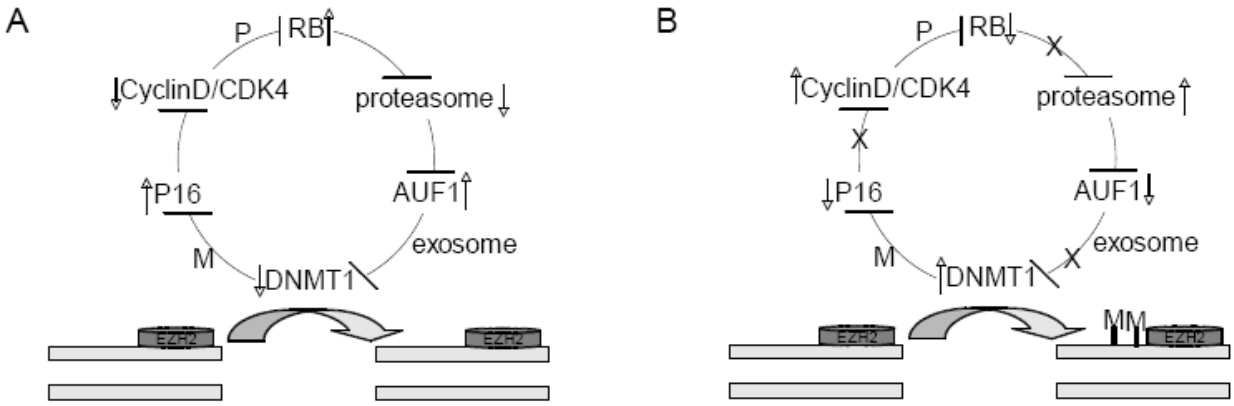


Figure 5. RB, AUF1, DNMT1 DNA methylation pathway. **(A)** Under normal conditions. P-phosphorylation; M-methylation; X-inhibited process; box-gene; EZH2- indicates a gene targeted with the histone methyltransferase EZH2. **(B)** Under conditions where RB is depleted or hyperphosphorylated excess DNMT1 is targeted to genes associated with EZH2 resulting in hypermethylation and gene silencing leading to cellular transformation.

Primer group and gene	Primer Direction	Sequence (5'-3')	Annealing Temperature (°C)
p16 ChiP Set 1(Bracken, Kleine-Kohlbrecher et al. 2007)	Forward	ACC CCG ATT CAA TTT GGC AG	56
p16 ChiP Set 1(Bracken, Kleine-Kohlbrecher et al. 2007)	Reverse	AAA AAG AAA TCC GCC CCC G	56
p16 ChiP Set 2(Bracken, Kleine-Kohlbrecher et al. 2007)	Forward	AGA GGG TCT GCA GCG G	58
p16 ChiP Set 2(Bracken, Kleine-Kohlbrecher et al. 2007)	Reverse	TCG AAG CGC TAC CTG ATT CC	58
p16 ChiP Set 3(Bracken, Kleine-Kohlbrecher et al. 2007)	Forward	GCC AAG GAA GAG GAA TGA GGA G	58
p16 ChiP Set 3(Bracken, Kleine-Kohlbrecher et al. 2007)	Reverse	CCT TCA GAT CTT CTC AGC ATT CG	58
p16 ChiP Set 4(Bracken, Kleine-Kohlbrecher et al. 2007)	Forward	CAA GCT GAT CTT CTC AGC ATT CG	58
p16 ChiP Set 4(Bracken, Kleine-Kohlbrecher et al. 2007)	Reverse	GCC AGA GAG AAC AGA ATG GTC AGA GCC A	58
CNR1 mDIP(Vire, Brenner et al. 2006)	Forward	GCAGAGCTCTCCGTAGTCAG	60
CNR1 mDIP(Vire, Brenner et al. 2006)	Reverse	AACAGGCTGGGGCCATACAG	60
WYNT1 mDIP(Vire, Brenner et al. 2006)	Forward	ACCCGTCAGCTCTCGGCTCA	63
WYNT1 mDIP(Vire, Brenner et al. 2006)	Reverse	TGCAGTTGCGGCGACTTTGG	63
KNCA mDIP(Vire, Brenner et al. 2006)	Forward	TGACGGTGATGTCTGGGGAG	60
KNCA mDIP(Vire,	Reverse	GGTTGCGGTCGAAGAAGTAC	60

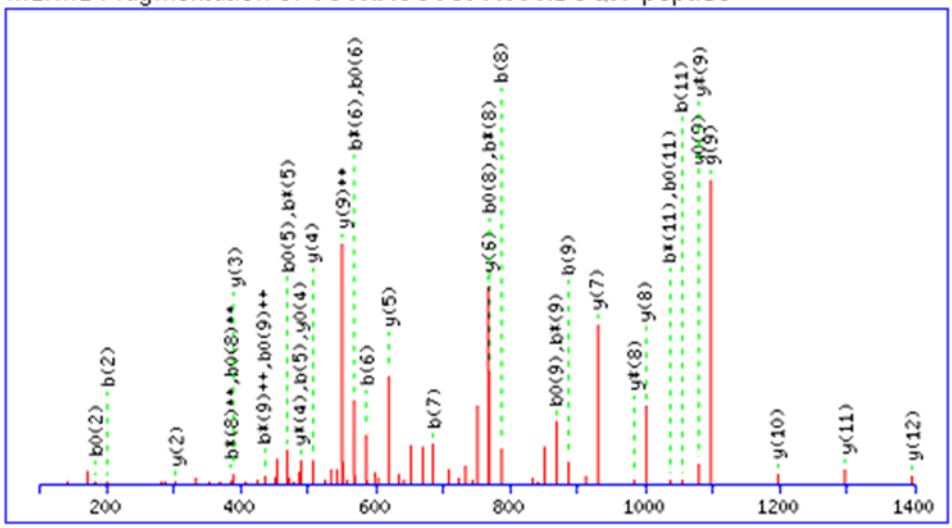
Brenner et al. 2006)			
β-Actin mDIP(Vire, Brenner et al. 2006)	Forward	CCA ACG CC AAAA CTC TCCC	58
β-Actin mDIP(Vire, Brenner et al. 2006)	Reverse	AGCCATAAAAGGCAACTTTCG	58
p16 Sequencing Outside(Sato, Fujita et al. 2002)	Forward	ATTTTAGGGGTGTTATAT	56
p16 Sequencing Outside(Sato, Fujita et al. 2002)	Reverse	CTACCTAATTCCAATTCCCCTACA	56
p16 Sequencing Nested(Sato, Fujita et al. 2002)	Forward	TTTTTAGAGGATTTGAGGGATAGG	56
p16 Sequencing Nested(Sato, Fujita et al. 2002)	Reverse	TTCCAATTCCCCTACAAA	56
CNR1 Sequencing Outside	Forward	AGGTGAAGTTTTTAAGAGTAG	58
CNR1 Sequencing Outside	Reverse	AAAACCATCTAAAATAAACTTCA	58
CNR1 Sequencing Nested	Forward	GGTTATGTGATATATTAGTGTT	58
CNR1 Sequencing Nested	Reverse	CTAAAAAAATAACCCACAAAAAA	58
AUF1 RT	Forward	C ACC ATG GAC TAC AAA GAC	56
AUF1 RT	Reverse	G TAC CGA TAT CAG ATC TAT CG	56
β-Actin RT	Forward	AGA TGT GGA TCA GCA AGC AGG AGT	56
β-Actin RT	Reverse	GCA ATC AAA GTC CTC GGC CAC ATT	56
DNMT1 RT	Forward	TTTGTATGTTGGCCAAAGCCCGAG	57
DNMT1 RT	Reverse	TTCATGTCAGCCAGGCCACAAAC	57
GAPDH RT	Forward	TGCACCACCAACTGCTTA	57
GAPDH RT	Reverse	GGATGCAGGGATGATGTTC	57
p16 RT	Forward	GGGGTTCGGGTAGAGGAGGTG	58
p16 RT	Reverse	CATGGTTACTGCCTCTGGTG	58
Hsp70 RT	Forward	CCCACCATTGAGGAGGTAGA	56
Hsp70 RT	Reverse	GGAATAAAAGGGCATCACTTG	56
EZH2 RT(Sudo, Utsunomiya et al. 2005)	Forward	TTGTTGGCGGAAGCGTGTAAAATC	60
EZH2 RT(Sudo, Utsunomiya et al. 2005)	Reverse	TCCCTAGTCCCGCGCAATGAGC	60
CNR1 RT	Forward	TGATTTCCAAGCCTCTCTGGCACT	60
CNR1 RT	Reverse	ATCTGGTGGTTGGGCCTATTTCCT	60

Table 1. Primer Sequences.

A

heat shock 70kDa protein 8 isoform 1 variant [Homo sapiens]
Score: **603**
Sequence Coverage: **29%**

MS/MS Fragmentation of **TVTNAVVTVPAYFNDSQR** peptide



B

1 MSKGPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNR**TTPS YVAFTDTER**L
51 IGDAAK**NQVA MNPTNTVFDA K**RLIGR**RFDD AVVQSDMK**HW PFMVVNDAGR
101 PKVQVEYKGE TK**SFYPEEVS SMVLTK**MKEI AEAYLGK**TVT NAVVTVPAYF**
151 NDSQRQATK**D AGTIAGLNVL RIINEPTAAA IAYGLDEK**VG AERNVLIFDL
201 GGGTFDVSIL TIEDGIFEVK **STAGDTHLGG EDFDNRMVNH FIAEFK**RKHK
251 KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 R**FEELNADLF R**GTLDPVEKA LRDAKLDK**SQ IHDIVLVGGS TR**IPKIQK**LL**
351 QDFFNGKELN K**SINPDEAVA YGAAVQAAIL SGDK**SENVQD LLLLDVTPLS
401 LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
451 KDNNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
501 ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQRDKVSSK**N SLESYAFNMK**
551 ATVEDEKLQG KINDEDKQKI LDKCNEIINW LDKNQTAEKE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVDTAXI
651 D

Table 2. Mass Spectrum of AUF1 interacting protein Hsp70. (A) MS/MS sequencing of one peptide identifying the protein Heat Shock 70 kDa (HSP70). Percent coverage for protein identification and peptide score are indicated. **(B)** Identified peptides are represented in bold in the total HSP70 protein sequence.

Discussion

AUF1 protein levels have previously been shown to significantly decrease as cells enter into S-phase (Torrisani, Unterberger et al. 2007). Our observation that the regulation of AUF1 protein is directed by Rb provides an insight into the cellular mechanism of control over DNMT1 and DNA methylation. By utilizing a system of gene specific methylation through EZH2 interaction with DNMT1 (Vire, Brenner et al. 2006), disruptions in Rb, a well-studied occurrence in cancer (Nevins 1992; Tanaka, Fujii et al. 1998; Vooijs and Berns 1999), can have wide repercussions on the status of genomic methylation.

In this study, we demonstrated the mechanism through which the AUF1 protein is regulated. Confirming the results from a previous study, we have shown that the cell cycle protein Rb regulates AUF1 at the protein level (Fig. 1B, C) in a manner that is dependent on the proteasome (Fig. 2B). While we do not discount the possible regulation of AUF1 at the level of transcription, mRNA stability and translation, the proteosomal regulation of AUF1 has been shown to have a large affect on the epigenome through the mRNA targets of DNMT1. This importance of Rb serving as a regulator of AUF1, and consequently DNMT1, can be seen in the account that Rb acts as a major checkpoint protein in the cell cycle. Inactivation of Rb, through phosphorylation (Korenjak and Brehm 2005), is an essential step for entry into S-phase. DNMT1 has previously been shown to increase in S-phase (Robertson, Uzvolgyi et al. 1999), where it has been shown to methylate nascent strands of DNA during DNA replication. However, it has been previously shown that when this regulation of DNMT1 in the S-phase is lost, cellular transformation can occur (Detich, Ramchandani et al. 2001). We propose that due to the regulation of AUF1 by Rb, when a oncogenic event such as Rb inactivation (Weinberg 1995; Ferres-Marco, Gutierrez-Garcia et al. 2006) occurs, we observe an increase in DNMT1 levels (Fig. 1B) (Szyf 2003), as well methylation of tumor suppressors (Fig. 3G) (Rhee, Bachman et al. 2002).

