



*Ministero dell'Istruzione, dell'Università e della Ricerca*

*Università degli Studi di Palermo*

**Dottorato di Ricerca in**
**Genomica e Proteomica nella ricerca Oncologica ed Endocrino-Metabolica**

**Ciclo XXII: 2008-2010**
***Settore Scientifico Disciplinare: BIO/18***

# DNMT1 SILENCING ELICITS DIFFERENT CELL CYCLE RESPONSES IN PRIMARY VERSUS TUMOR CELLS AND IS ASSOCIATED WITH ANEUPLOIDY GENERATION

**Tesi di Dottorato di:**
*Dott.ssa Viviana Barra*

**Coordinatore:**
*Prof. Salvatore Feo*

**Tutor:**
*Prof. Aldo Di Leonardo*

# Cell cycle

The cell cycle, or cell-division cycle, is the series of events that take place in a cell leading to its duplication. In eukaryotic cells the cell cycle can be divided in two periods: interphase and mitosis (M) phase. In interphase cells grow, accumulating nutrients needed for mitosis and to duplicate its DNA. In the mitosis phase cells split themselves into two distinct cells, often called daughter cells. The interphase consists of three distinct phases: $G_1$ phase, synthesis phase (S), $G_2$ phase; M phase is itself composed of two tightly coupled processes: karyokinesis, in which the cell chromosomes are segregated between the two daughter cells, and cytokinesis, in which the cell cytoplasm divides in half forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called $G_0$ phase (fig.1).

Before a cell can enter cell division, it needs to take in nutrients. All of the preparations are done during the interphase. The first phase within interphase, from the end of the previous M phase until the beginning of DNA synthesis is called $G_1$ (G indicating *gap*), or the growth phase. During this phase the biosynthetic activities of the cell, which had been considerably slowed down during M phase, resume at a high rate. This phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication.

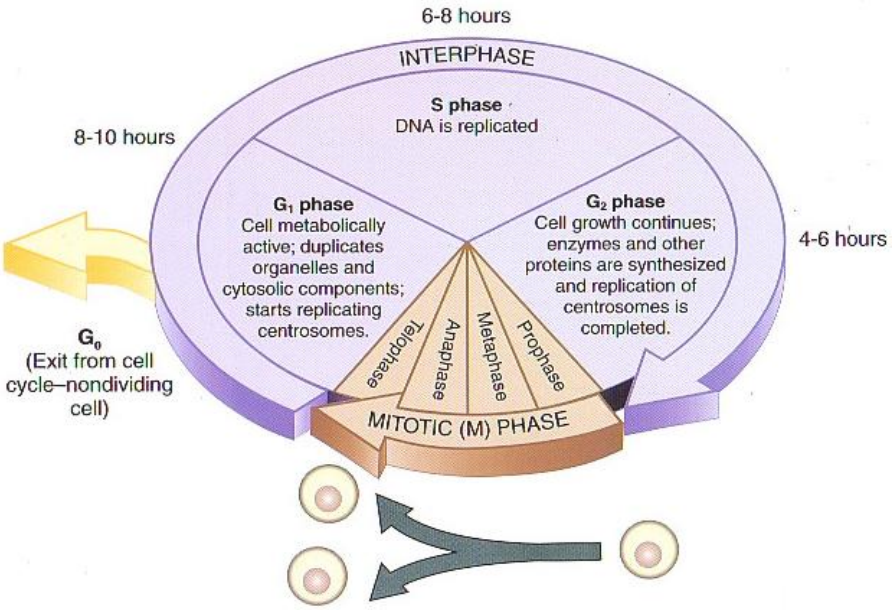



*Figure 1. Schematic view of cell cycle phases.*

Duration of $G_1$ is highly variable, even among different cells of the same species. The ensuing S phase starts when DNA synthesis commences; when it is complete all of the chromosomes have been replicated. Thus, during this phase, the amount of DNA in the cell has effectively doubled, though the ploidy of the cell remains the same because each chromosome has two sister chromatids. Rates of RNA transcription and protein synthesis are very low during this phase. An exception to this is histone production, most of which occurs during the S phase. Then the cell enters the $G_2$ phase, which lasts until the cell enters mitosis. Again, significant biosynthesis occurs during this phase, mainly involving the production of microtubules, which are required during the process of mitosis. The process of mitosis is complex and highly regulated. The sequence of events is divided into stages, corresponding to the completion of one set of activities and the start of the next. These stages are prophase, metaphase, ana-

phase and telophase. During mitosis the pairs of chromosomes condense and attach to fibers that pull the sister chromatids to opposite sides of the cell. The cell then divides in cytokinesis, to produce two identical daughter cells (fig. 1).

The Prophase, from the ancient Greek *πρό* (before) and *φάσις* (stage), is a stage of mitosis in which the chromatin condenses into a highly ordered structure called a chromosome in which the chromatin becomes visible and sister chromatids are attached to each other at a DNA element called the centromere. The two microtubule organizing center, an important organelle called centrosome, are pushed apart to opposite ends of the cell nucleus by the action of molecular motors acting on the microtubules. The nuclear envelope breaks down to allow the microtubules to reach the kinetochores on the chromosomes, marking the end of prophase.

In the following metaphase, from the ancient Greek *μετά* (between), chromosomes align in the middle of the cell before being separated into each of the two daughter cells. The centromeres of the chromosomes convene themselves on the *metaphase plate*, or *equatorial plate*, an imaginary line that is equidistant from the two centrosome poles. This alignment is due to the counterbalance of the pulling powers generated by the opposing kinetochores. Only after all chromosomes have become aligned at the metaphase plate, when every kinetochore is properly attached to a bundle of microtubules, does the cell enter anaphase. It is thought that one unattached or improperly attached kinetochore generates a signal that activates the mitotic Spindle Assembly Checkpoint (SAC), to prevent premature progression to anaphase, even if most of the kinetochores have been attached and most of the chromosomes have been aligned. During anaphase, from the ancient Greek *ἀνά* (up), each chromatid moves to opposite

poles of the cell, the opposite ends of the mitotic spindle, near the centrosomes. During early anaphase, or Anaphase A, the chromatids abruptly separate and move toward the spindle poles, thanks to the shortening of spindle microtubules. When the chromatids are fully separated, late anaphase, or Anaphase B, begins. This involves the polar microtubules elongating and sliding relative to each other to drive the spindle poles to opposite ends of the cell. Anaphase B drives the separation of sister chromatids to opposite poles through three forces. Kinesin proteins that are attached to polar microtubules push the microtubules past one another. A second force involves the pulling of the microtubules by cortex-associated cytosolic dynein. The third force for chromosome separation involves the lengthening of the polar microtubules at their plus ends. During telophase, from the ancient Greek "τελος" (end), two daughter nuclei form in the cell. The nuclear envelopes of the daughter cells are formed from the fragments of the nuclear envelope of the parent cell. Cytokinesis usually occurs at the same time that the nuclear envelope is reforming, yet they are distinct processes.

The passage of a cell through the cell cycle is well controlled by molecular events that check that each process occurs in a sequential fashion and it is impossible to reverse the cycle. Among the main players in animal cell cycle are cyclins, $G_1$ cyclins (D cyclins), S-phase cyclins (cyclins E and A), mitotic cyclins (B cyclins), whose levels in the cell rise and fall with the stages of the cell cycle, and cyclin-dependent kinases (Cdks), $G_1$ Cdk (Cdk4), S-phase Cdk (Cdk2), M-phase Cdk (Cdk1). Cdks levels in the cell remain fairly stable, but each must bind the appropriate cyclin in order to be activated. These complexes add phosphate groups to a variety of protein substrates that control processes in the

cell cycle. Another player is the anaphase-promoting complex (APC) also called the cyclosome, that triggers the events leading to cohesin destruction thus allowing the sister chromatids to separate, and degrades the mitotic B cyclins.

A rising level of $G_1$-cyclins bind to their Cdks and signal the cell to prepare the chromosomes for replication. The retinoblastoma tumor suppressor pRb plays also a critical role in regulating G1 progression. PRb has been shown to bind and regulate a large number of cellular proteins, including members of the E2F family of transcription factors [1]. E2F factors regulate the expression of many genes encoding proteins involved in cell cycle progression and DNA synthesis, including cyclins E and A, cdk1, B-myb, dihydrofolate reductase, thymidine kinase, and DNA polymerase α. Binding of pRb to E2F inhibits E2F transcriptional activation capacity and, in at least some cases, converts E2F factors from transcriptional activators to transcriptional repressors. Phosphorylation of pRb by D-type cyclin kinases results in the dissociation of pRb from E2F and the expression of the above mentioned E2F-regulated genes (fig. 2). Through the activation of E2F, cyclin E is the next cyclin to be induced during the progression of cells through G1. The rising level of S-phase promoting factor (SPF), A and E cyclins bound to Cdk2, enters the nucleus and prepares the cell to duplicate its DNA and its centrosomes. As DNA replication continues, cyclin E is destroyed, and the level of mitotic cyclins begins to rise in $G_2$. M-phase promoting factor, B cyclins with the Cdk1, initiates assembly of the mitotic spindle, breakdown of the nuclear envelope, cessation of all gene transcription, condensation of the chromosomes taking the cell to metaphase.

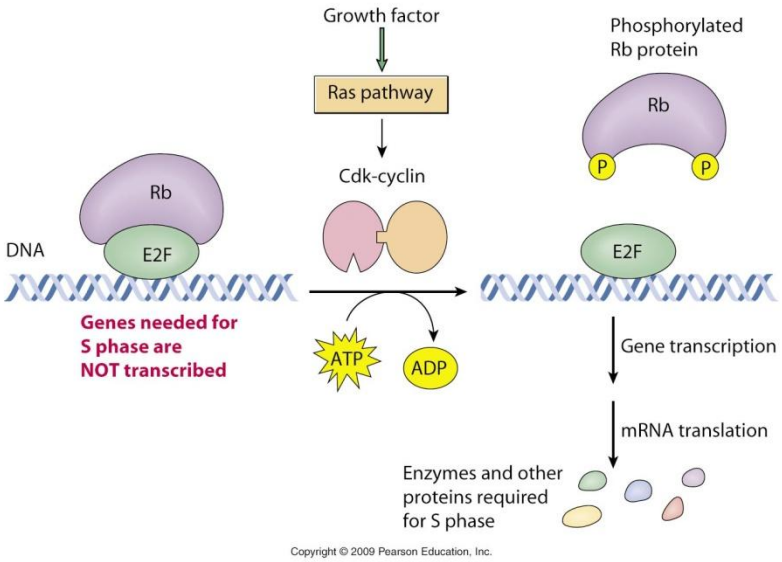



*Figure 2. G1 checkpoint: G1 Cdk-cyclin complex controls G1 checkpoint through pRb phosphorylation that prevents the binding and inactivation of transcription factor E2F. When E2F is then active, allows the transcription of numerous factors that trigger S-phase.*

At this point, the M-phase promoting factor activates the anaphase-promoting complex which allows the sister chromatids to separate and move to the poles, completing mitosis. Moreover, APC turns on synthesis of $G_1$ cyclins (D) for the next turn of the cycle and degrades geminin, a protein that has kept the freshly-synthesized DNA in S phase from being re-replicated before mitosis. This is the only one mechanism by which the cell ensures that every portion of its genome is copied once and only once during S phase.

Separation of sister chromatids depends on the breakdown of the cohesins that has been holding them together. Cohesin breakdown is caused by a protease called separase, also known as separin. Separase is kept inactive until late metaphase by an inhibitory chaperone called securin. Anaphase begins when APC

destroys securin by tagging it with ubiquitin for deposit in a proteasome, thus ending its inhibition of separase and allowing separase to break down cohesin.

**Cell cycle checkpoints**

To ensure the fidelity of cell division in eukaryotic cells there are control mechanisms known as checkpoints. These checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. Multiple checkpoints have been identified, with some of them are less understood than others.

The first checkpoint, called also restriction point, is located at the end of the cell cycle's $G_1$ phase, just before entry into S phase, making the key decision of whether the cell should divide, delay division, or enter a resting stage. The restriction point is controlled mainly by action of the CKI- p16 (CDK inhibitor p16). Two families of CKI exist: the Cip/Kip family (p21Cip1/WAF1 ($p21^{waf1}$), p27Kip1 (p27) and p57Kip2 (p57)) that can act on most cyclin/cdk complexes and even on some kinases unrelated to cdks [2], and the INK4 family (p16INK4a (p16), p15INK4b (p15), p18INK4c (p18), and p19INK4d (p19)) that specifically interacts with cdk4 and cdk6 but not other cdks [3]. P16 inhibits the CDK4/6 and ensures that it can no longer interact with cyclin D1 to cause the cell cycle progression. When growth is induced, the expression of this cyclin is so high that they do bind. The new CDK/cyclin complex now phosphorylates pRb which relieves the inhibition of the transcription factor E2F. E2F is then able to allow G1-S phase transition (fig. 2).

Accurate duplication of eukaryotic genome is a challenging task, given that environment of cell growth and division is rarely ideal. Cells are constantly under the stress of intrinsic and extrinsic agents that cause DNA damage or interference with DNA replication. To cope with these assaults, cells are equipped with DNA maintenance checkpoints to arrest cell cycle and facilitate DNA repair pathways. DNA maintenance checkpoints include the DNA damage checkpoint, that recognize and respond to DNA damage, and the DNA replication checkpoint, that monitors the fidelity of copying DNA [4]. DNA damage checkpoint ensure the fidelity of genetic information both by arresting cell cycle progression and facilitating DNA repair pathways. Studies on many different species have uncovered a network of proteins that form the DNA damage checkpoints. Central to this network are protein kinases of ATM/ATR family [5]. These kinases sense DNA damage and phosphorylate Chk1 or Chk2 initiating a signal transduction that culminates in cell cycle arrest, by activating tumor suppressor protein TP53 or by inactivation of the Cdc25 phosphatase, in order to induce DNA repair.

In response to DNA damage, the TP53 protein is stabilized and activated as a transcription factor. The $p21^{waf1}$ gene promoter contains a TP53-binding site that allows TP53 to transcriptionally activate the $p2^{waf1}$ gene. Induction of $p21^{waf1}$ inhibits cell cycle progression, blocking G1 transition by inhibiting a variety of cyclin/cdk complexes and by halting DNA synthesis through PCNA binding [3] . Cell Division Cycle 25 (CDC25) phosphatases dephosphorylate and activate cyclin-dependent kinase CDK–cyclin complexes, such as CDK2–cyclin E at the G1–S transition or CDK1–cyclin B at the entry into mitosis, thus allowing catalysis and substrate phosphorylation. The phosphorylation induced by the

ATM/ATR pathway inhibits CDC25 and consequently inactivates CDK–cyclin complexes (fig. 3).

Damaged template, protein complexes bound to DNA, and poor supply of dNTPs are among the many obstacles that must be overcome to replicate genome. All of these situations can stall replication forks. Stalled forks pose grave threats to genome integrity because they can rearrange, break, or collapse through disassembly of the replication complex [6]. The pathways that respond to replication stress are signal transduction pathways that are conserved across evolution [7,8]. Atop of these pathways are also ATM/ATR family kinases. These kinases together with a trimeric checkpoint clamp (termed 9-1-1 complex) and five-subunit checkpoint clamp loader (Rad17-RFC2-RFC3-RFC4-RFC5) senses stalled replication forks and transmit a checkpoint signal [4]. One of major functions of replication checkpoint is to stabilize and protect replication forks [9]. The protein kinases Chk1 and Chk2 are critical effectors of the replication checkpoint [10].

In order to prevent transmission of DNA damage to daughter cells, the cell cycle is arrested via inactivation of the CDC25 phosphatase (fig. 3). The spindle assembly checkpoint (SAC) is an active signal produced by improperly attached kinetochores. In the absence of kinetochore microtubules in prometaphase, several activities converge on the creation of the mitotic checkpoint complex (MCC), which is an anaphase-promoting complex/cyclosome (APC/C) inhibitor composed of BUB3 together with MAD2 and MAD3 bound to Cdc20. The Aurora-B, Cyclin-Dependent Kinase-1 (CDK1) and budding uninhibited by benzimidazole (BUB)1 kinases might stimulate directly the formation of the MCC.

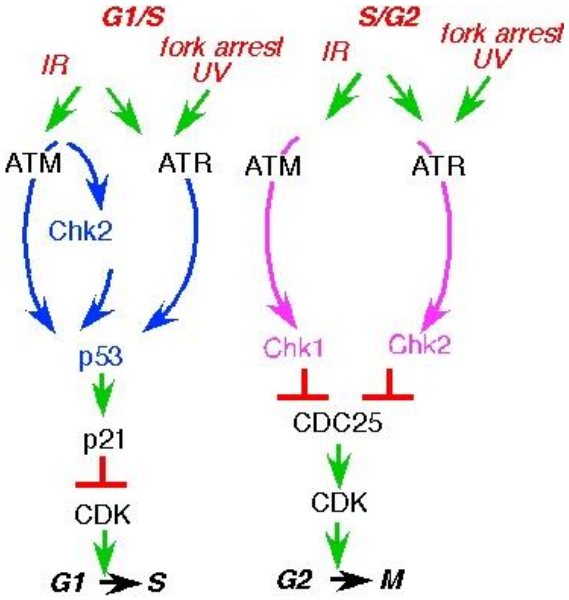



*Figure 3. DNA maintenance and DNA damage checkpoints.*

The closed MAD2 (C-MAD2)–MAD1 complex is recruited to unattached kinetochores and recruits open MAD2 (O-MAD2). This O-Mad2 changes its conformation to C-Mad2 and binds Mad1. The Mad1/C-Mad2 complex is responsible for the recruitment of more O-Mad2 to the kinetochores, which changes its conformation to C-Mad2 and binds Cdc20 in an auto-amplification reaction. This sequestration of Cdc20 is essential to maintain active the spindle assembly checkpoint. In a separate branch of the SAC response, centromere protein (CENP)-E binds and activates BUBR1 at unattached kinetochores. On microtubule–kinetochore attachment, a mechanism of 'stripping' based on the poleward-directed microtubule-motor activity of the dynein–dynactin complex starts removing SAC proteins from kinetochores. The ability of CENP-E to activate

BUBR1 also subsides on formation of stable microtubule–kinetochore attachments. All this results in APC activation and anaphase progression [11].