Certain considerations were taken into understanding the exact mechanism through which Rb can regulate AUF1, DNMT1 and the epigenome. We observed similar effects on AUF1 and DNMT1 by Rb in two sets of untransformed cell lines (Fig. 1B, D), eliminating any cell line specific effect. Using untransformed cell lines instead of immortalized cells allowed us to observe cell cycle regulation without the potential effect of gene mutation and the alteration of cellular function brought on by immortalization and cellular transformation (Fridman and Tainsky 2008). Furthermore we used two types of siRNAs, targeting different regions of Rb mRNA, as well as control siRNA, removing any non-specific effects that siRNA may have on the cell cycle (Fig. 1D). Additionally, we detected that the depletion of Rb did not significantly affect the proportion of fibroblasts in S-phase (Fig. 1F) nor did it lead to cell death (Fig. 1E) with the time frame of our observations. This eliminates any possible effects that an alteration in cell number and cycle may have on levels of AUF1 and DNMT1. Previous studies have found an associated relationship between Rb and DNMT1, with E2F serving as a transcriptional factor working directly on the DNMT1 promoter (Kimura, Nakamura et al. 2003; McCabe, Low et al. 2006), as well as a direct interaction between Rb and DNMT1, leading to an inhibition in DNMT activity (Pradhan and Kim 2002). A study by Agoston et al. revealed that increased DNMT1 protein levels were associated with dysfunction of Rb in several cancer cell lines, suggesting a relationship between DNMT1 and Rb at the level of regulation (Agoston, Argani et al. 2007). While we must consider all levels of regulation, we determined that Rb regulation over AUF1 and DNMT1 occurred independently of transcription (Fig. 2A). This reveals that Rb maintains multiple levels of regulation over DNMT1, through transcription of DNMT1 in S-phase, as well as stabilization of DNMT1 mRNA.

The precise mechanism through which AUF1 is regulated remains to be fully understood, however we observed that Hsp70 mediated the regulation of Rb over AUF1 through siRNA knockdown of Hsp70 (Fig. 2C). Previous studies have examined the mechanism through which AUF1 protein is regulated. One study determined that the AUF1 protein can be marked for proteosomal degradation through ubiquitination

(Laroia, Cuesta et al. 1999), while another showed that Hsp70 controls AUF1 stability (Laroia and Schneider 2002). These studies give strength to Hsp70 serving as the main regulator of AUF1 protein stability, which we further implicated through our observed interaction of Hsp70 with AUF1 (Fig. 2E and Table 2). We propose that Hsp70 regulation over AUF1 is inhibited by Rb and inactivation of Rb leads to Hsp70 mediated degradation of the AUF1 protein.

As previously mentioned, we observed the regulation of Rb over AUF1 and DNMT1 within a period of 48 hours. While this time frame appears rapid, disruption of this system has immediate consequences at the global (Fig. 3A, B) and promoter level (Fig. 3E, G and Fig. 4D, E and F). This suggests that the regulation of Rb over AUF1, and consequently DNMT1 mRNA, are tightly associated.

Finally, we established that loss of regulation of AUF1 by Rb lead to an increase in genomic methylation levels. This genomic hypermethylation occurred at levels similar to previously observed hypermethylation in AUF1 siRNA treated fibroblasts (Torrisani, Unterberger et al. 2007). These increases in global methylation lead to an increase in methylation of the promoter of tumor suppressor *p16/INK4a* (Fig. 3E, F and G). As previously mentioned, inactivation of p16 is a necessary step for overcoming checkpoint inhibition of S-phase entry.

We observed that increases in methylation of promoters were mediated by EZH2 in a specific manner. Specifically, increased association of EZH2 with DNMT1 (Fig. 4B) in Rb depleted cells led to increased methylation of the *CNR1* promoter (Fig. 4D, E). This confirms previous studies (Vire, Brenner et al. 2006) that determined that EZH2 association with DNMT1 leads to changes in methylation of promoters, and that EZH2 selectively recruits DNMT1 to specific areas of the genome. Our observation that an increase in DNMT1 levels leads to unnatural changes in methylation leave us to suggest that DNMT1 protein levels may serve as a limiting step in abnormal changes in methylation. We propose that the increased association of DNMT1 to EZH2 led to the

abnormal reworking of a gene specific targeting system, leading to the methylation of genes, including p16, a known EZH2 target (Sato, Fujita et al. 2002).

This study presents a novel mechanism through which the disruption of Rb regulation over AUF1 and DNMT1 levels can lead to gene specific methylation, mediated by EZH2. These gene specific methylation events were followed by a decrease in gene expression of the targeted promoters (Fig. 3H, 4G). We propose that under healthy conditions, active Rb prevents AUF1 degradation, leading to increased levels of AUF1 protein (Fig. 5A). AUF1 increases *DNMT1* mRNA instability, leading to minimal levels of DNMT1. This prevents any unnatural hypermethylation of the genome. Under conditions where Rb is inactivated or deleted, AUF1 protein degradation increases, leading to increased DNMT1 mRNA and protein. Increased DNMT1 in the cell leads to gene specific hypermethylation by utilizing EZH2 to target gene promoters such as p16, CNR1 and WYNT1 (Fig. 5B) leading to their subsequent silenced expression. In particular, methylation of the p16 promoters could have consequences for the cell such as growth proliferation and cellular transformation (Serrano, Gomez-Lahoz et al. 1995). All the events discussed in this study have been previously shown to be necessary elements for tumorigenesis, including Rb inactivation, increased DNMT1, p16 promoter methylation and decreased expression of p16, however we have shown for the first time the exact mechanism through which these events are interconnected.

Acknowledgements

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Connecting Text

We originally observed that AUF1 levels decreased as the cell cycle entered into S-phase in chapter 4. We determined that these changes occurred through proteasomal degradation controlled by Rb and Hsp70. These results confirmed previous studies that observed that AUF1 was regulated by the proteasome and Hsp70 (Laroia, Cuesta et al. 1999; Laroia, Sarkar et al. 2002) as well as our own results suggesting that AUF1 was regulated in the cell cycle (Torrisani, Unterberger et al. 2007). Depletion of Rb led to a decrease in AUF1 protein levels, implying that Rb positively regulated AUF1 and upon inactivation the AUF1 protein was destabilized. This decrease in AUF1 lead to an increase in DNMT1 protein levels as well as genomic methylation levels. We observed an increased association of DNMT1 with EZH2, previously shown to interact (Vire, Brenner et al. 2006). In this context we observed that DNMT1 and EZH2 mediated methylation of EZH2 specific promoters. These results suggest that upon Rb inactivation, DNMT1 levels increase due to AUF1 absence and associates abnormally with EZH2, leading to methylation of specific gene promoters.

This chapter revealed the potential effects that disruption of the natural regulation of AUF1 and DNMT1 can have on genomic methylation. We observed increased levels of DNMT1 upon Rb inactivation, both conditions have been determined to be associated with progression of cancer (Murphree and Benedict 1984). To determine how the effects of DNMT1 upregulation can be reversed in cancer we examined the mechanism through which depletion of DNMT1 affects cancer cells. Previous studies have shown that depletion of DNMT1 induces a replication arrest (Knox, Araujo et al. 2000) and genes involved in the DNA damage checkpoint (Milutinovic, Zhuang et al. 2003) in a manner that is independent of DNA methylation (Milutinovic, Brown et al. 2004). We next examined how DNMT1 depletion induced

replication arrest by examining if DNMT1 depletion activates the DNA damage checkpoint signaling pathway and by determining the mechanism of this induction.

Chapter 6

DNA methyltransferase 1 knockdown activates a replication-stress checkpoint

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DNA methyltransferase 1 (DNMT1) is an important component of the epigenetic machinery and is responsible for copying DNA methylation patterns during cell division. Coordination of DNA methylation and DNA replication is critical for maintaining epigenetic programming. Knock down of DNMT1 leads to inhibition of DNA replication but the mechanism has been unclear. Here we show that depletion of DNMT1 with either specific antisense or siRNA activates a cascade of genotoxic-stress checkpoint proteins, resulting in phosphorylation of checkpoint kinase (Chk)-1 and (Chk)-2, γH2AX foci formation and CDC25a degradation, in an ATM-Rad3-related (ATR) dependent manner. This response is sensitive to DNMT1 depletion, independent of the catalytic domain of DNMT1, but not to loss of DNA methylation *per se* as indicated by the lack of response to 5-aza-CdR which causes demethylation but does not result in disappearance of DNMT1 from the nucleus. The effect of DNMT1 knock down augments the response induced by the classic replication-stress inducer hydroxyurea

suggesting a possible combination of chemotherapeutic agents causing DNA damage and DNMT1 inhibitors in anticancer therapy. siRNA knock-down of ATR blocks the response to DNMT1 depletion, DNA synthesis continues in the absence of DNMT1 resulting in global hypomethylation. We therefore suggest that the response to DNMT1 knockdown described here protects mammalian cells from compromising their epigenetic integrity and ensures proper inheritance of the epigenome during cell division.

Maintenance of the epigenome is crucial for normal gene expression and preservation of cell identity. The epigenome is composed of chromatin, which is fashioned by remodelling complexes and modification enzymes as well as of a pattern of covalent modification of DNA by methylation. During cell division replication of both the genetic and epigenetic, information is faithfully conserved. Due to a preference for hemi-methylated DNA, DNA methyltransferase-1 (DNMT1) is believed to be responsible for copying the DNA methylation pattern within the mother cell and maintaining the pattern of genomic methylation within the daughter cell (Szyf 1991). Several lines of evidence suggest a role for DNMT1 in cellular transformation (MacLeod and Szyf 1995) (Wu, Issa et al. 1993; MacLeod, Rouleau et al. 1995; Slack, Cervoni et al. 1999; Bigey, Ramchandani et al. 2000), indeed DNMT1 has been observed as being upregulated in multiple human cancers(Issa, Vertino et al. 1993; Robertson, Uzvolgyi et al. 1999), though some colorectal cancers have been observed to have loss of function mutations(Kanai, Ushijima et al. 2003). DNMT1 has therefore been proposed as a target for anti-cancer therapy (Szyf 1994). Indeed, preclinical studies using antisense to DNMT1 have already shown reversion of tumour growth both *in vitro* (Fournel, Sapieha et al. 1999) and *in vivo* (Ramchandani, MacLeod et al. 1997). Taken together these data suggest an important role for DNMT1 in both maintaining the epigenome and controlling cell cycle.

A loss of DNMT1 during replication would result in a loss of epigenetic information. Thus, it has been proposed that cells have developed multiple ways to coordinate the transfer of genetic and epigenetic information from mother to daughter cell during cellular division (Szyf 2001). Proposed mechanisms include cell cycle regulation of DNMT1 expression at both the transcriptional and posttranscriptional level (Szyf, Bozovic et al. 1991). However, such mechanisms only explain how cells are able to synthesize sufficient amounts of DNMT1 under normal conditions. We have shown that a knockdown of DNMT1 leads to a reduction in firing of origins of DNA replication (Knox,

Araujo et al. 2000) and S-phase (period of DNA synthesis) cell cycle arrest (Milutinovic, Zhuang et al. 2003). The mechanisms responsible for DNA replication inhibition following DNMT1 knockdown have been unknown. This type of response is also seen when replication forks are stalled during cell division. Cells respond to the appearance of single stranded DNA (Zou and Elledge 2003) that arise from stalled replication forks during DNA replication or DNA Damage by activating ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) effector kinases (Bao, Tibbetts et al. 2001) to initiate a signalling pathway (Falck, Petrini et al. 2002) that involves activation of the checkpoint kinases (ChK) leading to phosphorylation and degradation of cell division control protein (CDC)-25a. As a consequence the downstream effect is the decreased capacity to load CDC45 onto replication origins, therefore leading to impaired recruitment of replication complexes and DNA replication arrest. In this paper we define the pathway responsible for DNA replication arrest in response to loss of DNMT1 and show that it is similar to the pathway elicited by hydroxyurea a classic inducer of the DNA replication stress checkpoint. We also show that once this response is blocked, loss of DNMT1 leads to genomic hypomethylation. We propose that this response evolved to protect the epigenetic information from challenges to the DNA methylation machinery during cell division.

MATERIALS AND METHODS

Plasmid construction

DNMT1 cDNA (NM_010066(start nucleotide 547(3'UTR absent))) in pTOT1 vector was digested with *Hind*III and *Sma*I and cloned into pBluescript M13 sk+(Stratagene) This plasmid was then digested with *Hind*III and annealed with the following primers containing a *Bssh*II restriction site (5'-AGCTTGGCGCGCCA-3' and 5-'ACCGCGCGGTTCGA-3'). pBluescript DNMT1 plasmid was mutated using Stratagene Quickchange II XL Site directed Mutagenesis Kit at nucleotide 2925 of DNMT1 cDNA creating a stop codon. The

following primers were used: sense 5'-CGCTTCTACTTCCTCGAGGTCTAGAATTCAAAGACCAAGAAC-3' and anti-sense 5'-GTTCTTGGTGTTTGAATTCTAGACCTCGAGGAAGTAGAAGCG-3'. Mutations were confirmed by sequencing. Plasmids pEF6-DNMT1 WT and pEF6-DNMT1 ΔCAT were created by digestion of pBluescript DNMT1 and pBluescript DNMT1 ΔCAT plasmids with *Sma*I/*Bssh*II and insertion into pEF6/HisB vector (Invitrogen).

Cell Culture, transfections and drug treatments

T24, a human bladder transitional carcinoma-derived cell line, A549, a human non-small cell lung carcinoma cell line, and NIH/3T3, a mouse fibroblast cell line, were obtained from the ATCC (HTB-4, CCL-185 and CRL-1658). Normal human fibroblasts and Seckel syndrome fibroblasts were obtained from the CCR (GM01887 and GM18366). T24 cells were maintained in McCoy's medium, A549 cells were grown in Dulbecco's modified Eagle's medium (low glucose), NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (high glucose), Normal human fibroblasts and ATR mutant fibroblasts were grown in minimum essential medium. The cells were supplemented with 10% fetal calf serum, 2mM glutamine, 100 units/ml penicillin, and 100ug/ml streptomycin. 18-24 hours prior to either antisense or siRNA treatment, cells were plated at a concentration $4x10^5$ cells in a 100mm tissue culture dish or $5x10^4$ cells in a 6 well plate or $1x10^4$cells in a 24 well plate. The phosphorothioate oligodeoxynucleotides used in this study were previously described(Milutinovic, Zhuang et al. 2003), human DNMT1 antisense oligonucleotide (DNMT1 AS, 5'-AAGCATGAGCACCGTTCTCC-3') and its mismatch control (Con AS, 5'-AACGATCAGGACCCTTGTCC-3'), which has a 6-base pair difference from DNMT1 AS (Fournel, Sapieha et al. 1999). The following siRNAs were used in this study, human DNMT1 siRNA (siDNMT1, Antisense strand 5'-GCAUGAGCACCGUUCUCCdTdT-3'), siRNA mismatch control (siControl, Antisense strand 5'-UGGAGAGCACCGUUCUCCdTdT-3'), human ATR siRNA (siATR (SMARTpool siRNA)) and mouse 3'UTR DNMT1 siRNA (simDNMT1, Antisense strand 5'-AAUAAAGCGUUCUGUCAACC-3') were obtained from

Dharmacon. Antisense oligonucleotides were transfected using Lipofectin (Invitrogen) in serum-free Opti-MEM. The oligonucleotide-containing Opti-MEM medium was removed from the cells and replaced with regular growth medium after 4h. The cells were harvested after 24h. siRNA was transfected using Lipofectamine (Invitrogen) in serum-free Opti-MEM. The oligonucleotide-containing Opti-MEM medium was removed from the cells and replaced with regular growth medium after 4h. Stable cell lines were produced using NIH/3T3 cells transfected with pBabe.puro and either pEF6, pEF6-DNMT1 WT or pEF6 DNMT1 ΔCAT using the $CaPO_4$ method, at a ratio of 1:20 of pBabe:pEF6 constructs. Cells were selected using Puromycin (3μg/mL) for 2 weeks. Individual clones were isolated and seeded to 24-well dishes and were screened for protein expression. For 5-aza-CdR treatment, cells were grown in regular culture medium in the presence of 10^{-6}M 5-aza-CdR (Sigma) dissolved in DMSO. The cells were treated for 24h.

Western Blot Analysis

50μg of nuclear protein, whole cell or chromatin bound extracts and 25ug of histone extracted protein extracts protein extract were fractionated on a 7.5%-12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane. Chromatin bound protein extraction was performed by sonication of nuclei pellet isolated by nuclear extaction. The following antibodies were used: human DNMT1 (New England Biolabs), mouse DNMT1 (Abcam, ab5208-100), CDC25a (Santa Cruz , F-6), CDC25b (Santa Cruz, C-20), β-Actin ((Sigma, A5316), PCNA (Santa Cruz, PC10), phospho-Chk2 (Thr68) (Santa Cruz, sc-16297-R), Chk2 (Oncogene, Ab-1), phospho-Chk1 (Ser 317) (Calbiochem, DR-1025), Chk1 (Santa Cruz , sc-8408), Xpress (Invitrogen, 46-0528), γH2A.X (Upstate, 05-636), and pan-Histone 3 (Upstate, #30374). Membranes were blocked in 5% milk in TBT with 0.05% Tween (TBST) for an hour, then exposed to antibody in the TBST 5% milk solution overnight at 4°C. The membranes were washed with TBST then exposed to either anti-rabbit HRP-IgG or anti-mouse HRP-IgG in TBST 5%

milk or TBST, respectively, for one hour at room temperature. The signal was then developed using ECL kit from Amersham (#135,136). Quantification was performed by measurement of relative optical density. To calculate the final signal for each sample, the ROD value of the antibody signal was divided by the ROD value of the band within the actin lane. To calculate the normalized final signal of the pChk2 samples, the ROD value of the pChk2 band was divided by the ROD value of the Chk2 band.

Immunocytochemistry and Confocal Microscopy

T24 cells were plated on coverslips in 24 well plates. After treatment the cells were washed with PBS and fixed with 4% paraformaldehyde. Cover slips were washed with PBS and blocked with 10% horse serum and 0.1%Triton X-100 (Sigma) in PBS (PBST) for 1h. Coverslips were stained with either □H2AX antibody (Upstate, JBW103), DNMT1 antibody (New England Biolabs) or ATR antibody (Santa Cruz Biotechnology, N-19) in PBST and 5% horse serum for an hour. The coverslides were washed with PBST and stained with either Alexa fluor 488 anti rabbit IgG, Alexa fluor anti rabbit 548, Alexa Fluor 633 anti-rabbit IgG or Alexa fluor 568 anti goat IgG (Molecular Probes). The cover slips were then washed with PBST and mounted on slides using Immuno-mount. 5 methyl-Cytosine staining was performed by fixation with 4% paraformaldehyde for 30 minutes, washed twice with PBS and blocked with 10% Fetal Bovine Serum in PBST for 2h. DNA was denatured in 2N HCl and 0.1%TritonX-100 in PBS for 45 minutes at 37^0C. The cells were neutralized with 0.1M Sodium Borate solution, washed with PBS and stained with a mouse monoclonal anti 5-methyl-Cytosine antibody (a gift from Dr. Alain Niveleau) in PBST. The cells were washed with PBS and stained with Alexa fluor 488 anti mouse IgG (Molecular Probes) PBS. The coverslips were washed with PBS and were mounted on slides using Immuno-count. DAPI staining was performed by staining cells with DAPI (Sigma) in PBS for 5 min and washed with PBS. The staining was analysed using LSM 520 Laser Scanning microscopy, version 2.5 (Zeiss). The levels of ATR, DNMT1,

γH2AX and 5-methyl Cytosine staining were quantified (MCID™ Elite Image Analysis Software, Imaging Research Inc. city).

Thymidine Incorporation

The rate of DNA synthesis was determined 24 hours after initiation of treatment with either ATR or DNMT1 siRNAs by measuring [^{3}H]-thymidine incorporation into DNA following a 6-h pulse with 66μCi/ml [^{3}H]-thymidine. Pulsed cells were washed five times with PBS and incubated in 10% Trichloroacetic acid at 4^{o}C for 12h. Cells were washed twice with 5% trichloroacetic acid for 30 minutes. The cells were lysed with 1% SDS in 1N NaOH and the level of [^{3}H]-thymidine incorporation was measured in a liquid scintillation counter.

5-Methylcytosine quantification by nearest neighbour analysis

Levels of 5-methylcytosine was quantified by nearest-neighbour analysis as described (Ramsahoye 2002). The intensity of 5-methylcytosine and cytosine mononucleotide spots was measured using a phospohimager screen and Image Quant image analysis program. Levels of unmethylated cytosine content are expressed as a percentage of [Cytosine]/[Cytosine + MethylCytosine].

RESULTS

DNMT1 knockdown activates a cascade of genotoxic-stress checkpoint proteins.

We have previously shown that DNMT1 knock down leads to inhibition of firing of origins of DNA replication (Knox, Araujo et al. 2000) and to an intra S-phase arrest of DNA replication (Milutinovic, Brown et al. 2004). The mechanisms responsible for these effects of DNMT1 knock down were unknown. Checkpoint kinases (ChKs) are known to mediate the response to replication stress (Falck, Petrini et al. 2002), which also results in inhibition of firing of origins of DNA replication and arrest of DNA synthesis. We reasoned that DNMT1 knock down might be targeting a similar pathway. We therefore examined the effect of DNMT1 depletion on the phosphorylation status of the Chk1 and Chk2 effectors and subsequent downstream CDC25a expression. Human bladder transitional carcinoma-derived T24 cells treated with DNMT1 (DNMT1 AS) antisense oligonucleotide, which targets the 5' UTR of human DNMT1 mRNA (Fig 5. F), showed significantly ($P<0.05$) decreased levels of DNMT1 protein in comparison to the cells treated with mismatch control oligonucleotide (AS Control) (Fig. 1 a,b). Although DNMT1 antisense significantly (*$P<0.05$) increased levels of Chk2 phosphorylation in comparison to the control treated cells (Fig. 1 a,c), absolute levels of ChK2 were not significantly (*$P<0.05$) altered by DNMT1 antisense treatment. DNMT1 antisense increased levels of Chk1 phosphorylation in comparison to the control treated cells (Fig. 1.I), while overall levels of Chk1 were not altered by DNMT1 antisense treatment. In addition, DNMT1 antisense significantly (*$P<0.05$) decreased levels of CDC25a protein in comparison to the cells treated with mismatch control oligonucleotide (Fig. 1 a,d). These results are consistent with the effect of replication stress (Chaturvedi, Eng et al. 1999), where activation of Chk2 by phosphorylation during DNA replication block leads

to degradation of the downstream effector CDC25a phosphatase (Falck, Mailand et al. 2001; Hassepass, Voit et al. 2003).

To exclude the possibility that the observed response was due to non-specific effects of antisense oligonucleotide treatment, we examined the effect of DNMT1 depletion using DNMT1 siRNA (siDNMT1), which targets the 5' UTR of human DNMT1 mRNA in a region separate from DNMT1 AS (Fig 5. f). Treatment of T24 cells with siDNMT1 significantly (*P<0.05) decreased levels of DNMT1 protein in comparison to the cells treated with the non-specific control siRNA (Fig. 1 e,f). Although, siDNMT1 significantly (*P<0.05) increased the levels Chk2 threonine-68 phosphorylation (Fig. 1 e,g), absolute levels of Chk2 were not significantly (*P<0.05) altered (Fig. 1 e). siDNMT1 significantly (P<0.05) decreased levels of CDC25a protein in comparison to the cells treated with non-specific siRNA control (Fig. 1 e,h**)**. To determine if the effect of DNMT1 depletion on CDC25 protein levels is not limited to the a isoform, we observed a decrease in protein levels of CDC25b (Fig 1 j), which has been implicated in the intra S-phase checkpoint(Manke, Nguyen et al. 2005). The fact that these checkpoint proteins were activated by two different methods of DNMT1 depletion supports the conclusion that checkpoint activation is due to the absence of DNMT1 rather than a side effect of the specific treatment.

To determine whether this cascade of events is common to cells derived from different cancer tissues we examined whether DNMT1 depletion would increase levels of Chk2 phosphorylation in the non-small cell lung carcinoma A549 cell line. Treatment of A549 cells with DNMT1 antisense oligonucleotide decreased levels of DNMT1 protein in comparison to the cells treated with the mismatch oligonucleotide control (Fig. 2). Although, DNMT1 antisense oligonucleotide increased levels of Chk2 threonine-68 phosphorylation, absolute levels of ChK2 were not altered. These results show that the causal relation between knock down of DMNT1 and checkpoint activation is not unique to T24 cells.

DNMT1 depletion induces γH2AX foci formation.

A hallmark in the cells response to replication stress is the formation of histone variant γH2AX foci, a characteristic marker of cells undergoing replication stress (Furuta, Takemura et al. 2003). We first examined the effect of DNMT1 depletion on histone variant γH2AX foci formation. Treatment of T24 cells with either DNMT1 AS or siDNMT1 lead to histone variant γH2AX foci formation in comparison to the cells treated with either the con AS or siCon, respectively (Fig. 3 a,b). To confirm the results seen through immunocytochemistry, we observed γH2A.X induction by western blot in DNMT1 depleted T24 cells compared to control by DNMT1 AS or siDNMT1 treatment(Fig. 3 c,d). These results lead us to conclude that induction of γH2AX foci formation occurs as a consequence of replication stress induced by depletion of DNMT1, which resembles studies that germ cells lacking DNMT3l are positive for γH2AX foci (Bourc'his and Bestor 2004).

ATR is necessary for the replication stress checkpoint.

In the replication stress pathway, ATR kinase is involved in the phosphorylation and foci formation of histone variant γH2AX (Cortez, Guntuku et al. 2001; Shiloh 2001). We tested whether ATR is required for the effect of DNMT1 depletion on histone variant γH2AX foci formation. Treatment of T24 cells with 100nM of ATR siRNA significantly (* $P < 0.05$) decreased ATR immunoreactivity in comparison to the cells treated with siCon (Fig. 4 a,b). T24 cells treated with siATR did not elicit γH2AX foci formation (Fig. 4 c). Cells treated with siDNMT1 lead to γH2AX foci (Fig. 4C), however when DNMT1 was depleted in cells treated with siATR γH2AX foci formation was significantly reduced (* $P < 0.05$) (Fig. 4 d). Replication arrest by drugs like aphidicolin and hydroxyurea cause DNA synthesis arrest that results in ATR activation(Ward and Chen 2001; Florensa, Bachs et al. 2003; Ishimi, Komamura-Kohno et al. 2003). It is possible therefore that the activation of ATR by DNMT1 knock-down is an indirect consequence of DNA synthesis

arrest, which was previously shown to be induced by DNMT1 depletion (Knox, Araujo et al. 2000), as well as a characterized intra S-Phase arrest (Milutinovic, Zhuang et al. 2003). This replication inhibition was relieved however when ATR was knocked down concurrently with DNMT1. To confirm if ATR is necessary for the replication arrest caused by DNMT1 depletion, we utilized fibroblast cells that were established from a patient with Seckel syndrome(Goodship, Gill et al. 2000). These cells have been characterized as having a mutation that resulted in an alteration in splicing leading to a loss of exon 9 and an introduction of a stop codon in exon 10(O'Driscoll, Ruiz-Perez et al. 2003) as a consequence ATR transcript and protein levels are markedly decreased. Control fibroblasts, established from an unaffected patient, elicited an induction of γH2A.X when treated with siDNMT1 compared to siCon (Fig. 4 f). However Seckel syndrome cells, when treated with siDNMT1 did not have an induction of γH2A.X. These results combined with the attenuated γH2A.X induction by ATR knockdown in siDNMT1 treated cells indicates that ATR is required for the replication arrest induced by DNMT1 depletion.