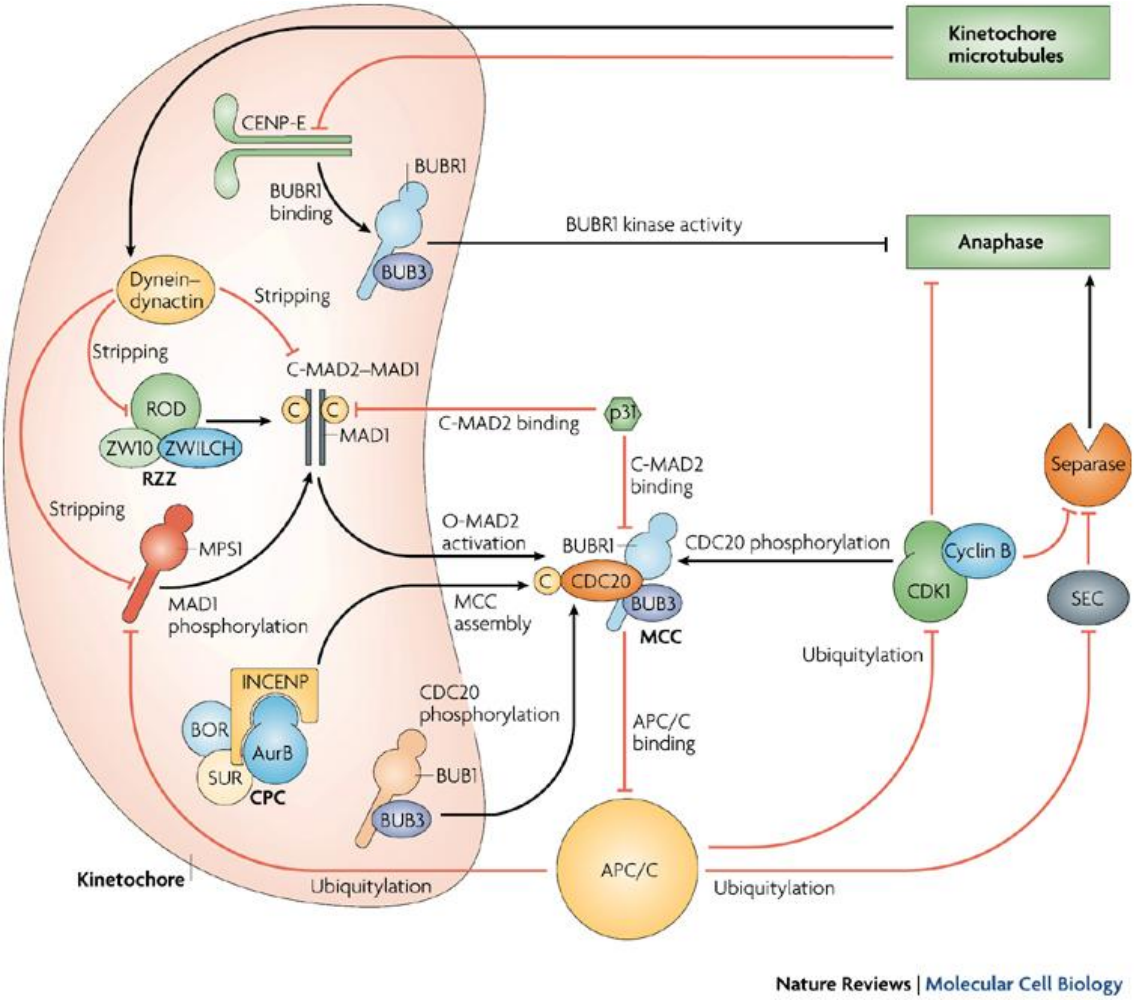



*Figure 4. The spindle assembly checkpoint (SAC) network [11].*

# Epigenetics

The term *epigenetics* was first proposed by Conrad Waddington to designate the study of the processes by which the genetic information of an organism, defined as the genotype, interacts with the environment in order to produce its observed traits, defined as the phenotype [12]. More recently, the term has been used to define  the study of heritable changes in genome function that occur without a change in DNA sequence, hence the name *epi-* (Greek: *επί-* over) -*genetics*. When Waddington coined the term the physical nature of genes and their role in heredity was not known; he used it as a conceptual model of how genes might interact with their surroundings to produce a phenotype. These two definitions are closer than they seem because an individual's cells all share the same linear sequence of DNA nucleotides, the genome, but different cell types are characterized by the presence of different chromatin flavors of this genome, the epigenomes, that specify the characteristic functions of each cell type and allows the maintenance of the memory of these functions through cell division. Epigenetic inheritance represents a critical mechanism that allows a remarkably stable propagation of gene activity states over many cell generations. The field of epigenetics has been receiving remarkable attention over recent years, owing to the awareness that epigenetic inheritance is essential for development and critical cellular processes such as gene transcription, and differentiation. Epigenetic mechanisms are versatile and adapted for specific cellular memory function not only during development but also during life-time.

Epigenetic mechanisms act to change the accessibility of chromatin to transcriptional regulation locally and globally via modifications of the DNA and by

modification or rearrangement of nucleosomes. In this way epigenetic programming is crucial in mammalian development, and stable inheritance of epigenetic settings is essential for the maintenance of tissue- and cell-type- specific functions [13]. With the exception of controlled genomic rearrangements, all other differentiation processes are initiated and maintained through epigenetic modifications. Therefore, epigenetic gene regulation is characterized overall by a high degree of integrity and stability. Perturbation of epigenetic balances may lead to alterations in gene expression, ultimately resulting in cellular transformation and malignant outgrowth.

Epigenetic information that fulfils the criterion of heritability are generally classified into two distinct types: DNA methylation and histone modifications (fig. 5).

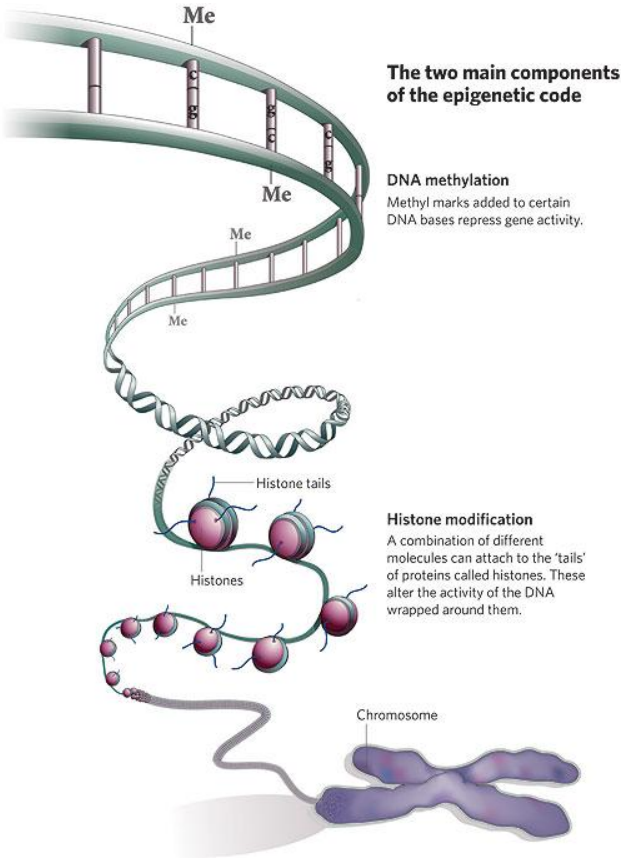



*Figure 5. Epigenetic mechanisms [14].*

**Histone modifications**

Genes may exist in two structural conditions, active or inactive state, independently of their sequence. Genes are found in the active state only in cells where they are expressed. The change of structure precedes the act of transcription and indicates that the gene is *transcribable*. This suggest that the acquisition of the active state must be the first step in gene expression. Active genes are found in domains of euchromatin with a preferential susceptibility to nucleases, and, in fact, hypersensitive sites are created at promoters before a gene is activated. More recently it has turned out that there is an intimate and continuing connection between initiation of transcription and chromatin structure. Indeed some activators of transcription directly modify histones; in particular, acetylation is correlated with gene activation. On the other hand some repressor of transcription function by deacetylating histones. So a reversible change in the histone structure in the vicinity of the promoter is involved in the control of gene expression. But the acetylation/deacetylation is only one of the numerous modifications that histones can be subjected to. Generally, histone modifications are distinguished in ATP-dependent and ATP-independent, depending on whether they need or not the energy derived by ATP hydrolysis to function.

ATP-dependent chromatin remodelers use the energy to disrupt protein-protein and protein-DNA contacts in order to release histones from chromatin. When histones are released from DNA other proteins, such as transcription factors and RNA polymerase, can bind. In vitro three types of remodelling changes have been described: histone octamers may slide along DNA; the spacing be-

tween histone octamers may be changed; and the most extensive change is that an octamer(s) may be displaced entirely from DNA to generate a nucleosome-free gap.

SWI/SNF was the first remodeling complex to be identified. SWI/SNF complexes can remodel chromatin *in vitro* without overall loss of histones or can displace histone octamers. Both types of reaction may pass through the same intermediate in which the structure of the target nucleosome is altered, leading either to reformation of a remodeled nucleosome on the original DNA or to displacement of the histone octamer to a different DNA molecule. The SWI/SNF complex alters nucleosomal sensitivity to DNAase I at the target site, and induces changes in protein-DNA contacts that persist after it has been released from the nucleosomes. The SWI2 subunit is the ATPase that provides the energy for remodeling by SWI/SNF.

Remodeling complexes do not themselves contain subunits that bind specific DNA sequences. This suggest that they are recruited by activators or repressors of transcription. The transcription factor Swi5p activates the *HO* locus in yeast. Swi5p enters nuclei toward the end of mitosis and binds to the *HO* promoter. It then recruits SWI/SNF to the promoter. Then Swi5p is released, leaving SWI/SNF at the promoter. This means that a transcription factor can activate a promoter by a *hit and run* mechanism, in which its function is fulfilled once the remodeling complex has bound [15] (fig. 6).

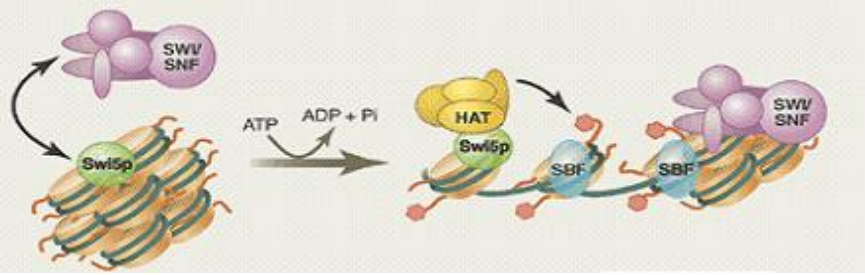



*Figure 6. Yeast HO promoter activation* [16].

It is not always the case, however, that nucleosomes must be excluded in order to permit initiation of transcription. Some activators can bind to DNA on a nucleosomal surface. Nucleosomes appear to be precisely positioned at some steroid hormone response elements in such a way that receptors can bind. Receptor binding may alter the interaction of DNA with histones, and even lead to exposure of new binding sites. The exact positioning of nucleosomes could be required either because the nucleosome *presents* DNA in a particular rotational phase or because there are protein-protein interactions between the activators and histones or other components of chromatin.

ATP-independent histone modifications are enzymes that induce post-translational modifications on histones. Histones can be acetylated/deacetylated, phosphorylated/dephosphorylated, methylated, SUMOylated or ADP-ribosylated, and every modification has a different meaning depending on its nature and position (histone code) [17]. The histone modifications may directly affect nucleosome structure or create binding sites for the attachment of nonhistone proteins that change the properties of chromatin [18,19]. Modifications take place in

the N-terminal tails of the histones, especially H3 and H4. The histone tails consist of the N-terminal 20 amino acids, and extend from the nucleosome between the turns of DNA. The modifications alter positive charge led by lysine and arginine (fig. 7). The histone modifications may directly affect nucleosome structure or create binding sites for the attachment of nonhistone proteins that change the properties of chromatin. Modification can be a local event, for example, restricted to nucleosomes at the promoter. Or it can be a general event, extending for example to an entire chromosome.

All core histones can be acetylated but the major targets are lysines in the N-terminal tails of histones H3 and H4. Acetylation occurs in two different situations: during DNA replication and when genes are activated.

When chromosomes are replicated, during the S phase of the cell cycle, histones are transiently acetylated before they are incorporated into nucleosomes.

It is known that histones H4 and H3 are acetylated when they are associated with one another in the $H3_2$-$H4_2$ tetramer. The tetramer is then incorporated into nucleosomes. Quite soon after, the acetyl groups are removed.

Acetylation is reversible. Each direction of the reaction is catalyzed by a specific type of enzyme. Enzymes that can acetylate histones are called histone acetyltransferases or HATs; the acetyl groups are removed by histone deacetylases or HDACs (fig. 7). There are two groups of HAT enzymes: group A describes those that are involved with transcription; group B describes those involved with nucleosome assembly. Two inhibitors have been useful in analyzing acetylation. Trichostatin A (TSA) and butyric acid inhibit histone deacetylases, and cause acetylated nucleosomes to accumulate.