Loss of DNMT1 leads to global hypomethylation once the DNA stress response pathway is blocked.

We then tested the hypothesis that the stress response elicited by DNMT1 depletion serves to protect the genome from disruption of the DNA methylation pattern by replication in the absence of maintenance methylation. We therefore examined the effect of a double knock down of ATR and DNMT1 on the global state of methylation. Using a nearest neighbour analysis to determine the cytosine levels in the ATR and DNMT1 siRNA treated T24 cells (Fig 4 g,h), the global cytosine levels were revealed to be unaltered in the siATR treated cells as compared to control treated cells, as well as the siDNMT1 treated cells as compared to control treated cells. However, the double knock down of siATR and siDNMT1 lead to a significant increase in the fraction of unmethylated cytosines in the genome (*$p < 0.05$) as compared to the control treated

cells as well as to single knock-downs of either ATR or DNMT1. Our data implies that the arrest of DNA replication following DNMT1 depletion prevents loss of DNA methylation. This data reveals the functional significance of the ATR mediated response to depletion of DNMT1.

DNMT1 depletion response is insensitive to global hypomethylation.

To determine whether DNMT1 depletion in the nucleus or demethylation of DNA signals the response described here we took advantage of the classic DNA methylation inhibitor 5-aza-2'-deoxycytidine (5azaCdR) (Milutinovic, Zhuang et al. 2003). The nucleoside analogue is incorporated in the DNA and inhibits the DNA methylation transfer activity of DNMT1 and other DNMTs by entrapment, but does not inhibit the *de novo* synthesis of DNMT1 protein or its residence in the nucleus (Fig. 5 d,e) (Jones 1985). 5azaCdR causes an immediate loss of global methylation. If the signal that triggers the response to DNMT1 depletion is global demethylation, then 5-azaCdR should elicit a response similar to the DNMT1 knockdown. We first compared the effect of DNMT1 knockdown and 5-aza-CdR on genomic methylation using previously characterized anti 5-methylcytosine antibodies. Levels of genomic methylation in T24 cells treated with siDNMT1 did not significantly differ in comparison to the cells treated with siCon (Fig. 5 a,b) whereas, T24 cells treated with 1µM 5-aza-CdR showed significant (*$p<0.05$) DNA demethylation, as indicated by the loss of 5-methyC immunoreactivity (Fig. 5 a,b). The cells were then harvested and nuclear protein levels were measured by western blot analysis. As expected, protein extracts of T24 cells treated with 5-aza-CdR showed no immunoreactivity to the DMNT1 primary antibody (Fig. 5 c), implying that the free *DNMT1* had been significantly depleted. However, when DNMT1 levels were examined in extracts prepared from chromatin bound protein, DNMT1 was not depleted by 5-azaCdR treatment (Fig. 5 c). These results are consistent with a mechanism of action of 5-azaCdR, where it traps DNMT1 in the nucleus(Schermelleh, Spada et al. 2005), rather than deplete DNMT1 protein levels through mRNA degradation like siRNA or antisense.

Although, levels of Chk2 threonine-68 phosphorylation remained undetected, absolute levels of ChK2 protein were not significantly (*p <0.05) altered by 5-aza-CdR treatment (Fig. 5 c). Since it has recently been shown that 5-azaCdR could cause degradation of DNMT1 protein in addition to its trapping on the DNA(Ghoshal, Datta et al. 2005), we further confirmed the presence of DNMT1 in the nuclei of 5-azaCdR treated cells by immunostaining (Fig. 5 d) for DNMT1. Although there was some small reduction of DNMT1 in T24 cells treated with 5-AzaCdR, as compared to control cells, DNMT1 was clearly present in contrast to T24 cells treated with DNMT1 siRNA where it was essentially absent. These results confirm previous findings that 5-azaCdR retards replication but does not initiate intra S-phase arrest (Milutinovic, Zhuang et al. 2003). This is consistent with the hypothesis that depletion of DNMT1 in the nucleus rather than DNA hypomethylation, initiates the replication arrest response described here.

DNMT1 depletion response is not dependent on the DNMT catalytic domain

To examine if the effect observed in this study are dependent on the catalytic function of DNMT1, we constructed a series of mouse DNMT1 expressing vectors, one containing the full length wild type DNMT1, as well as a second containing a DNMT1 with a stop codon causing a deletion of the catalytic domain (Fig. 5 f). Stable NIH/3T3 cell lines were produced expressing these constructs and validated by examination of the Xpress epitope (Fig. 5 f). Utilizing the absence of the 3'UTR in the pEF6-DNMT1 WT and pEF6-DNMT1 ΔCAT, compared to the endogenous mouse DNMT1, we utilized siRNA specific for mouse DNMT1, simDNMT1, (Fig. 5 f) that would deplete the cells of endogenous DNMT1 (Fig. 5 g) but that would not affect exogenous Xpress-tagged DNMT1 protein levels (fig. 5 f). Furthermore we examined the effect of the presence of exogenous DNMT1, in an endogenously depleted DNMT1 cell. Empty vector cells depleted of DNMT1 were observed to have an induction of γH2A.X, which corresponds with our observations seen in T24 cell lines (Fig 3). Cells with pEF6-DNMT1 WT did not have an induction of γH2A.X, as well as cells with pEF6-DNMT1 ΔCAT. This leads us to

conclude that presence of exogenous DNMT1 rescues the cells from the replication arrest checkpoint induced by a DNMT1 depletion regardless of whether the catalytic domain is present, suggesting that this checkpoint is independent of the catalytic function of DNMT1.

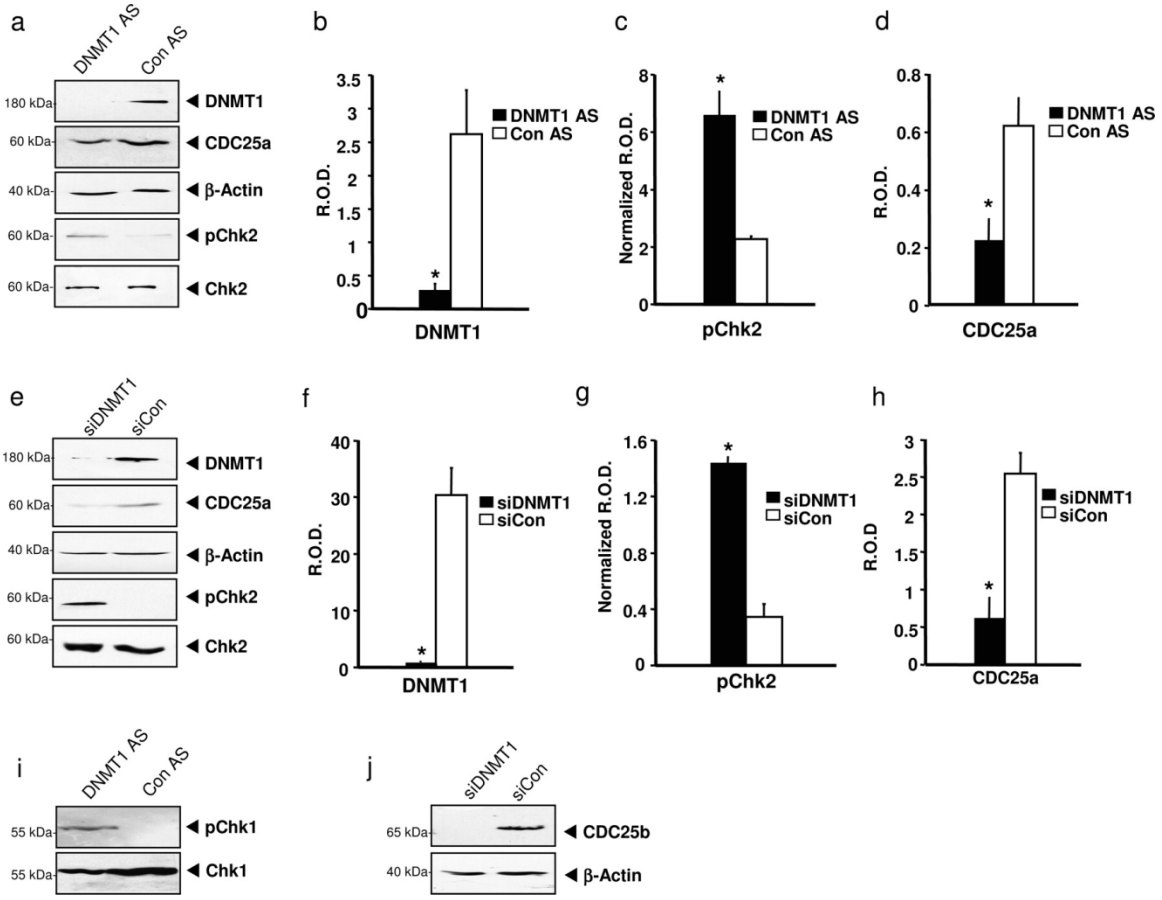


Figure 1. DNMT1 knockdown activates a cellular response utilizing components of the replication stress checkpoint. (A) Representative western blot analysis of protein levels of DNMT1, CDC25a, β-Actin, pChk2 and Chk2, in nuclear extracts prepared from T24 cells treated with either 50nM of DNMT1 antisense (DNMT1 AS) or its mismatch control (Con AS) for 24h. **(B,D)** Relative optical density of DNMT1 and CDC25a (ROD; mean ± S.E.M.) immunoreactivity (n=3, **P*<0.05) (student's t-test). **(C)** Normalized relative optical density of pChk2. (Relative ROD; mean ± S.E.M) immunoreactivity (n=3; **P*<0.05) (student's t-test). **(E)** Representative western blot analysis of protein levels of DNMT1 and replication stress checkpoint response proteins; CDC25a, β-Actin, pChk2 and Chk2 in nuclear extracts prepared from T24 cells treated with either 120nM of DNMT1 siRNA (siDNMT1) or its siRNA control (siCon) for 24h. **(F,H)** Relative optical density of DNMT1 and CDC25a (ROD; mean ± S.E.M.) immunoreactivity (n=3, **P*<0.05) (student's t-test). **(G)** Normalized relative optical density of pChk2 (Relative ROD; mean ± S.E.M) immunoreactivity (n=3; **P*<0.05) (student's t-test). Protein levels were compared to β-Actin loading control, except pChk2, which was compared to Chk2 protein levels. **(I)** Western blot analysis of protein levels of pChk1 and Chk1 in nuclear extracts from T24 cells treated with either 50nM of DNMT1 antisense (DNMT1 AS) or its mismatch control

(Con AS) for 24h. **(J)** Western blot analysis of protein levels of CDC45b and β-Actin in nuclear extracts from T24 cells treated with either 120nM of siDNMT1 or siCon for 24h.

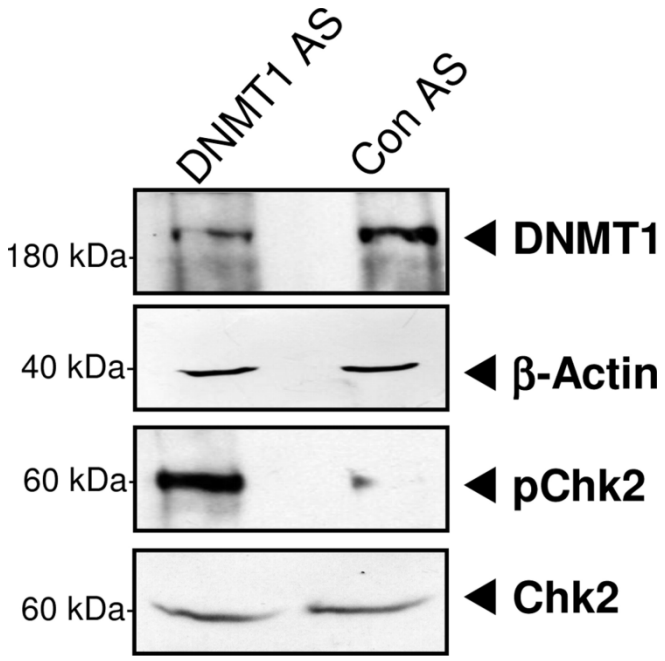


Figure 2. Knockdown of DNMT1 protein levels in A549 cells induce pChk2. (A) Western blot analysis of protein levels of DNMT1, β-Actin, Chk1 and Chk2 in nuclear extracts prepared from A549 cells which were treated with either 120nM of DNMT1 AS or Con AS, to determine if DNMT1 depletion dependent replication stress checkpoint induction is cell line specific.

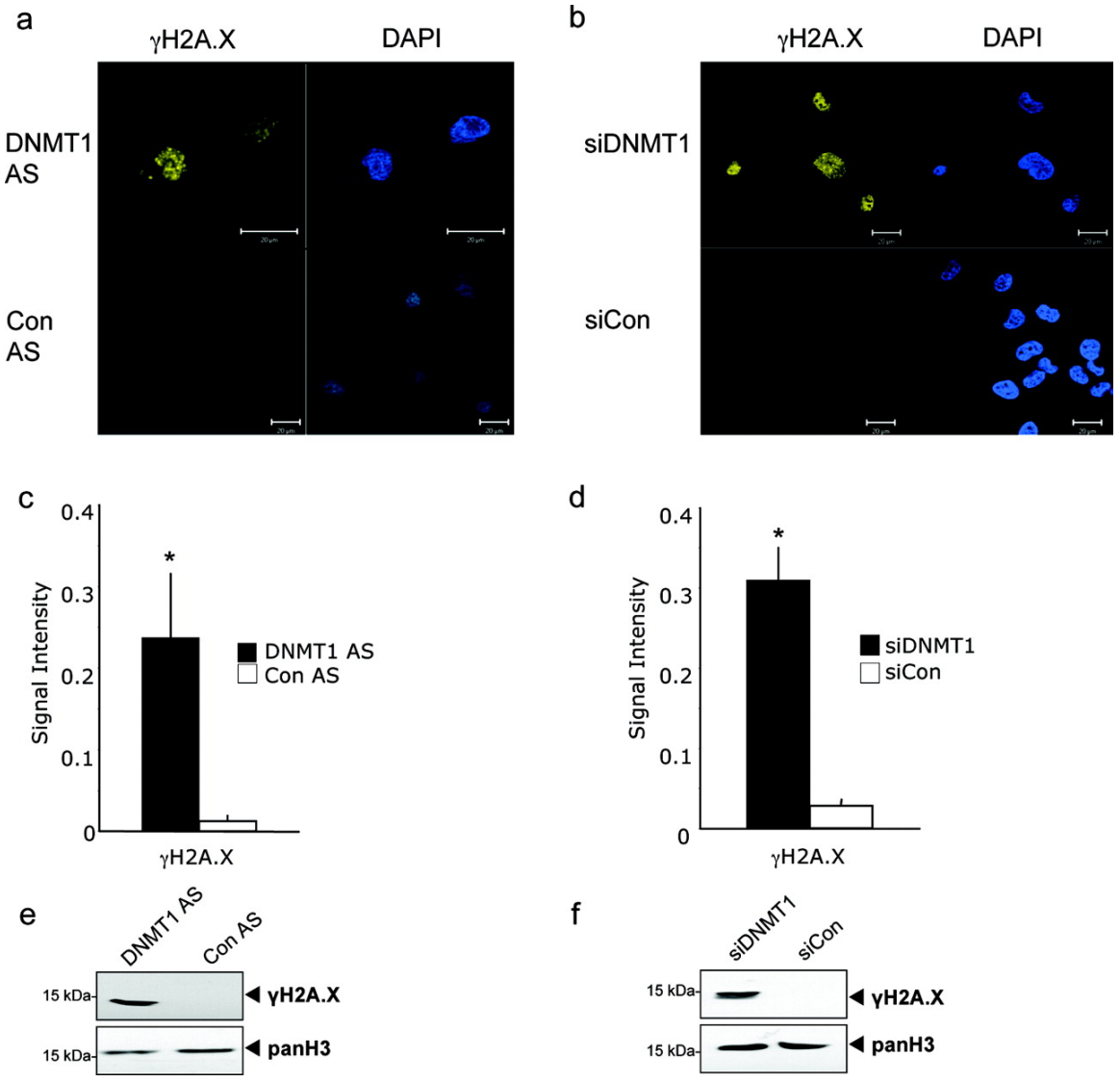
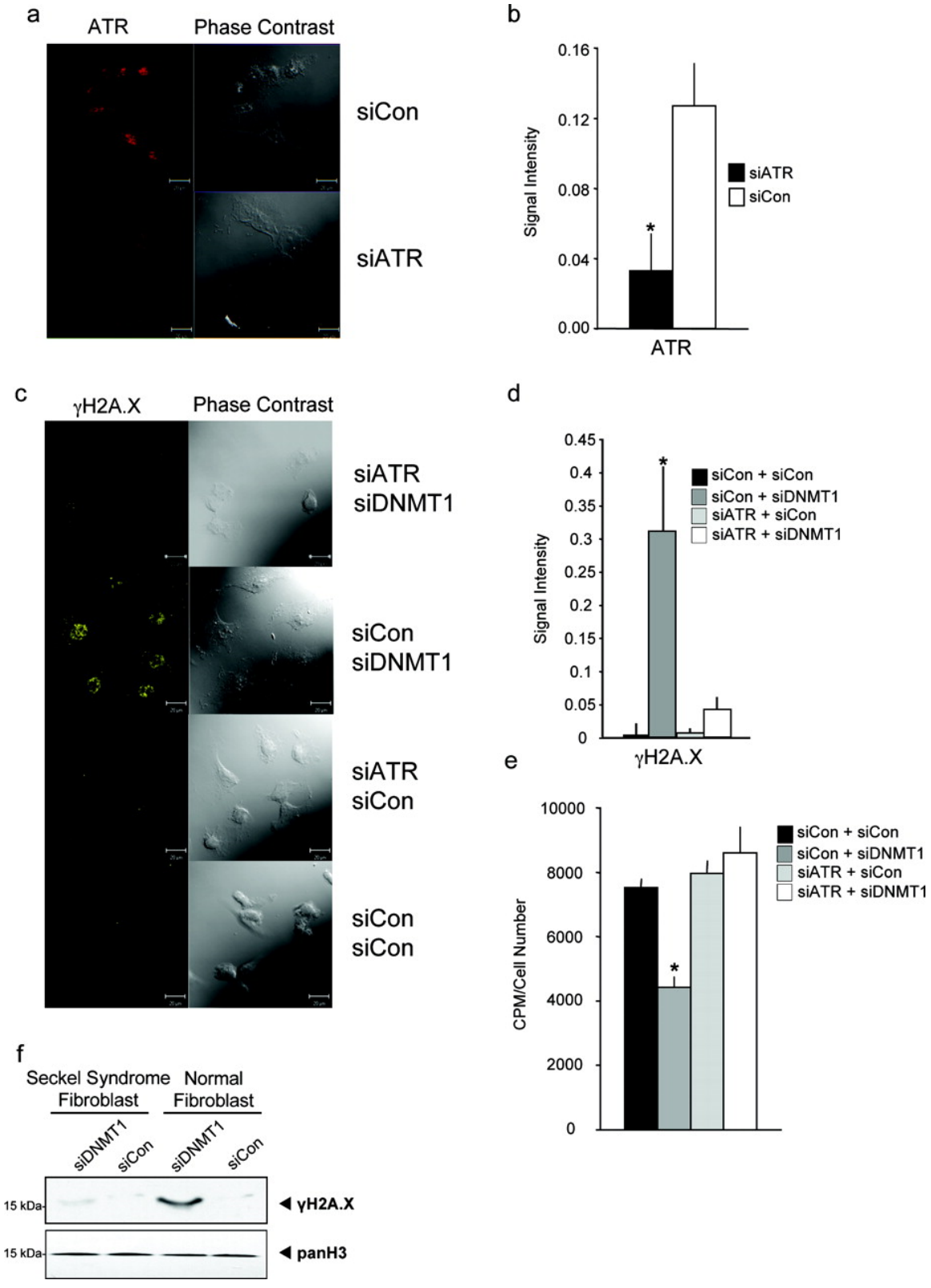


Figure 3. DNMT1 depletion induces γH2AX foci formation. (A) T24 cells treated with 50nM of DNMT1 AS or Con AS for 24h were co-immunostained with the anti-γH2AX antibody, which recognizes phosphoSer139-H2AX and DAPI to visualize DNA. **(B)** T24 cells treated with 120nM of siDNMT1 or siCon for 24h were co-immunostained with the anti-gH2AX antibody to recognize phospho-H2AX and DAPI to recognize DNA. **(C)** Western blot analysis of protein levels of γH2A.X and pan-H3 in histone extracts prepared from T24 cells which were treated with either 50nM of DNMT1 AS or Con AS for 24h. **(D)** Western blot analysis of protein levels of γH2A.X and pan-H3 in histone extracts prepared from T24 cells which were treated with either 120nM of siDNMT1 or

siCon for 24h. **(e)** Western blot analysis of protein levels of γH2AX and pan-H3 in histone extracts prepared from T24 cells which were treated with 50 nM DNMT1 AS or Con AS for 24 h. **(f)** Western blot analysis of protein levels of γH2A.X and pan-H3 in histone extracts prepared from T24 cells which were treated with 120 nM siDNMT1 or siCon for 24 h.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Signal Intensity
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γH2A.X
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CPM/Cell Number
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siCon + siCon
siCon + siDNMT1
siATR + siCon
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*
f
Seckel Syndrome Fibroblast
Normal Fibroblast
siDNMT1
siCon
siDNMT1
siCon
15 kDa
γH2A.X
15 kDa
panH3