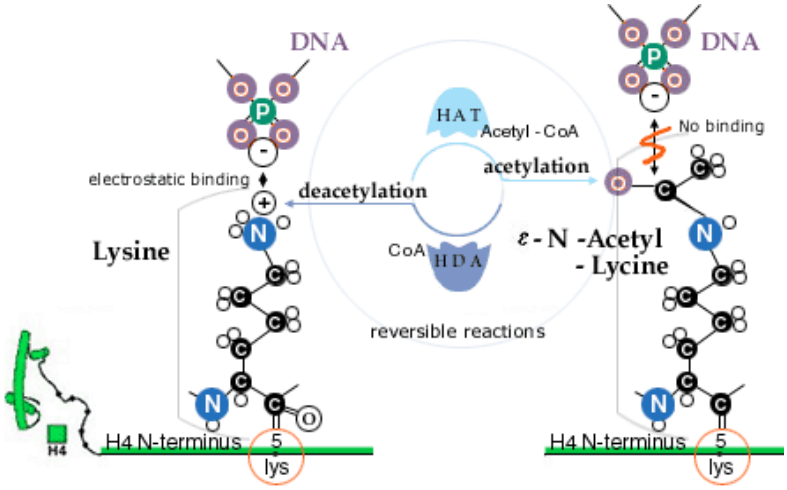



*Figure 7. Acetylation of lysines. Lysine has a positively charged amino group in its side chain that can be acetylated by HATs. This reaction can be reversed by HDACs. The positive charge on the histone tails should increase the potential for electrostatic interactions with the negatively charged phosphate backbone of DNA.*

One of the first general activators to be characterized as an HAT was p300/CBP, a co-activator that links an activator to the basal apparatus. p300/CBP acetylates the N-terminal tails of H4 in nucleosomes. Another co-activator, called PCAF, preferentially acetylates H3 in nucleosomes. p300/CBP and PCAF form a complex that functions in transcriptional activation. Acetylation may be necessary to *loosen* the nucleosome core. At replication, acetylation of histones could be necessary to allow them to be incorporated into new cores more easily. At transcription, a similar effect could be necessary to allow a related change in structure, possibly even to allow the histone core to be displaced from DNA. Alternatively, acetylation could generate binding sites for other proteins that are required for transcription. In either case, deacetylation would reverse the effect.

Histone methylation is considered as a process that maintains epigenetic memory. In particular, methylation of lysines of tails extending from the nucleosomes is a crucial event for transcriptional regulation, X chromosome inactivation, DNA methylation and heterochromatin formation at centromeric and telomeric regions of chromosomes [20,21]. This reaction is catalyzed by Histone Methyl-Transferases (HMTs), and can induce both the activation and the repression of gene transcription.

Methylation of H3 $_{9}$Lys is a feature of condensed regions of chromatin, including heterochromatin as seen in bulk and also smaller regions that are known not to be expressed. The histone methyl-transferase enzyme that targets this lysine is called SUV39H1. Other histone methyl-transferases act on arginine. In addition, methylation may occur on $_{79}$Lys in the globular core region of H3; this may be necessary for the formation of heterochromatin at telomeres [17].

**DNA methylation**

DNA methylation refers to a covalent modification of the cytosine base located at 5' to a guanine base in a CpG dinucleotide.

Around 2-7% of the cytosines of animal cell DNA are methylated and the majority of the CG sequences are methylated. Usually the C residues on both strands of this short palindromic sequence are methylated, a condition known as fully methylated. But after DNA replication each daughter duplex has one methylated strand and one unmethylated strand. Such a site is called hemi-methylated. The perpetuation of the methylated site now depends on what hap-

pens to hemimethylated DNA. If methylation of the unmethylated strand occurs, the site is restored to the fully methylated condition. However, if replication occurs first, the hemimethylated condition will be perpetuated on one daughter duplex, but the site will become unmethylated on the other daughter duplex.

The methylation consists in the addition of a methyl group to the 5 position of the cytosine pyrimidine ring. The transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to cytosine in CpGs is catalyzed by several DNA methyltransferase (DNMTs) (fig. 8).

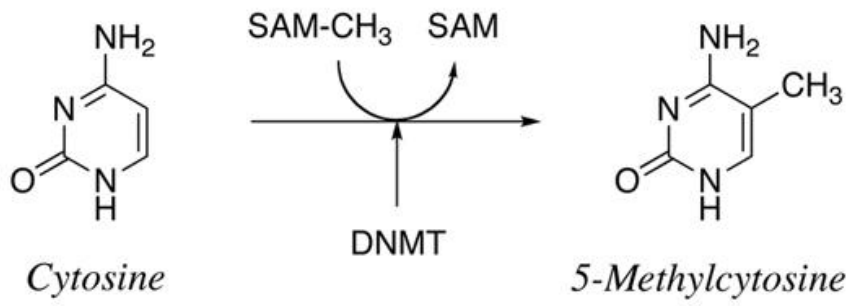



*Figure 8. Conversion of cytosine to 5-methylctosine by methyl-transferase (DNMT). DNMT catalyses the transfer of a methyl group (CH3) from S-adenosyl methionine (SAM) to the 5-carbon position of cytosine.*

There are two types of DNA methylase, whose actions are distinguished by the state of the methylated DNA. To modify DNA at a new position requires the action of the de novo methylase, which recognizes DNA by virtue of a specific sequence. It acts only on unmethylated DNA, to add a methyl group to one strand. There are two *de novo* methylases (Dnmt3A and Dnmt3B) in mammals; they have different target sites, and both are essential for development.

A maintenance methylase acts constitutively only on hemimethylated sites to convert them to fully methylated sites. Its existence means that any methylated site is perpetuated after replication. There is one maintenance methylase (Dnmt1) in mammals, and it is essential: mouse embryos in which its gene has been disrupted do not survive past early embryogenesis [22]. Maintenance methylation is virtually 100% efficient.

DNMT1, DNMT3A and DNMT3B have a regulative domain at the N-terminus and a catalytic one at the C-terminus, separated by a little fragment made up with GK dinucleotide repeats (fig. 9).

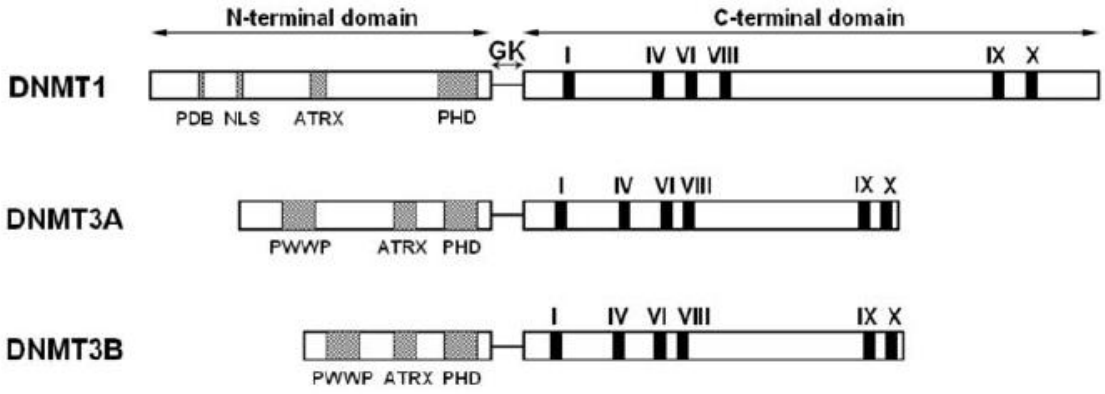



*Figure 9. Members of DNMT family [23].*

The catalytic activity of DNMT1 needs the interaction between the two domains and the C-terminus alone is useless. On the contrary the C-terminus of DNMT3A and DNMT3B is functional alone too [23]. Methylation has various types of targets. Gene promoters are the most common target. The promoters are methylated when the gene is inactive, but unmethylated when it is active. The presence of $m^5$CpG dinucleotides in the first gene exon or promoter may have

an effect on gene transcription in a direct or indirect manner. The direct mechanism involves the interference of $m^5$CpG dinucleotides with transcription factors binding to a promoter. However, the indirect mechanism of gene regulation is preceded by DNA binding $m^5$CpG dinucleotide-specific proteins which block the interaction of transcription factors with certain DNA sequences [24]. These protein suppressors of promoters mainly include MBDs ($m^5$CpG binding Domain proteins) and $m^5$CpG-binding proteins (MeCPs) [25]. These proteins are able to form complexes with HDAC, co-repressor (Sin3a) and ATP-dependent chromatin remodeling proteins, which are involved in the stabilization of heterochromatin structure. The absence of Dnmt1 in mouse causes widespread demethylation at promoters, and it is assumed this is lethal because of the uncontrolled gene expression [22]. Satellite DNA is another target. Mutations in Dnmt3B prevent methylation of satellite DNA, which causes centromere instability at the cellular level. Mutations in the corresponding human gene cause the ICF (Immunodeficiency, Centromeric instability, Facial abnormalities) syndrome [26].

Once a site has been methylated, there are two possible ways to generate demethylated sites. One is to block the maintenance methylase from acting on the site when it is replicated. After a second replication cycle, one of the daughter duplexes will be unmethylated. The other is actively to demethylate the site. It is known that active demethylation can occur to the paternal genome soon after fertilization [27], but it is not known what mechanism is used.

The pattern of germ cells methylation is established in each sex during gametogenesis by a two stage process: first the existing pattern is erased by a genome-wide demethylation; second the pattern specific for each sex is then imposed. All allelic differences are lost when primordial germ cells develop in

the embryo; irrespective of sex, the previous patterns of methylation are erased, and a typical gene is then unmethylated. In males, the pattern develops in two stages. The methylation pattern that is characteristic of mature sperm is established in the spermatocyte. But further changes are made in this pattern after fertilization. In females, the maternal pattern is imposed during oogenesis, when oocytes mature through meiosis after birth. The specific pattern of methyl groups in germ cells is responsible for the phenomenon of imprinting, which describes a difference in behaviour between the alleles inherited from each parent.

Finally, it is clear that a generalized mechanism of transcriptional control in response to epigenetic modifications exists. In the silent promoter, genomic sequencing and chromatin immunoprecipitation experiments clearly display a hypermethylated sequence enriched with MeCP2-HDAC repressor complexes. Inhibitors of DNA methylation and histone deacetylase activity can alleviate this dominant repression pathway. Histone hyperacetylation mediated by deacetylase inhibitors have no effect on the transcription levels of the hypermethylated gene (fig. 10).

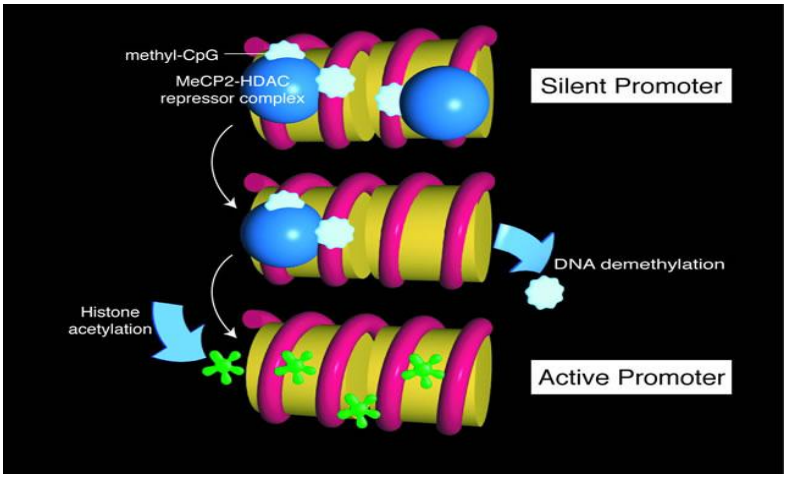



*Figure 10. Generalized mechanism of transcriptional control in response to epigenetic modifications. Nucleosome structures are simplified in this model by illustrating the DNA coil (red) wrapped around the histone core (yellow)* [19].