Figure 4

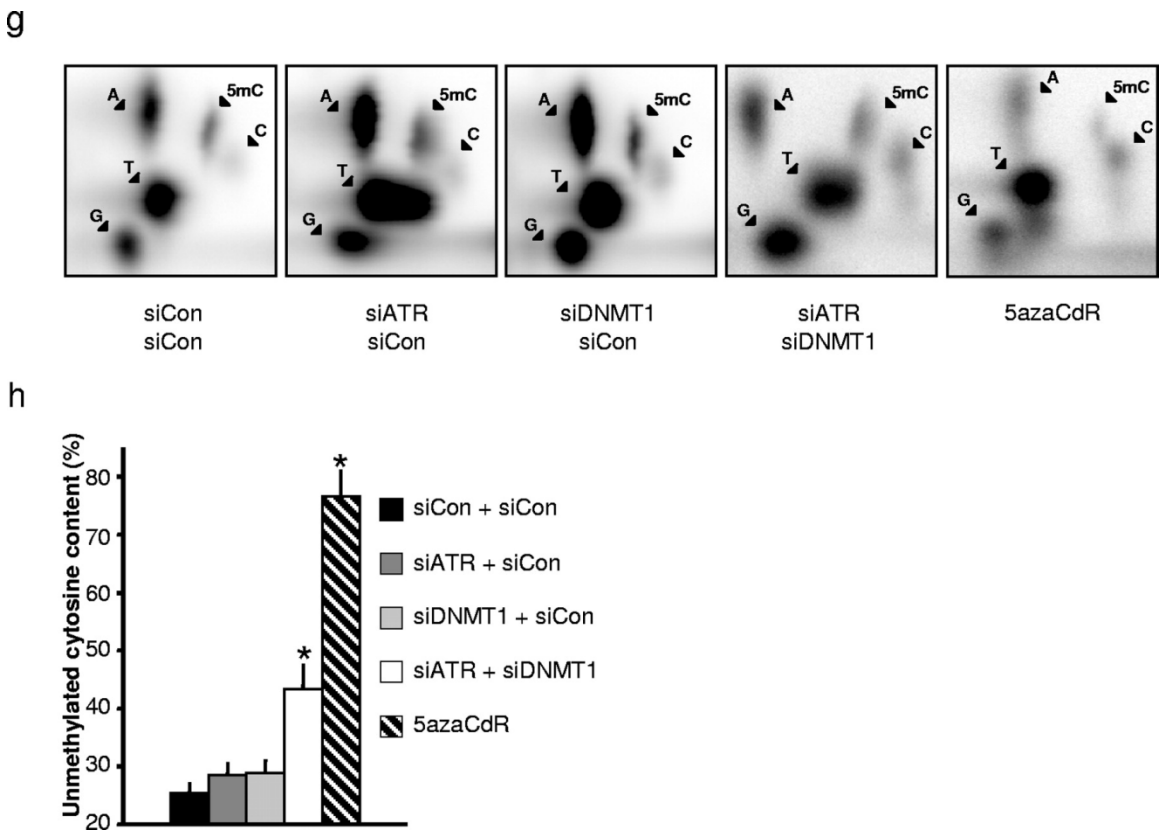
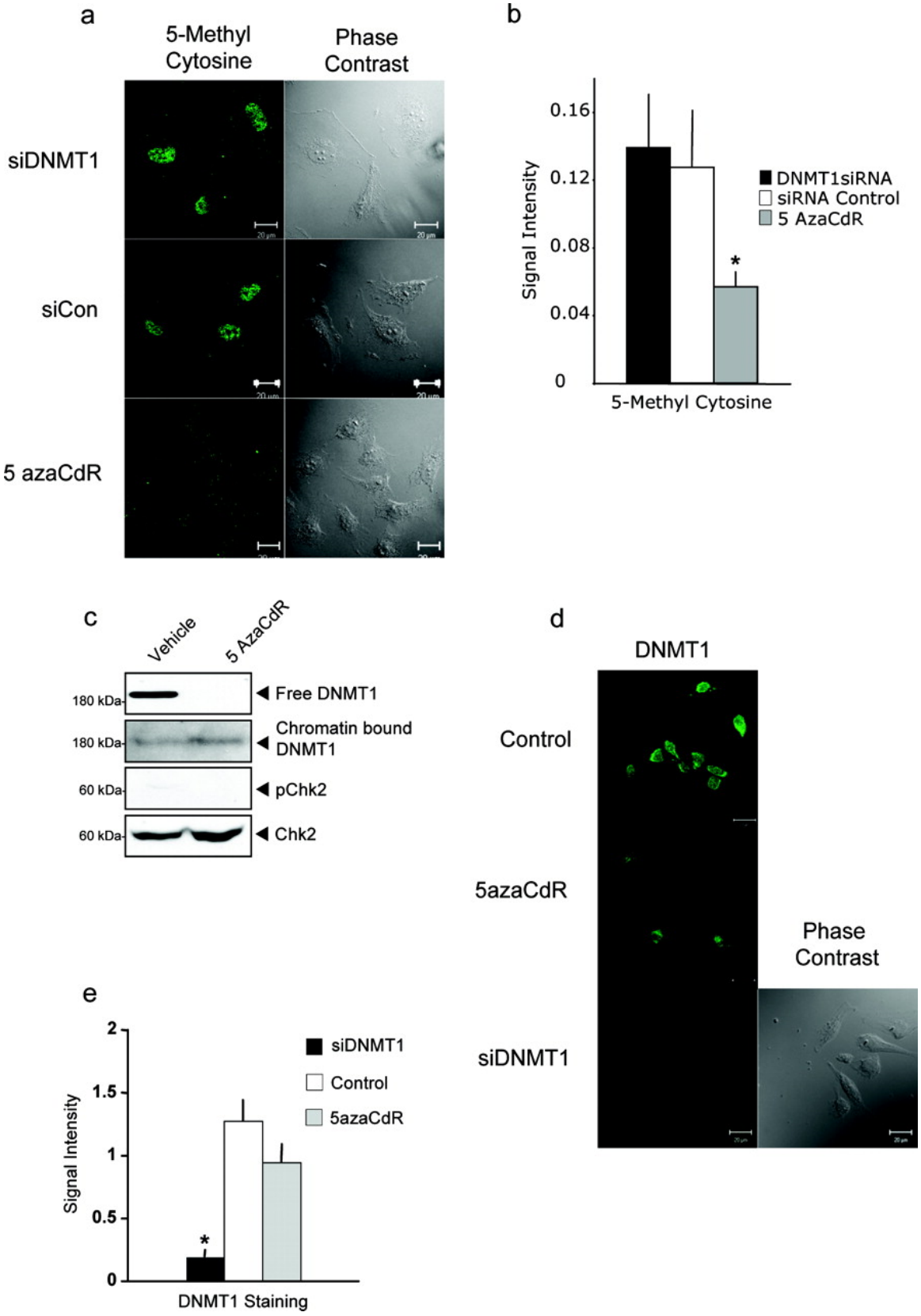


Figure 4 (continued). Replication stress induction due to DNMT1 depletion is dependent on ATR. (A) Representative confocal microscopic images of T24 cells treated with 100nM of ATR siRNA (siATR) or siCon for 24h immunostained with the anti-ATR antibody. **(B)** Quantification of ATR immunoreactivity of T24 cells treated with 100nM of siATR or siCon for 24h stained with the anti-ATR antibody. (Signal Intensity; mean ± S.E.M.) (n=3; *P< 0.05) (student's t-test). **(C)** Representative confocal microscopic images of T24 cells treated with 100nM of siATR or siCon for 24h followed by treatment with 120nM siDNMT1 or siCon control for 24h immunostained with the anti-γH2AX antibody to recognize phosphoSer139-H2AX. **(D)** Quantification of ATR immunoreactivity of T24 cells treated with 100nM of siATR or siCon for 24h followed by treatment with 120nM siDNMT1 or siCon for 24h and stained with the anti-γH2AX antibody. (Signal Intensity; mean ± S.E.M.) (n=3; *P< 0.05) (paired t-test). **(E)** Quantification of incorporated [^{3}H]-thymidine in T24 cells treated with 100nM of control siRNA, 100nM of ATR siRNA, 120nM of DNMT1 siRNA or 100nM ATR and 120nM DNMT1 siRNA (n=3; *P< 0.05) (paired t-test). **(F)** Western blot analysis of protein levels of γH2A.X and pan-H3 in histone extracts prepared from normal human fibroblast cells and Seckel syndrome fibroblasts cells which were treated with either 100nm of siDNMT1 or siCon for 48h. **(G)** Representative images of Cytosine and Methylcytosine of treatment groups Control, siATR, siDNMT1, siDNMT1 and siATR, 1μM 5azaCdR determined by nearest neighbour analysis. **(H)** Quantification of % of unmethylated cytosine content of treatment groups (n=3; *P< 0.05) (paired t-test).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siCon
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b
Signal Intensity
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siRNA Control
5 AzaCdR
*
5-Methyl Cytosine
c
Vehicle
5 AzaCdR
180 kDa
Free DNMT1
180 kDa
Chromatin bound DNMT1
60 kDa
pChk2
60 kDa
Chk2
d
DNMT1
Control
5azaCdR
siDNMT1
Phase Contrast
e
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0
siDNMT1
Control
5azaCdR
*
DNMT1 Staining

Figure 5

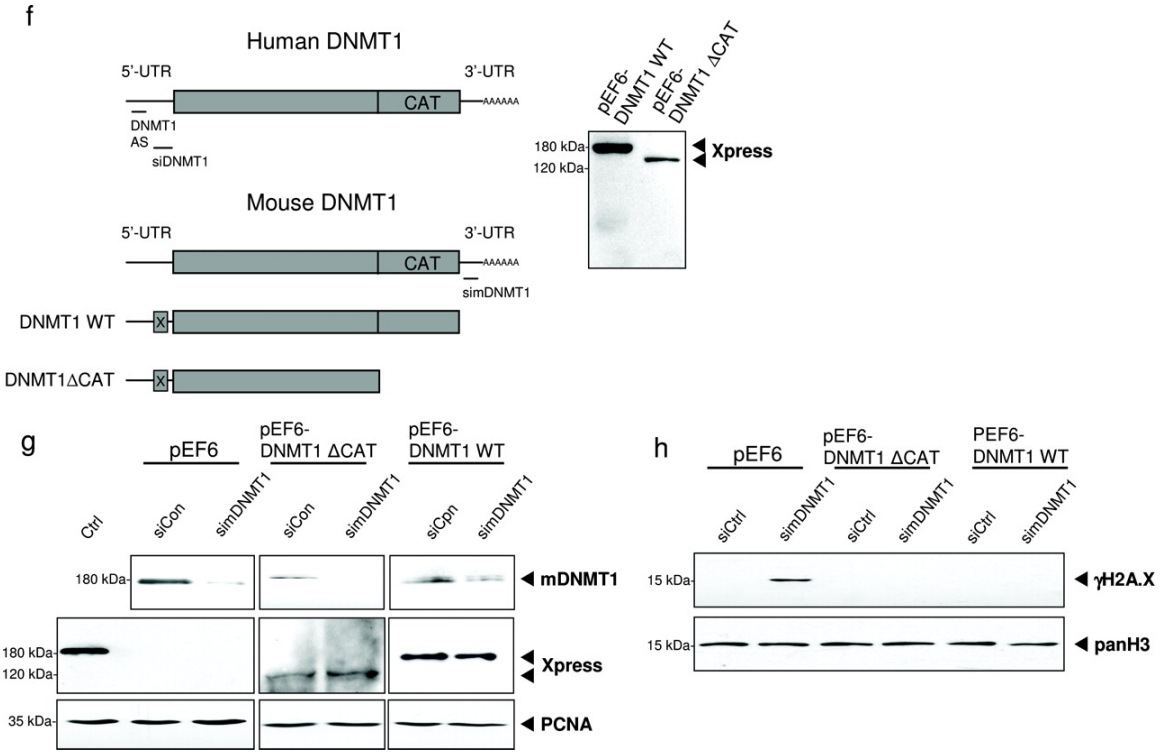

Figure 5 (continued). Depletion of DNMT1 does not induce genomic hypomethylation while genomic hypomethylating agents do not induce replication stress. (A) Representative stained T24 cells treated with 120nM siDNMT1, siCon or 1μM 5-azaCdR immunostained with the anti-5-methyl-Cytosine antibody. **(B)** Quantification of 5-methyl cytosine immunoreactivity of T24 cells treated with 120nM siDNMT1, siCon or 1μM 5-azaCdR (intensity; mean ± S.E.M.) (n=3; *P<0.05) (paired t-test). **(C)** 5-azaCdR treatment depletes free DNMT1 but not chromatin bound DNMT1 but does not induce replication stress. Western blot analysis of protein levels of whole cell DNMT1, chromatin bound DNMT1, pChk2 and Chk2 in whole cell extracts prepared from T24 cells were treated with 1μM 5-azaCdR or vehicle control (DMSO). **(D)** Representative T24 cells treated with 1μM 5azaCdR for 24h or 120nM siDNMT1 were stained with the anti-DNMT1 antibody and visualized by phase contrast microscopy. **(E)** Quantification of DNMT1 immunoreactivity of T24 cells treated with 1μM 5-azaCdR for 24h, 120nM siDNMT1 or control cells (n=3; *P<0.05) (paired t-test). **(F)** DNMT1 constructs and siRNAs specific for the different UTR regions of DNMT1 used in this study. Human DNMT1 AS and siDNMT1 target the human 3'UTR but do not have overlapping regions. Mouse DNMT1 siRNA (simDNMT1) targets the 3'UTR present in the endogenous mouse DNMT1. DNMT1-WT has an Xpress epitope present in the 5'UTR, and has no 3'UTR. DNMT1-ΔCAT has an Xpress epitope present in the 5'UTR, a deletion in the catalytic doman, as well as no 3'UTR. Western blot analysis of levels of Xpress-tagged proteins in nuclear extracts preapred from NIH 3T3 cells stably transfected with either pEF6 DNMT1-WT or pEF6 DNMT1ΔCAT. **(G)** Western blot analysis of protein levels of endogenous mouse DNMT1 (mDNMT1), Xpress and PCNA in nuclear extracts prepared

from either control cells, HEK 293 transiently transfected with pEF6 DNMT1-WT, or NIH 3T3 cells stably transfected with pEF6 empty vector, pEF6 DNMT1-WT or pEF6-ΔCAT which were treated with either 100nm of simDNMT1 or siCon for 48h. **(H)** Western blot analysis of protein levels of γH2A.X and pan-H3 in histone extracts prepared from NIH 3T3 cells stably transfected with pEF6 empty vector, pEF6 DNMT1-WT or pEF6-ΔCAT which were treated with either 100nm of simDNMT1 or siCon for 48h.

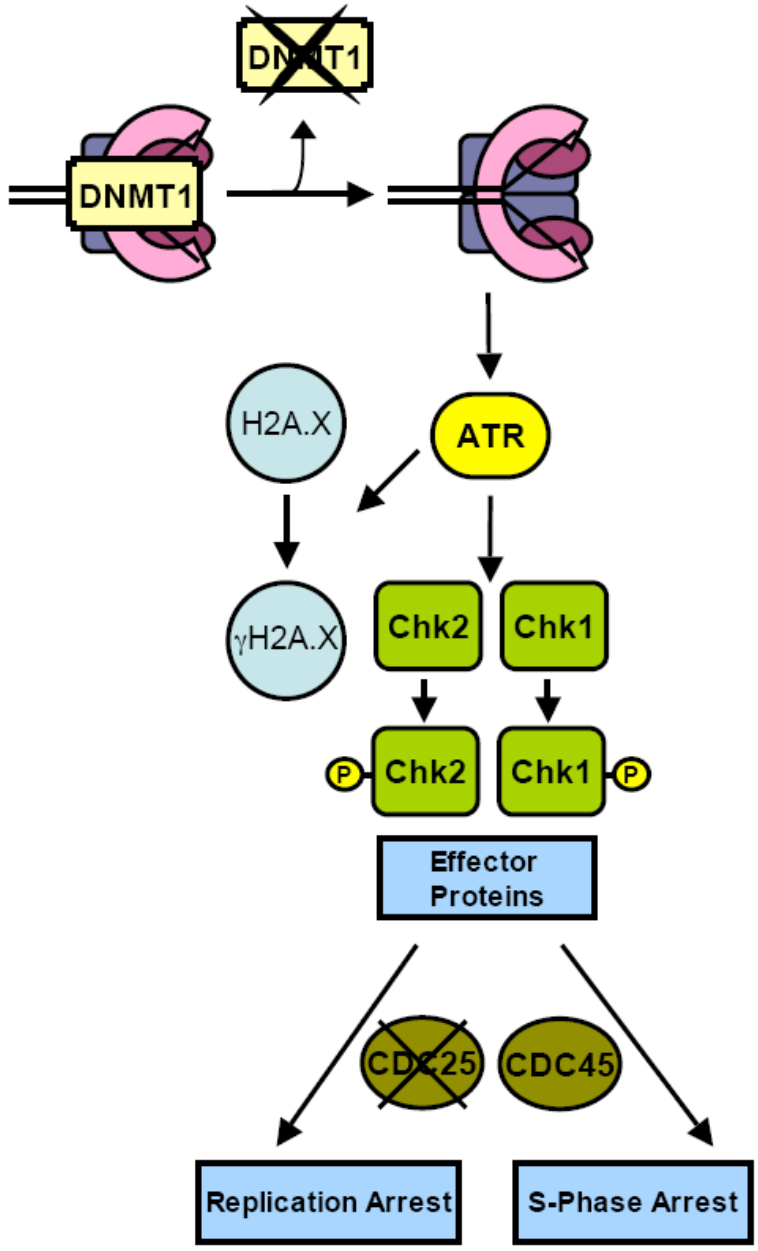

Figure 6. Model of replication arrest checkpoint through DNMT1 depletion. DNMT1 is normally present in the replication fork. Depletion of DNMT1 similar to depletion of other fork resident proteins, leads to activation of ATR and the downstream pathway; phosphorylation of γH2AX and Chk1/Chk2, leading to CDC25a phosphorylation and degradation. CDC25a phosphatase activity is required for activation of CDC45(Falck, Petrini et al. 2002) thus degradation of CDC25a leads to decreased CDC45 loading onto the replication origin. This leads to an overall decreased capacity for the replication complex to load onto origins, leading to an arrest in DNA replication as well as a block in progression of S-Phase.

DISCUSSION

Loss of DNMT1 during replication would result in loss of epigenetic information, triggering reprogramming gene expression of a subset of genes. Loss of DNA methylation could result in activation of silenced genes similar to an activating mutation or rearrangement of enhancer and promoter elements, which could be a consequence of a genotoxic challenge. Additionally, a loss of epigenetic information could lead to hypomethylation of repetitive sequences, which could lead to activation of retrotransposable elements, leading to chromosomal rearrangements, an event similar to the effects of DNA damage. Thus, it stands to reason that a cell must protect itself from epigenetic challenge as it protects itself from genotoxic challenges. Therefore, cells have developed different strategies to prevent such epigenetic erasure. We depleted DNMT1 protein levels using antisense to DNMT1, as well as two different DNMT1 siRNAs targetting separate regions of DNMT1 mRNA. We have shown that DNMT1 depletion initiates a cell response, which utilizes similar effectors to the replication stress check-point induced by DNA damage and replication fork arrest and is dependent on the upstream kinase ATR. Cells utilize common downstream effectors to respond to different upstream events. Indeed, DNMT1 depletion initiates the same signalling pathway common to the DNA damage response (Zhou and Elledge 2000; Shiloh 2003). This cellular response provides a mechanism for the replication arrest induced by DNMT1 depletion.

The ATR dependent cellular response described here is sensitive to DNMT1 depletion rather than demethylation *per se* since a similar response is not triggered by a DNA demethylation agent (Fig. 5 c). ATR is known to be activated by a wide variety of genotoxic challenges as well as uncoupling of DNA replication proteins from the replication fork (Pohler, Otterlei et al. 2005).

DNMT1 depletion might cause loss of DNA methylation as previously observed in *dnmt1*-/-mice. We have recently shown however that DNMT1 depletion leads to very limited genomic demethylation (Milutinovic, Zhuang et al. 2003). We previously proposed that the intra S-phase arrest in DNA replication protected the cells from demethylation upon reduction in DNTMT1 levels (Milutinovic, Zhuang et al. 2003). Similarly, DNMT1 knockout by homologous recombination in human colorectal cancer cells resulted in very limited inhibition of DNA methylation suggesting that other DNMTs compensate for the loss in DNMT1 (Rhee, Bachman et al. 2002; Ting, Jair et al. 2004). Nevertheless this data does not exclude the possibility that a small level of hemimethylated DNA generated in the course of synthesis of nascent DNA in the absence of DNMT1 signalled activation of checkpoint signalling pathway. This hypothesis is not supported however by the lack of Chk2 activation in response to the DNA methylation inhibitor 5-azaCdR (Fig. 5 c). Although 5-azaCdR induced extensive global hypomethylation as shown in (Fig. 4 f,g and 5 a), it did not elicit the phosphorylation of Chk2 similar to DNMT1 knock down (Fig. 5 c). This is consistent with our previous observations that short term 5-azaCdR treatment did not induce an intra S-phase cell cycle arrest (Milutinovic, Zhuang et al. 2003). Thus, our data suggests that it is the absence of the DNMT1 protein from the fork, which elicits this epigenetic checkpoint. This might be a general rule for other epigenetic proteins such as CAF1 and histone modifying enzymes (Gaillard, Martini et al. 1996; Hoek and Stillman 2003; Ye and Adams 2003; Ye, Franco et al. 2003). It is possible that a global mechanism exists which senses the absence of the full complement of chromatin modifying enzymes in the fork and activates ATR leading to a checkpoint resulting in immediate arrest of DNA replication in the S phase of the cell cycle.

Our study unravels many of the components of the pathway leading from DNMT1 depletion to S-phase arrest. DNMT1 depletion in the replication fork leads to ATR activation and as a consequence its downstream effectors such as Chk1 and Chk2 are activated by phosphorylation, leading to phosphorylation and degradation of cell division control protein (CDC)-25a phosphatase and therefore decreased capacity of its

substrate CDC45 to load onto replication origins leading to an overall arrest of replication and S-Phase (Fig. 6). The model proposes a potential two phase effect of DNMT1 depletion, where the immediate effect of DNMT1 depletion causes an arrest in DNA replication through the response outlined in this study, as well as a transcriptional effect previously described (Milutinovic, Zhuang et al. 2003; Milutinovic, Brown et al. 2004) whereby a DNMT1 depletion causes induction of stress response genes. The data presented here shows that cells possess a mechanism to sense impending epigenetic catastrophe such as loss of DNMT1 during DNA replication. Malfunctioning of this response could lead to replication in the absence of DNMT1 and loss of DNA methylation.

ACKNOWLEDGEMENTS

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Chapter 7

General Discussion

The aim of this thesis is to examine the regulation of DNMT1 within the cell cycle and the impact of this regulation on cellular status. More specifically, this thesis defines: 1) The mechanism through which DNMT1 mRNA levels are regulated during the different phases of the cell cycle; 2) How AUF1, which regulates the cell-cycle specific expression of DNMT1, is itself regulated with the cell cycle; 3) The impact of deregulation of the cell-cycle phase specific expression of DNMT1 on genomic DNA methylation; and 4) the impact that DNMT1 depletion has on DNA replication and the cell cycle in cancer cells. The findings described in the previous chapters provide evidence for the cell-cycle-phase specific regulation of DNMT1 mRNA, by a protein known as AUF1. RB regulates AUF1 protein levels, linking nodal cell-cycle regulatory circuits and DNMT1. Disruption of regulation of DNMT1 at several levels leads to site-specific methylation. The polycomb protein EZH2 targets DNMT1 to specific positions in the genome. Surprisingly, depletion of DNMT1 in cancer cells induces a DNA damage replication arrest pathway suggesting a novel mechanism of action for DNMT inhibitors in cancer therapy. The following discussion chapter summarizes the results of this thesis and expands on how these results might potentially revise our basic understanding of what occurs during the initial steps of cellular transformation. The potential therapeutic implications of these findings for treatment of cancer will also be discussed.

7.1. DNMT1 regulation

As previously discussed in the literature review, it has been observed that DNMT1 mRNA levels vary throughout the cell cycle. As the cell enters into S-phase and

DNA replication begins, DNMT1 mRNA levels increase and as a consequence DNMT1 protein levels are elevated. This is thought to occur in order to provide sufficient DNMT1 molecules to copy the methylation pattern onto the nascent DNA strands. Upon completion of replication, the cell enters into G_2-phase and continues through the cell-division cycle. DNMT1 mRNA levels decrease and remain at low levels until the cell enters into S-phase. This biphasic nature of DNMT1 is believed to maintain the levels of DNMT1 during the S-phase of the cell cycle and to preclude aberrant *de novo* methylation of non-replicating DNA during other phases of the cycle. Interestingly, although DNMT1 mRNA and protein levels change through the cell cycle, DNMT1 transcription remains constant. Previous work by Detich determined that a post-transcriptional regulatory element in the 3'UTR of DNMT1 mRNA was responsible for the cell cycle phase-specific regulation of DNMT1 mRNA levels (Detich, Ramchandani et al. 2001). A 40 kDa protein was found to bind this element in G_1-phase and was proposed to facilitate the destabilization of DNMT1 mRNA. Interestingly, when this regulatory element in DNMT1 mRNA was deleted, levels of DNMT1 were stabilized at similar levels throughout the cell cycle and the cells expressing this DNMT1 deletion mutant underwent cellular transformation. This study reveals that this regulation of DNMT1 mRNA serves to protect the cells from transformation and that disruption of this level of regulation could harm the integrity of the cell. In chapter 4 we identified the 40kDa protein that bound DNMT1 mRNA as AUF1. We discovered that AUF1 bound DNMT1 mRNA and destabilized it through recruitment of the exosome complex. Furthermore AUF1 destabilized DNMT1 in the cell cycle specific manner since expression of AUF1 protein was also regulated with the cell cycle. AUF1 levels were at their highest in G_0/G_1-phase thus explaining the decrease in DNMT1 mRNA during this phase, while AUF1 protein levels decreased upon entry into S-phase, leading to a subsequent stabilization and increase in DNMT1 mRNA levels. When AUF1 was depleted by siRNA targeting, we observed an increase in overall DNMT1 levels and consequently genomic hypermethylation.

DNMT3b mRNA but not DNMT3a mRNA is also regulated in a cell cycle specific manner (Robertson, Keyomarsi et al. 2000). We were unable to show that AUF1 negatively regulated DNMT3b mRNA. Understanding cell cycle specific DNMT3b mRNA regulation is important since DNMT3b might be involved in aberrant methylation of tumor suppressor genes in cancer. While AUF1 does not play a role in destabilizing DNMT3b mRNA, other members of the hnRNP family could be involved. In total there have been over 100 hnRNPs identified. Furthermore, another protein involved in mRNA stability could play a role in DNMT1 and DNMT3b mRNA stability. HuR is a protein that binds ARE in the 3'UTR of target mRNA and stabilizes the mRNA from degradation by blocking the factors needed for degradation (Peng, Chen et al. 1998). In fact HuR has been shown to compete for similar regions of a target mRNA with AUF1 and has the opposite effect of AUF1 on stability of similarly targeted mRNAs (Blaxall, Pende et al. 2002; Lal, Mazan-Mamczarz et al. 2004). HuR could play a role in the increase of DNMT1 (and DNMT3b) mRNA as the cell enters into S-phase through binding and stabilizing the mRNA. It is possible that the balance between AUF1 and HuR could dictate DNMT1 mRNA stability. In G_1-phase we observed increased AUF1 protein, which could tilt the balance towards destabilizing DNMT1 mRNA. Perhaps in S-phase the decreased AUF1 in conjunction with an increase of HuR could lead towards increase stability of DNMT1. More work is required to understand how AUF1 levels vary with the progression of the cell cycle, as well as the role of HuR in regulation of DNMT1 mRNA.

Deregulation of DNMT1 is a common event in cancer. It is possible that impairment of AUF1 could lead to the increased levels of DNMT1 that are observed in cancer. Some studies suggest that alterations in AUF1 could lead to changes in expression of target genes in neoplastic lung tissue (Blaxall, Dwyer-Nield et al. 2000) and in breast cancer (Mazan-Mamczarz, Hagner et al. 2008), However more work is required to understand the role of AUF1 in cancer and how alterations of mRNA stability can play a role in cancer phenotype.