These results clearly demonstrate that histone hyperacetylation is not an overriding mechanism of control over DNA methylation. However, 5azaCitidine induced DNA demethylation causes some release of MeCP2-HDAC complexes from promoter chromatin and hyperacetylation of histone tails by TSA can reactivate the gene. Stable transcription complexes form on hyperacetylated chromatin and allow the basal machinery access for transcriptional activation [19] (fig. 10). Moreover it is also evident that the epigenetic factors cooperate in order to establish an active or inactive chromatin domain. The order of recruiting is alternative and it may happen that DNA methylation favours histone modification and vice versa [28] (fig. 11).

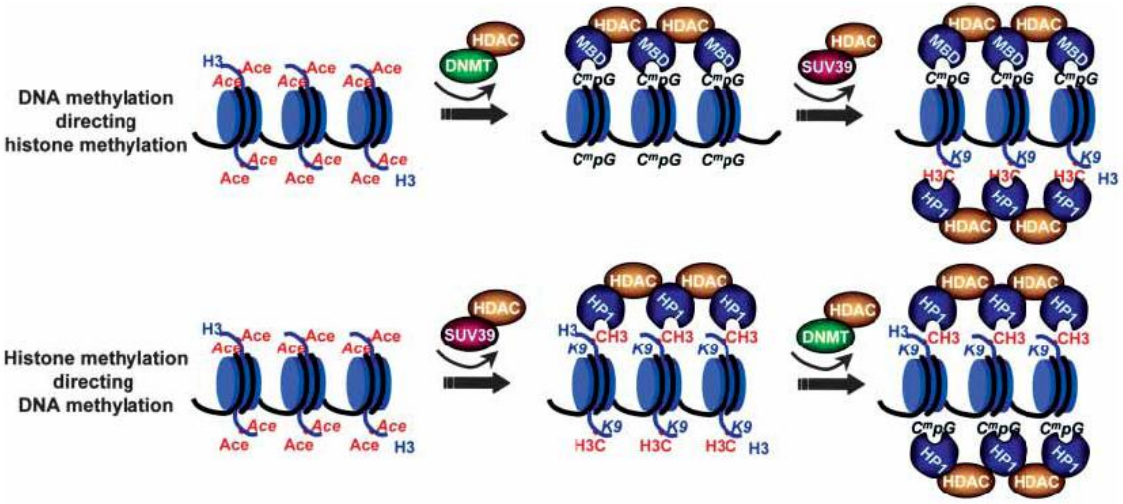



*Figure 11. Integrative epigenetic gene repression. (Top) DNA methylation drives histone modification; (Bottom) Histone modification directs DNA methylation* [28].

**DNA methyl-transferase 1 (DNMT1)**

DNMT1 is the major enzyme responsible during replication for maintenance of the DNA methylation pattern. During the replication of eukaryotic genomic DNA, approximately 40 million $m^5$CpG dinucleotides are converted into the hemimethylated state in the newly synthesized DNA strand. These hemimethylated CpG sites must be methylated precisely to maintain the original DNA methylation pattern. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA strands directly after the replication round [29] (fig. 12). DNMT1 displays a 5- to 40-fold higher activity *in vitro* for hemimethylated DNA than for unmethylated DNA [29,30]. However, this enzyme also exhibits *de novo* methylation activity in vitro [31] or stimulated by DNMT3A [32] .

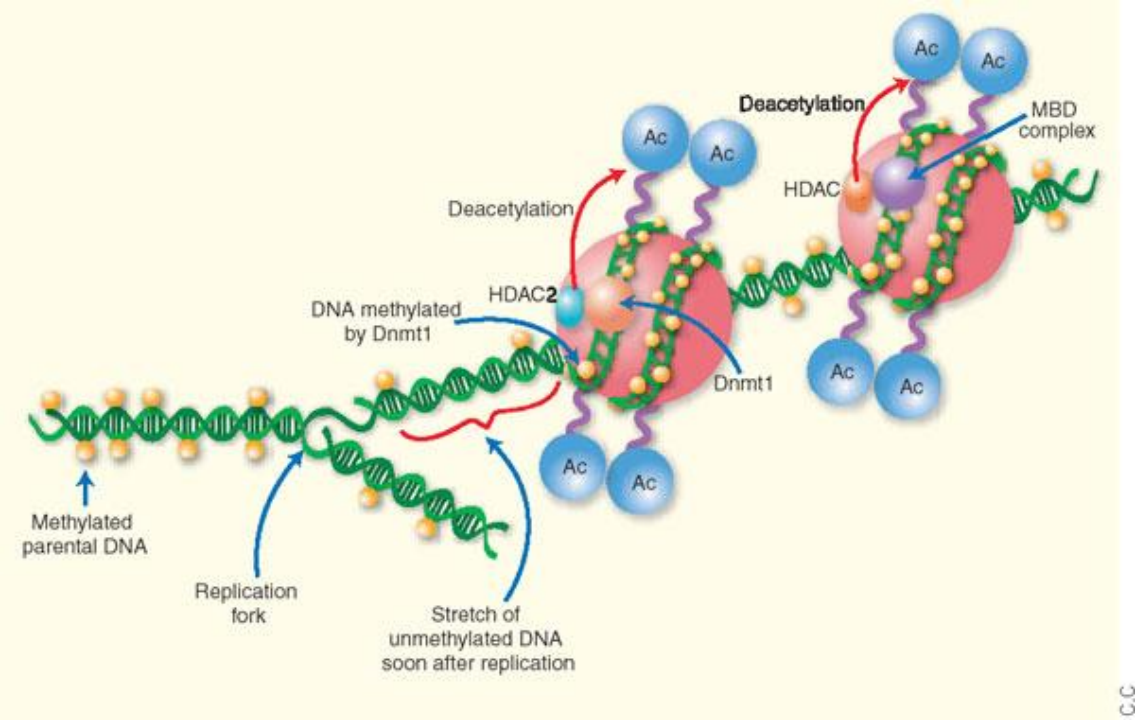



*Figure 12. Hypothetical models by which CpG methylation can contribute to the propagation of a silent chromatin structure. Dnmt1 recognizes hemimethylated DNA, which is generated during the replication of methylated DNA, and binds DNA at replication forks with HDAC2* [33].

Its structure indicates that the DNMT1 gene could have been formed during the fusion of a prokaryotic DNMT gene with a mammalian DNA binding protein gene [34,35]. Mammalian DNMT1 is also composed of at least three major structural elements. The first 621 amino acids of the N-terminus are not essential for DNMT1 activity [36]. However, the N-terminal DNMT1 domain is essential for discrimination between hemimethylated and unmethylated DNA strands and is responsible for a decrease in *de novo* methylation activity. The charge-rich motif of the N-terminal domain of DNMT1 interacts with DMAP1 and represses transcription without the participation of HDAC [37]. The DNMT1 N-terminal domain can also interact with other proteins, including the proliferating cell nuclear antigen (PCNA) [38], retinoblastoma protein pRb [39], HDAC1 or HDAC2 [40]. The N-terminus of DNMT1 can also recognize the MBD1, MBD3, MeCP2 and HP1

proteins. The interaction of DNMT1 with numerous protein suppressors of promoters suggests that this DNA methyl-transferase is also a crucial element of the transcription suppression complex.

The ATRX zinc finger motifs and the PCNA-binding domain (PBD) of DNMT1 also interact with DNA and PCNA respectively during replication. This causes a better presentation of targeting sequences (TS) which stabilizes the replication foci and induces an increase in the biosynthesis rate of new DNA strands [41].

The primary structure of human DNMT1 suggests that the entire catalytic site of this enzyme is composed of 500 amino acids and is located at the C-terminal domain [36].

Although DNMT1 is constitutively expressed in mammalian cells, regulation of its activity has to be coordinated with biological processes and therefore must be tightly regulated in the cell cycle. DNMT1 promoter is a TATA-less one and therefore its transcriptional activity is highly dependent on the binding of transcription factors to the basal promoter region. The E2F-binding site located within the transcription initiation region is critical for the regulation of DNMT1 transcription in proliferating cells via the E2F1/pRb pathway [42]. Moreover, it has been described that the APC/β-catenin pathway negatively regulates DNMT1 expression in human cancer cells [43]. Several groups had also independently reported an inverse relationship between DNMT1 and $p21^{waf1}$ expression in mammalian cells, which strongly suggest that the two proteins may be linked in a regulatory pathway. DNMT1 has been shown to repress $p21^{waf1}$ in transcriptional regulation [44], through UHRF1 (Ubiquitin-like, containing PHD and RING

fingers domains 1) [45], and vice versa p21$^{waf1}$ negatively regulates DNMT1 expression by modulating p300 [44]. Together these pathways may play a pivotal role to ensure regulated DNMT1 expression and DNA methylation in mammalian cell division.

DNMT1 is an essential gene during mammalian development [22], probably because it is fundamental for both DNA replication [38] and maintaining methylation. It has most recently been implicated also in cancer, since it is found deregulated in lots of human cancers, such as colorectal [46], breast [47], lung [48] and retinoblastoma [49] cancers, but its role on tumor progression is not exactly understood yet.

**Epigenetics and Cancer**

Cancer is traditionally viewed as a primarily genetic disorder, however it is nowadays recognised as a genetic and epigenetic disease. Genetic changes and aneuploidy are associated with alterations in DNA sequence, and they are a hallmark of the malignant process. Epigenetic alterations are universally present in human cancer and result in heritable changes in gene expression and chromatin structure over many cell generations without changes in DNA sequence, leading to functional consequences equivalent to those induced by genetic alterations. Aberrant epigenetic events affect multiple genes and cellular pathways in a non-random fashion and this can predispose to induction and accumulation of genetic changes in the course of tumour initiation and progression [50]. The first link between epigenetics and cancer was demonstrated in 1983, when it was shown that the genomes of cancer cells are hypomethylated relative to their normal counterpart [51]. Since that discovery, a plethora of studies re-

ported aberrant epigenetic patterns, accompanied by silencing of tumour suppressor genes and other cancer-associated genes in a variety of human cancers. One important conclusion that emerged from numerous screenings is that epigenetic events, similar to genetic changes, are associated with virtually every step of tumour development and progression. In addition, in many cancer types, epigenetic changes were found to occur early and precede genetic changes in the course of tumour development [50].