7.2. AUF1 regulation

We observed that DNMT1 regulation was linked to the fact that AUF1 protein levels varied in the cell cycle. We next examined how the AUF1 protein itself was regulated. In chapter 5 we determined that the AUF1 protein was regulated through the actions of Hsp70 and Rb. Specifically, Rb positively upregulated AUF1 and upon Rb depletion AUF1 protein was degraded in a proteasome dependent manner. The exact mechanism of AUF1 protein degradation has yet to be fully elucidated. This degradation was dependent upon the presence of Hsp70. Previous studies have shown that AUF1 is regulated by the proteasome and Hsp70 (Laroia, Cuesta et al. 1999; Laroia, Sarkar et al. 2002), which was confirmed by our first study. We proposed that the mechanism of AUF1 regulation occurred as follows: In the G_1-phase Rb remains active and prevents AUF1 degradation. Entry into S-phase is characterized by inactivation of Rb function through phosphorylation (Murphree and Benedict 1984). Inactive Rb can no longer prevent the degradation of AUF1. Therefore AUF1 protein levels decrease as the cell enters into S-phase, and consequently DNMT1 levels increase. These observations establish a clear link between a critical cell cycle protein and AUF1 regulation. More interestingly, this association between Rb and AUF1 ties DNMT1 regulation into cancer. Rb inactivation is a common mutation that is found in cancer. We propose that upon Rb mutation in cancer, AUF1 protein stability decreases leading to increased DNMT1. We postulate that deregulation of the normal interrelationship between Rb, AUF1 and DNMT1 is a critical step in cancer development.

The studies performed in chapter 4 and 5 describe a mechanism for regulation of the epigenome with the cell cycle. Previous studies have shown that methylation patterns vary within the period of one cellular division (Brown, Fraga et al. 2007). Relative levels of DNA methylation were determined to be at their lowest during G_1,

while they increased upon entry into S-phase. It was determined that this increase in DNA methylation occurred at specific gene promoters without an alteration of the DNA methylation patterns of the repetitive elements. These observed alterations in DNA methylation patterns could be controlled by the cell-cycle variation in the levels of DNMT1 by Rb and AUF1. Site-specific changes in DNA methylation might be regulated by DNMT1-EZH2 interaction as observed in chapter 5.

7.3. Site-specific methylation

We observed in chapter 5 that deregulation of the Rb-AUF1 resulted in an increase in global DNA methylation levels. While cancer is characterized by global hypomethylation and regional hypermethylation, we focused here on DNMT and DNA methylation. We observed that changes in DNA methylation in response to a global increase in DNMT1 occurred at specific promoters. We determined that upon increase of DNMT1 in response to Rb depletion there was increased interaction between DNMT1 and EZH2, confirming a previous study that demonstrated physical interaction between DNMT1 and EZH2 (Vire, Brenner et al. 2006). This increased association led to the specific methylation of known EZH2 targets KCNA and CNR1 (Vire, Brenner et al. 2006), but not the promoter of the housekeeping gene β-actin. This gene-specific methylation was dependent on EZH2. These results indicate that depletion of Rb leads to site-specific methylation as a consequence of elevated DNMT1 levels associating with EZH2 and methylating EZH2 target sites. Interestingly, we observed that the promoter of tumour suppressor p16/INK4a was methylated in response to Rb depletion. This reveals a potential mechanism for methylation of the p16 gene promoter in cancer. Previous studies (Ohm, McGarvey et al. 2007; Schlesinger, Straussman et al. 2007) have observed that genes in untransformed cells that are marked with trimethylation of H3K27 become methylated in cancer, suggesting that EZH2, the histone methyltransferase responsible for H3K27, could mediate the DNA methylation of these genes through association with DNMT1. But it was unclear why these sites remained unmethylated in normal cells but

became methylated in cancer. We have shown in chapter 5 a mechanism whereby disruption of an Rb pathway, a common event in cancer, leads to increased methylation of sites marked for EZH2 association.

7.4. DNMT1 and replication arrest

Anti-cancer therapies that target DNMT1 are being examined by several groups as treatment for cancer. Previous studies revealed that specific knock down of DNMT1 in cancer cells using antisense oligonucleotides led to replication arrest and induction of genotoxic stress response genes (Knox, Araujo et al. 2000; Milutinovic, Zhuang et al. 2003). These effects were different than the effects caused by pharmacological inhibition of DNA methylation using the classical DNA methylation inhibitor 5-azaC. This suggested that inhibition of DNMT1 might exert an anticancer effect by a DNA demethylation independent mechanism. In chapter 6 we demonstrated that depletion of DNMT1 by a sequence-specific siRNA activated the replication arrest checkpoint, or the DNA damage checkpoint, whose function is to halt DNA replication and the cell cycle when damage to genome is detected or improper replication complexes are formed (Callegari and Kelly 2007). The endpoints of this pathway are either repair of the DNA damage or alternatively induction of programmed cell death if the repair fails (Lopes, Foiani et al. 2006). Induction of an apoptotic pathway upon DNMT1 depletion was originally hypothesized when DNMT1 genetic knockout mice where shown to be embryonic lethal (Li, Bestor et al. 1992). Our data suggests that the cell senses the lack of DNMT1 in the replication fork as genomic damage. The mechanism is still unclear but it might involve protein-protein interactions. After publication of this chapter other studies have replicated our work and determined that depletion of DNMT1 in human cancer cells led to induction of γH2A.X, a marker for pathway induction, and cell death through induction of the pathway (Chen, Hevi et al. 2007). Possible explanations for the biological role of the induction of DNA damage response upon DNMT1 depletion are as follows.

The first hypothesis is that induction of this pathway may serve to protect the integrity of the epigenome. If DNMT1 were depleted from the cell, DNA methylation sites would gradually be lost through passive demethylation. This would ultimately lead to deregulation of gene expression and aberrant activation of retrotransposons and repetitive elements. One interesting aspect of this study was that induction of the pathway upon DNMT1 depletion occurred independently of DNA methylation. This suggests that the DNA damage pathway responds to loss of DNMT1 protein in the fork as opposed to loss of DNA methylation. The fact that the response senses lack of DNMT1 protein rather than loss of DNA methylation ensures a quicker response preempting DNA methylation loss rather than responding to it after it had occurred. Ultimately this pathway may protect against other challenges to the epigenome as well. Studies have shown that HDAC inhibition sensitizes the cells to γH2A.X foci formation (Geng, Cuneo et al. 2006; Zhang, Adachi et al. 2006). Knockout of DNM3L revealed that meiotic catastrophe occurred in spermatocytes in DNMT3L (Bourc'his and Bestor 2004), suggesting growth arrest and cellular death response to the absence of a DNMT. Most recently a study has shown that depletion of the HATs p300, CBP and P/CAF by siRNA led to H2AX phosphorylation and foci formation as well as mitotic arrest (Ha, Kim et al. 2009). The induction of cell cycle arrest and H2AX phosphorylation upon depletion of these epigenetic enzymes support the hypothesis that there are cognate cellular responses to epigenetic challenge.

The second hypothesis for the role of induction of the damage response pathway in response to DNMT1 depletion relates to the role of DNMT1 in cancer. A study in 2005 postulated that the replication arrest checkpoint serves as a barrier to prevent tumourigenesis upon induction of oncogenes (Bartkova, Horejsi et al. 2005). They observed in urinary bladder, breast, lung and colon cancer that as cancer progressed from normal tissue to early superficial lesions (Ta), earliest invasive (T1) and more advanced primary carcinomas (T2–4) markers of the DNA damage response checkpoint were induced well before other molecular hallmarks of cancer, p53 mutation and genomic instability, occurred. They determined that formation and progression of

cancer requires the removal of this checkpoint. The results of our study in chapter 6 show that increased levels of DNMT1 prevent the induction of the DNA damage response pathway by DNA damaging agents. We propose that overexpression of DNMT1 in cancer, in addition to its known role in silencing tumour suppressor genes, serves to inhibit the replication arrest checkpoint/DNA damage pathway induced in response to oncogene activation and thus allows cellular transformation to go on. The exact mechanism of how DNMT1 blocks the DNA damage checkpoint remains unclear; however work is currently being done in our laboratory to determine the mechanisms through which DNMT1 blocks the DNA damage response pathway.

The results of this thesis have allowed us to propose the following model on the involvement of DNMT1 deregulation in cancer. Inactivation of Rb, through mutation or gene silencing, leads to increased degradation of AUF1 (Figure 7.1.). This in turn leads to increased stability of DNMT1 mRNA and consequential increases in DNMT1 protein and enzymatic activity. Increased DNMT1 commandeers EZH2 and leads to site-specific methylation of tumour suppressor genes, which prevents the inhibition of cellular growth. In addition elevated DNMT1 levels serve to inhibit the induction of the replication arrest/DNA damage checkpoint and further prevent any inhibitory block on cellular transformation. Ultimately these two events eliminate two major inhibitory systems that would normally protect from cellular transformation.

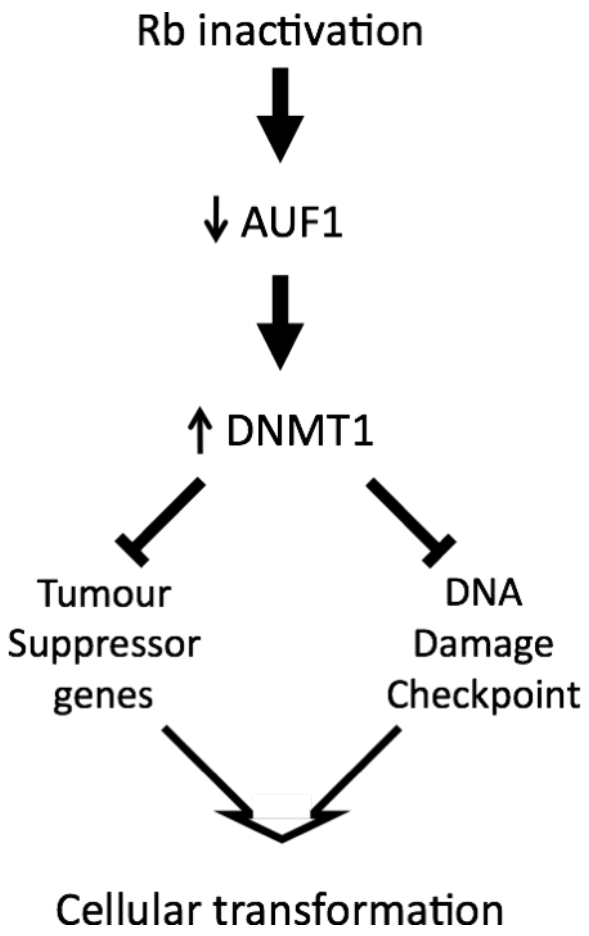

Figure 7.1. Thesis model. Rb inactivation leads to decreased AUF1 levels. DNMT1 increases and methylates tumour suppressor genes and blocks the DNA damage checkpoint. These actions facilitate cellular transformation.

7.5. Future directions in therapy

The results of this thesis allow us to consider new possibilities in targeting the epigenome in cancer therapy. Classically, the DNA methylation activity of DNMT has been targeted. One new direction is to focus on the activities that target DNMTs to

specific areas of the genome rather than overall methylation. By causing global hypomethylation using general DNA methylation inhibitors, we bear the risk of induction of oncogenes and metastatic genes through demethylation. In chapter 5 we investigated the role of DNMT1 and EZH2 in methylating specific promoters. This however does not discount other possible DNMT1 containing complexes. As mentioned in section 2.8, other complexes between DNMTs and targeting proteins have been discovered. By utilizing therapies that target these complexes we can alleviate site-specific methylation. By targeting methylation of tumour suppressor genes without affecting silenced metastatic genes, we can take advantage of the anti-growth properties of DNMT inhibitors without inducing metastasis. Understanding all the potential complexes that the DNMTs and demethylases are involved in would allow us to delineate the scope of DNA methylation events controlled by these complexes. Additionally, further research is needed to understand how histone marks or DNA sequences serve as the recruitment marks for gene-specific DNA methylation.

The presence of a replication arrest pathway that responds to loss of DNMT1 independent of DNA methyltransferase function provides another approach to take advantage of the growth inhibition consequences of DNMT1 depletion while avoiding re-activation of metastatic genes. By targeting the DNMT1 protein rather than its catalytic activity, we could induce cell cycle arrest, DNA replication arrest and ultimately cell death without causing demethylation. Targeting specific proteins through siRNA and antisense technology has been shown to be difficult. Due to toxic side effects and difficulty in delivering the RNA molecules to their specific tissues, more research is needed to develop drugs that can deplete DNMTs specifically. More work is also required to understand how the cell can detect the absence of DNMT1. We hypothesize that this occurs as a result of an unknown protein-protein interaction. Drugs that could target and block this interaction could potentially induce this pathway. However, blocking protein-protein interactions by small molecules is not an easy task. Developing antisense oligonucleotides that deplete DNMT1 or siRNA based reagents might be the preferred route. Furthermore, by understanding whether other DNMTs, DNA

demethylases and histone modifying enzymes can induce this pathway could provide us with a wider range of epigenetic targets for cancer treatment.

Concluding Remarks

In conclusion, the studies presented in this thesis provide support for targeting DNMT1 in anti-cancer therapy by showing that the regulation of DNMT1 serves as a critical step in the progression of cellular transformation. Together, this work adds to the knowledge of how DNMT1 regulation occurs in the cell cycle, and contributes to unraveling the consequences for the epigenome and promoter methylation when this regulation is impaired. I also determined the mechanism by which DNMT1 depletion arrested the cell cycle and prevented cancer growth. As a whole, the results of this thesis help our understanding of cancer development by showing that deregulation of DNMT1, through common events in cancer, can lead to site-specific methylation events, as well as inhibition of a nodal genotoxic checkpoint.

CHAPTER 8

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