Imbalance of histone acetylation/deacetylation in gene promoter regions contributes to the deregulation of gene expression and has been associated with carcinogenesis and cancer progression. Both, histone acetylases and deacetylases have central roles in regulating the access and recruitment of transcription factors to DNA regulatory elements and in the regulation of other post-translational modifications at the lysine residues. The aberrant targeting of HAT or HDAC activity to specific gene promoters can result from the fusion of transcription factors with protein domains that retain co-repressor or co-activator binding capacity. Acute promyelocytic leukemia and acute myeloid leukemia are caused by chromosomal translocations leading to the expression of transcription factors fused to the nuclear receptor RAR or to the zinc finger nuclear protein ETO, respectively, which contain co-repressor interaction domains. The progression of these leukemias is linked to the abnormal recruitment of the N-CoR/SMRT co-repressor complex containing histone deacetylase activity which acts by blocking differentiation and allowing uncontrolled growth of hematopoietic cells. More recent studies demonstrate that the transcriptional repression of target genes by

fusion proteins in leukemia is reinforced by epigenetic modifications such as DNA methylation [17].

DNA methylation aberrations are also a key event in cancer. There are several distinct mechanisms by which aberrant levels and patterns of methylated CpG dinucleotides may trigger genetic changes and contribute to tumourigenesis. These include the increased mutability of methylated cytosines, silencing of tumour-suppressor genes, activation of oncogenes and genomic instability.

It has long been hypothesized that the presence of methylated CpG sequences *per se* are the major cause of mutability in mammalian genomes. The importance of the 5-methylcytosine (5-mC) of the CpG sites in cellular functions is reflected by the fact that it is referred to as the "fifth" base of DNA that forms "the DNA methylation code" [52]. However, 5-mC constitutes a threat to the genome due to its intrinsic instability. Because it is prone to spontaneous hydrolytic de-amination under physiological conditions, 5-mC is considered as a potent endogenous mutagen. The spontaneous hydrolytic de-amination of 5-mC results in thymine. Thus, C to T transition will be fixed after DNA replication [50].

Methylation pattern defects include genome wide hypomethylation and localised aberrant hypermethylation of CpG islands. These imbalances can be present together in a single tumour, though the net effect is usually a decrease in total methylation levels. Whether genome hypomethylation and CpG island hypermethylation are linked by a common underlying mechanism or result from distinct abnormalities in the cancer cell is currently unknown. However, we do know that hypomethylation and hypermethylation occur at specific but distinct

sites within the cancer cell genome, suggesting different aetiologies (fig. 13). Hypermethylation in tumor cells occurs at CpG islands, most of which are unmethylated in normal somatic cells, and the resulting changes in chromatin structure effectively silence transcription (fig. 13). Genes involved in cell-cycle regulation (p16 [53], p21[waf1] [54]), tumor invasion (E-caderin [55]), DNA repair (hMLH1[56]), cell signalling, transcription (BRCA [57]) and apoptosis are found aberrantly hypermethylated and silenced in nearly every tumor type [52,58]. This provides tumor cells with a growth advantage and allows them to metastasize . In tumors with a well defined progression, such as colon cancer, aberrant hypermethylation is detectable in the earliest precursors lesions, indicating that it directly contributes to transformation and is not a late event that arises from genetic alterations [58,59].

Hypomethylation in tumor cells is primarily due to the loss of methylation from repetitive regions (fig. 13) of the genome causing both oncogenes and mobile DNA activation and genome instability.

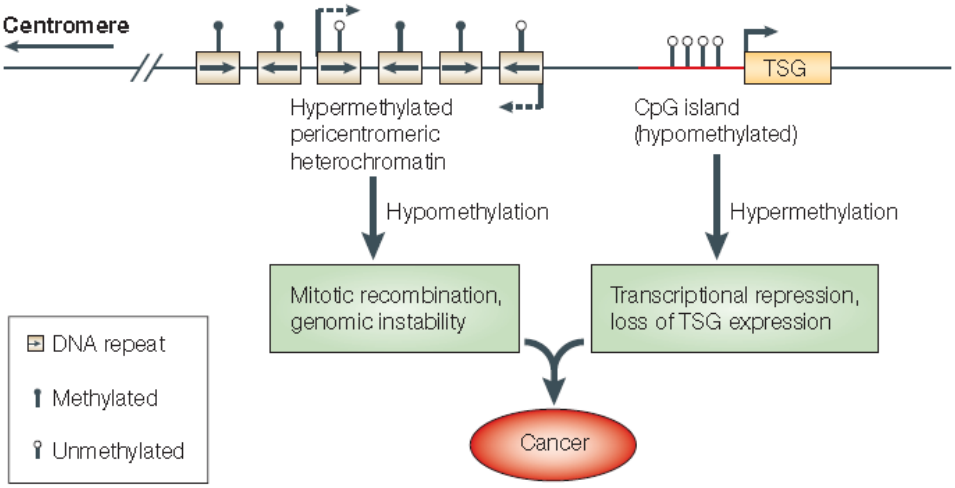



*Figure 13. DNA methylation and cancer* [58]

Hypomethylation in human cancers is causally related to transcriptional activation of a large group of genes of the *MAGE*, *GAGE*, *CTAG/LAGE*, and *SAGE* families. These unrelated gene families are located on the X chromosome and their cellular function is unknown. The promoters of *MAGE* type genes have an intermediate density of CpGs and may constitute a unique class of promoters that fall somewhere between the constitutively unmethylated CpG island promoter and the conditionally methylated CpG poor promoter. *MAGE* promoter demethylation, possibly as a consequence of genome wide hypomethylation, leads to transcriptional activation of *MAGE* genes in cancer cells [52].

In fact, DNA hypomethylation in tumors has been associated with transcriptional activation of an unexpectedly low number of genes, suggesting that most tissue-specific genes use regulatory mechanisms other than DNA methylation for selective repression. It is now considered that the major contribution of genome hypomethylation to tumor development is the enhancement of genomic instability [60], and that hypomethylation of retrotransposons and pericentromeric repeats is responsible for this genomic destabilization [61].

The timing of genomic DNA hypomethylation and relationship with promoter specific hypermethylation in cancer are important step in determining the mechanism and its significance in carcinogenesis. A number of studies found no significant correlation between the presence of DNA hypomethylation and hypermethylation in a variety of cancers, suggesting that the two events are not connected [62]. However, since global DNA hypomethylation has been observed in most cancers assayed, including some benign tumours, it is believed by some authors to constitute an early event in transformation, possibly via karyotypic instability [63,64].

# Aneuploidy and Tumorigenesis

Aneuploidy, an alteration in the number of whole chromosomes, is recognized as a trait of cancer cells. It occurs in 90% of solid tumors and 75% of hematopoietic cancers [65]. Even in 1914, German biologist Theodor Boveri postulated that aneuploidy, arising from altered cell division might lead to oncogenesis.

Whether aneuploidy is a cause or consequence of cancer is one of the long-standing question in cancer biology and still subject of debate. Part of the difficulty in understating the role of aneuploidy in cancer lies in the big paradox that an abnormal number of chromosomes is initially disadvantageous for mammalian cells [66,67]. As a consequence only in rare cases, such as trisomy 21, imbalanced number of chromosome is compatible with life. However, many studies indicate that the effects of chromosome gains and losses are not the same and organisms have much less tolerance for chromosome losses that for gains. The likely reason is that losses of chromosomes means a reduction in gene dosage and consequently in protein stoichiometry imbalances [68]. It is then clear that the effects of aneuploidy are more complex than expected and that aneuploidy can drive tumorigenesis, but it does not necessarily do so and it depends on the cell context [67].

Commonly aneuploidy is the result of chromosomal instability characterized by gain or loss of entire chromosomes (W-CIN), but the way in which it is generated is not well clarified. Lots of studies suggest that CIN originates from the alteration of genes involved in chromosomal segregation [69]. Alteration in the spin-

dle assembly checkpoint and in the replication of centrosomes have been associated to chromosomal instability and aneuploidy in cancer [70,71].

It has been now accepted that chromosome biology is largely affected by epigenetic marks within chromosomes. The highly degree of compaction, no accessibility to transcription and recombination machinery as are other chromosome regions and structured nucleosome arrays are all characteristics of heterochromatin that depend on epigenetic marks. Although for some time the repetitive DNA contained in heterochromatin was considered as “junk”, recent evidence indicates that heterochromatin can also play important roles during chromosome segregation [72].

**Centromere and epigenetics**

Central to the accurate mechanism of chromosomes segregation during mitosis is the assembly of a single site for microtubule attachment, called the kinetochore, on each sister chromatid. Kinetochores form the primary interface between chromosomes and the mitotic spindle and mediate microtubule-dependent chromosome movements during mitosis. Kinetochores also sense errors in chromosome attachment to the mitotic spindle and respond by activating the SAC until all chromosomes achieve bipolar spindle attachment. It is the centromere that directs kinetochore assembly, so its structure is fundamental to recruit a running kinetochore. The centromere was first named by Walther Flemming and described cytologically as the primary constriction on vertebrate chromosomes and was later characterized as a chromosomal region that had a reduced recombination frequency [73]. All centromeres share the same chromatin

composition characterized by the incorporation of the histone 3 variant centromere protein A (CENP-A) within nucleosomes of centromeric chromatin and the AT-rich repeats called alpha-satellite DNA (α-satellite) [73,74]. Current experimental evidences indicate that epigenetic factors modifying centromeric chromatin contribute to centromere assembly. CENP-A containing chromatin is usually embedded within a large domain of heterochromatin, called pericentric heterochromatin, which is also required for the accurate segregation of chromosomes during mitosis. Alteration in centromeric and pericentric chromatin epigenetics has been correlated with kinetochore disfunction and chromosomal instability [75-77].

**DNA methylation and chromosome instability**

Cells with reduced DNA methylation levels appear to be more susceptible to undergoing chromosomal loss, gain, or rearrangement, probably because hypomethylation reduces chromosomal stability. Pericentromeric regions are characterized by highly repetitive DNA segments termed classical satellites 2 and 3, which are mainly non-transcribed and highly methylated. These regions contain large amounts of methylated constitutive heterochromatin. Somatic cells from patients with ICF, a rare genetic disease in which some mutations in the Dnmt3b gene have been detected [26], exhibit hypomethylated pericentromeric regions associated with chromosomal rearrangements, centromere under-condensation, and the formation of micronuclei [78]. Apart from ICF syndrome observation that reveals a connection between DNA demethylation and centromere dysfunctions, it was demonstrated a direct correlation between DNA methylation

and centromere formation in mammals. The DNA of a functional centromere is indeed maintained in an overall hypermethylated state, consistent with the general heterochromatic and transcriptionally ‘‘silent’’ characteristics of this structure. Importantly it seems that pockets of hypomethylation within the centromere provide the necessary chromatin environment to allow transcription to take place without compromising the overall ‘‘heterochromatic’’ state and function of the centromere [79].

Pericentromeric regions have also been found hypomethylated in some cancers, including hepatocarcinoma, breast, urothelial, and ovarian cancer, and this condition was associated with poor prognosis [72]. As expected, recently the chromosomal instability observed in colorectal cancer cells has been directly associated to genome-wide hypomethylation. This evidence suggests that DNA hypomethylation is likely to induce a cascade effect with direct implications in the determination of the tumor progression [60].

# Research Purpose

Genomic instability is a characteristic of the majority of human tumors and is considered a driving force for tumorigenesis. However, when during cancer development genome instability arises and what are its molecular basis are questions still unresolved. Various forms of genome instability have been described that are characterized by an increased rate of a number of different genetic alterations [80,81]. Most cancers show a form that is called chromosomal instability (CIN), which refers to the high rate of numerical and structural chromosome changes found in cancer cells compared to normal cells. The presence of CIN has also been found in cancer cells grown in vitro. Numerical CIN is characterized by gains and losses of whole chromosomes (aneuploidy) during cell proliferation. Mutations in genes encoding mitotic regulators [82] and in genes controlling centrosome numbers and tumor suppressors [83-87] have been suggested as molecular defects underlying aneuploidy. However, some of these genes controlling the above processes were not found mutated in aneuploidy cells, instead their expression was reduced suggesting the involvement of possible epigenetic alterations. Thus, epigenetic alterations should be considered as a cause of aneuploid cells generation. The regulation of chromatin structure is a dynamic and complex process, modulated by epigenetic mechanisms (histone modifications and DNA methylation). Malfunctioning of these processes can cause gene expression alteration and could compromise important events such as chromosome condensation and segregation. Imbalance in cytosine methylation is a recurrent event in human sporadic cancers. Alteration of methylation

patterns includes genome-wide hypomethylation, that could affect chromosome condensation, as well localized aberrant hypermethylation of CpG islands, especially in the promoter region of tumor suppressor genes provoking their silencing. Thus, methylation pattern alterations could be directly implicated in tumor initiation/progression. Hypomethylation and hypermethylation occur at specific but different sites within the cancer cell genome and can precede malignancy [52]. Global genome hypomethylation in breast, ovarian, cervical and brain tumors increases with increasing malignancy [52].However, it is still object of investigation the mechanism(s) that correlates hypomethylation with tumor initiation-progression. Several hypotheses have been proposed, including chromosomal instability induced by hypomethylation of pericentromeric regions [88]. Enzymes directly involved in DNA methylation are known as DNA methyl-transferases, or DNMTs, namely DNMT1, DNMT3a and DNMT3b. DNMT1 differs from the other two human DNA methylases, DNMT3a and DNMT3b mainly because it is unable to methylate DNA with both strands unmethylated (*de novo* methylation) [23]. DNMT1 is able to restore DNA methylation patterns during cell divisions and it has been recently implicated in genomic stability [23,89]. In addition DNMT1 was found deregulated in different kind of human tumors suggesting its involvement in tumor initiation/progression. However, the DNMT1's role in human cancer cells remains controversial. To investigate DNMT1 implication in the generation of chromosomal instability (aneuploidy) I evaluated the effects of its depletion by RNA interference in primary human fibroblasts (IMR90) and in near diploid tumor cells (HCT116).

Moreover I wanted to study the effect of DNMT1 overexpression on the methylation of specific gene promoters in normal human cells (IMR90 and MCF10).

# Results

**Evaluation of DNMT1 post-transcriptional silencing in human cells.**

*SiRNAs targeting DNMT1 induce its downregulation in human cells.*

In order to deplete *DNMT1* transiently by RNAi in IMR90 and HCT116 cells I used a specific siRNA (siDNMT1) [90] targeting a portion of *DNMT1* transcript at different concentrations: 80nM and 100nM for IMR90 cells, 60nM and 80nM for HCT116 and 80nM in MCF10A cells (fig. 14). Microscopy observation of IMR90 cells transfected with siDNMT1 at 100nM revealed the presence of vacuole-like structure within the cytoplasm, likely indicative of cytotoxicity (fig. 14). To avoid this side effect the 80nM concentration of siDNMT1 was used for the remaining experiments in IMR90 cells.

MCF10A cells were severely affected by siDNMT1 at 80nM, and at 72 hours post transfection the majority of cells were dead and in suspension; the few living cells appeared thinner than control cells (fig. 14).

Real time RT-PCR, using cDNA generated from mRNA extracted 72 hours post-transfection from all cellular types, was performed to quantitatively determine DNMT1 knockdown extent obtained 72 hours after siRNAs transfection. The results indicate a significant knockdown (about 80%) using the 80nM concentration (fig. 15 A). The experiment was run.

To determine whether mRNA decreased levels correlated with Dnmt1 protein reduction, protein were extracted from cells treated with 80nM siRNA targeting *DNMT1* and from untransfected cells (control). Western blotting and immuno-probing revealed a large decrease of Dnmt1 protein level in IMR90 and only its partial reduction in HCT116 cells (fig. 15 B).

In order to reduce further Dnmt1 protein in HCT116 cells for other experiments I performed two rounds of siDNMT1 transfection separated by 48 hours of recovery (HCT-siDNMT1-2t). *DNMT1* post-transcriptional silencing was assessed by Real Time RT-PCR and western blotting revealed a massive decrease of the protein (fig. 15 A, B).

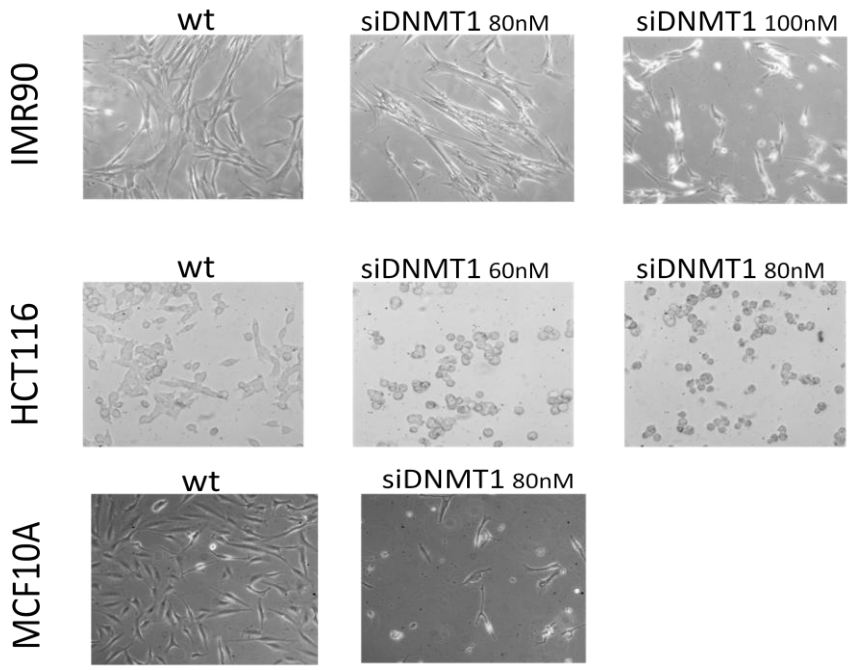



*Figure 14. Morphology of IMR90, HCT116 and MCF10A cells after 24 hours post-transfection of siDNMT1 at concentrations of different concentrations (80 and 100nM for IMR90; 60 and 80nM for HCT116; 80nM for MCF10A).*

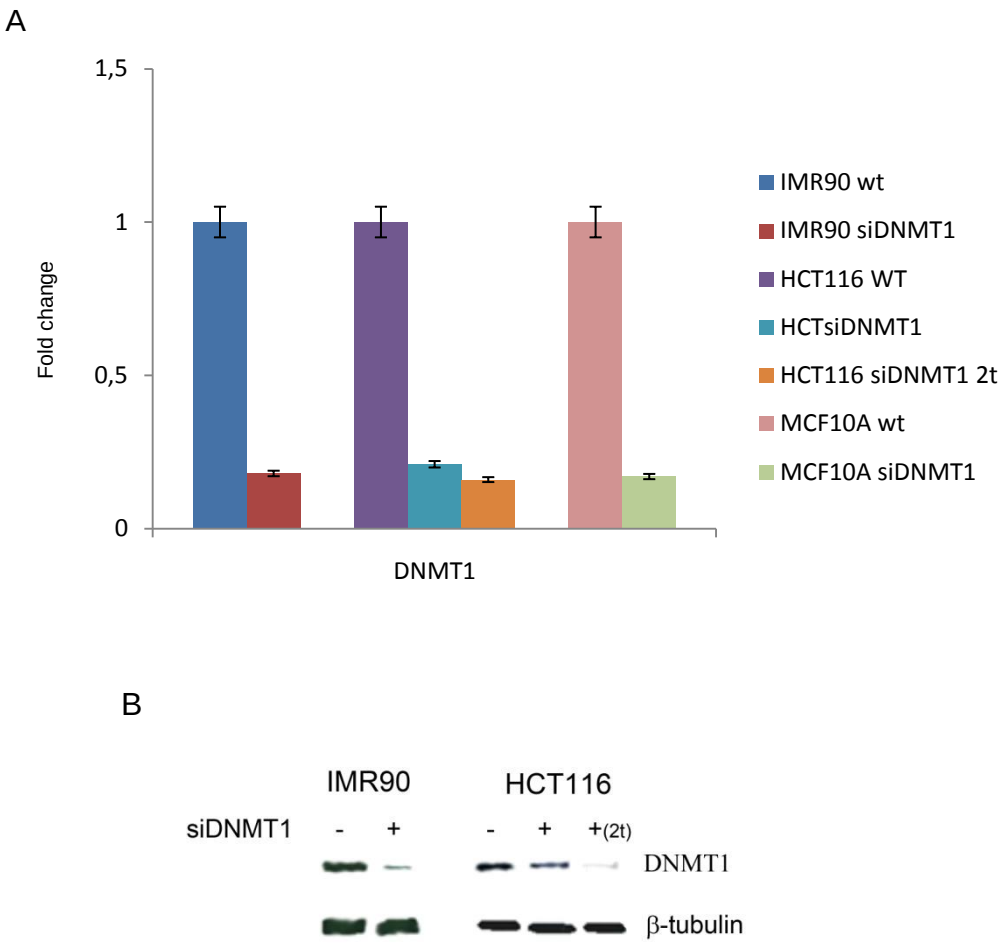



*Figure 15. A) Real Time RT-PCR analysis of IMR90, HCT116 and MCF10A cells showing decrease of DNMT1 transcriptional reduction after 72 hours from transfection of two different concentration of siRNAs targeting DNMT1, and of HCT116 cells after a double round with siDNMT1 80nM (HCT siDNMT1 2t). B) Western Blot analysis showing the reduction of Dnmt1 protein in both IMR90 and HCT116 cells treated with siDNMT1 80nM and in HCT116 siDNMT1 2t, consistent with Real Time RT-PCR results.*

*Transient DNMT1 downregulation induces aneuploidy, alterations of centromeric structure and micronuclei formation.*

To ascertain if epigenetic alterations might be implicated in the generation of chromosomal instability, I investigated if transient DNMT1 post-transcriptional silencing could induce aneuploidy in normal diploid cells. To this aim I did cytogenetics analyses in IMR90 and HCT116 cells treated for 72 hours with siDNMT1 or left untreated. The cytogenetics analysis showed that DNMT1 post-transcriptional silencing induced a high percentage of aneuploid cells in both cell strains. However, very few metaphases were observed in IMR90 siDNMT1 cells (mitotic index 0,3%), despite the treatment with colcemid for 8 hours, and the majority of IMR90 siDNMT1 metaphases were hyperdiploid (78%). On the contrary mitotic index of HCT siDNMT1 cells was not compromised and mitotic cells showed increase of hypodiploid cells (62%) (fig. 16 A, B). I also found anomalies of centromeric and pericentromeric region that seemed to be less stained with Giemsa in respect to the one of control cell, suggesting the presence of decondensed chromatin at that level (fig. 16 C).

DAPI staining of interphase nuclei showed high percentage of IMR90 siDNMT1 cells with micronuclei (37,6% of observed nuclei) (fig. 16 D).

I checked the number of centrosomes in order to exclude the involvement of centrosomes amplification in the generation of aneuploid cells, one of the known causes of aneuploidy induction in normal cells. Immunofluorescence microscopy analysis against γ-tubulin, the most important component of centro-

somes showed absence of supernumerary centrosomes (fig. 16 D), indicating that the observed aneuploidy was not induced by centrosomes amplification.

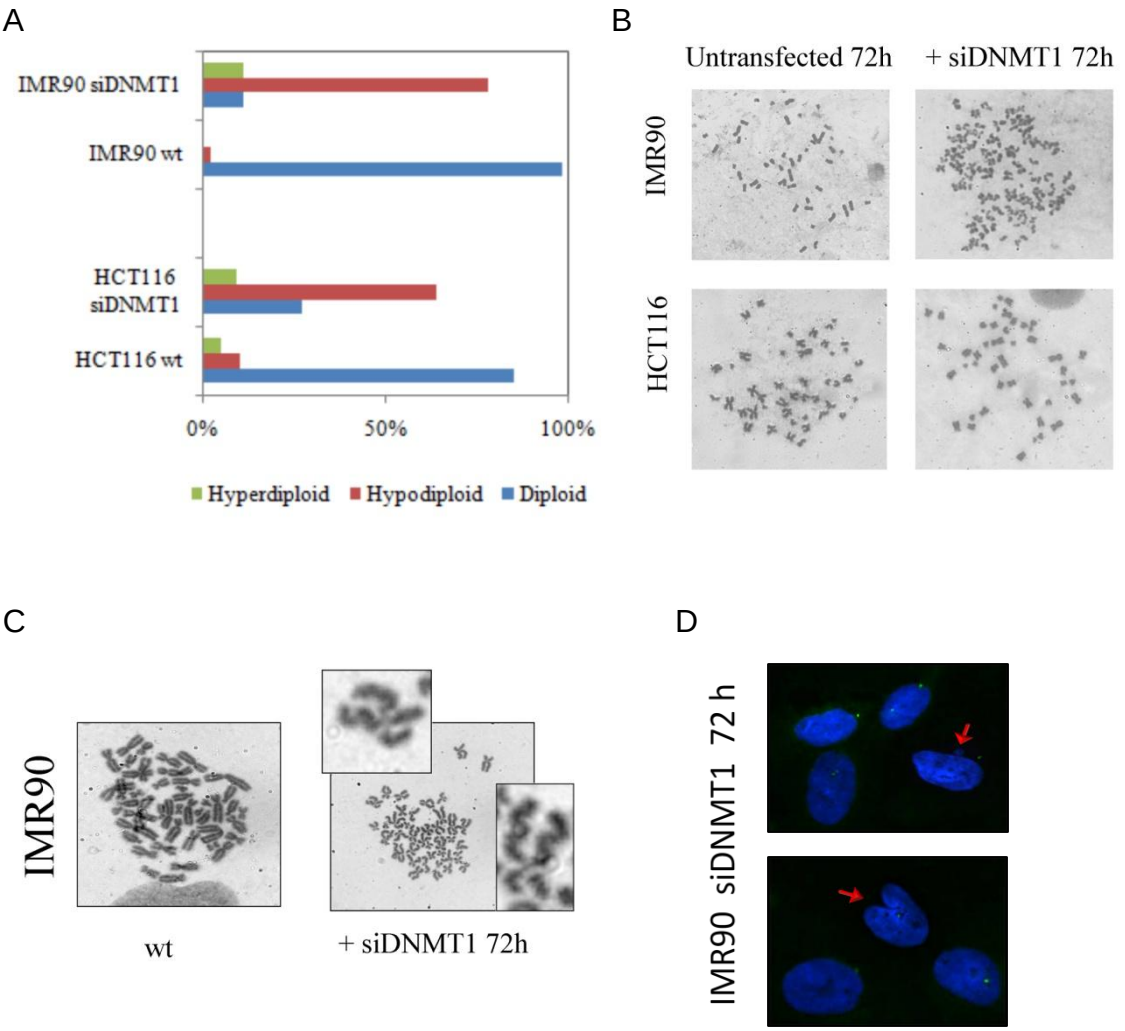



*Figure 16. A) Histogram showing percentages of both aneuploid and diploid metaphases. Both IMR90 and HCT116 cells acquired aneuploidy after 72 hours from siDNMT1 transfection. B) Pictures of metaphase spreads of both untransfected and siDNMT1 transfected IMR90 and HCT116 cells. On the left panels are example of IMR90 and HCT116 diploid metaphases (2N); on the right panels example of IMR90-siDNMT1 hyperdiploid metaphase and HCT116 siDNMT1 hypodiploid metaphase. C) Pictures of metaphase spreads of IMR90 wt and siDNMT1 cells. IMR90-siDNMT1 cells show alterations in chromosomal pericentromeric region where chromatin seemed to be decondensed in comparison to control (see inserts). D) IMR90-siDNMT1 nuclei stained with DAPI and detected with anti-$\beta$ tubulin antibody (green spots) showing the presence of morphological alterations, micronuclei and centrosomes.*

*Transient DNMT1 downregulation provokes cell growth inhibition.*

It is conceivable that DNMT1 depletion, causing defects in DNA methylation, will be sensed by the cell as a stress signal eliciting a cell cycle response (arrest/delay). Indeed, early effects (72 hours) of DNMT1 post-transcriptional silencing resulted in slowing down cell proliferation in both IMR90 siDNMT1 and HCT siDNMT1, as I just observed by their cellular morphology and by the cellular density/dish that was lower than the one showed by control cells (fig. 17 A). By counting cell numbers every 24 hours from siRNA transfection I found that IMR90-siDNMT1 cell numbers did not vary during the first 72 hours of silencing. However, these cells resumed proliferation and at 144 hours post-transfection the cellular density/dish was almost 100%. On the contrary, HCT-siDNMT1 cells duplicate once in 72 hours after transfection of siRNAs targeting DNMT1 (fig. 17 A). These observations suggest that DNMT1 depletion caused a transient cell cycle arrest in IMR90 cells (72h) and slowed-down proliferation in HCT116 cells.

To address this further I did cytofluorimetry analysis of IMR90-siDNMT1 and HCT116-siDNMT1 cells at 72 hours post-transfection to compare them with control cells (untreated/asynchronous). Cytofluorimetry analysis showed a large decrease in the number of S-phase BrdU positive IMR90-siDNMT1 cells and accumulation of the cells in the G1-phase indicative of a G1 arrest. On the contrary, HCT116-siDNMT1 cells did not arrest in G1 but they accumulated in S phase delaying the G2/M phase entry (fig. 17 B).

These results suggest a different response to DNMT1 downregulation of normal versus tumor cells. Moreover the cell cycle block in IMR90 cells was transient because cells re-entered cell cycle after 144 hours from transfection.

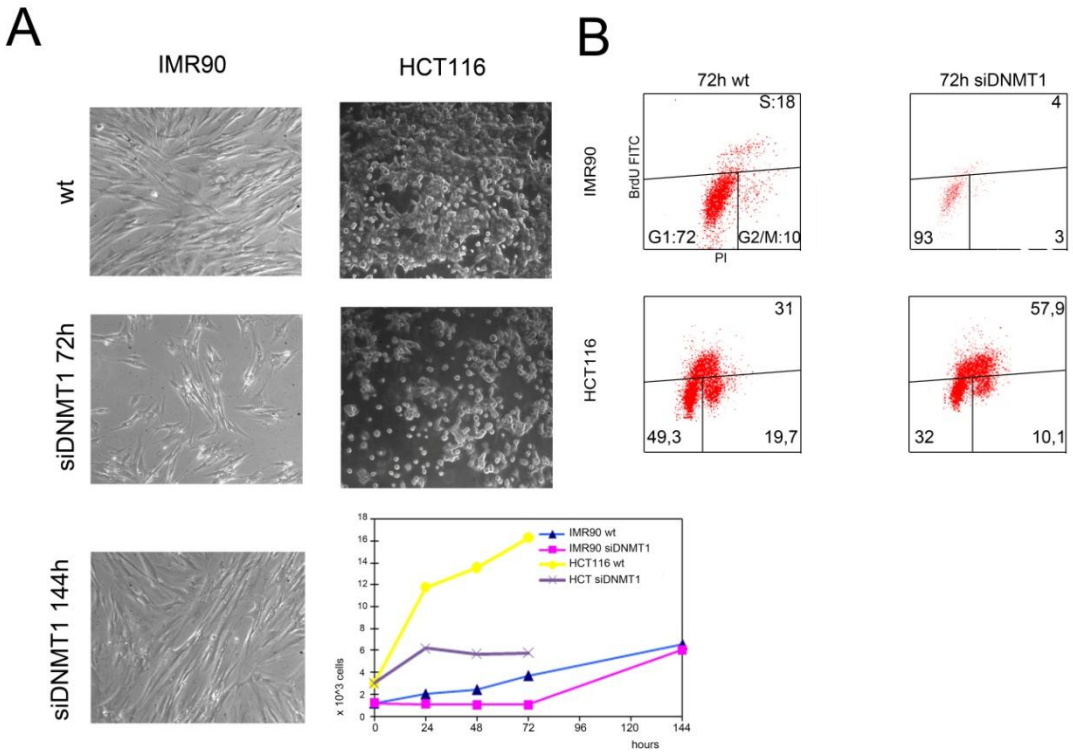
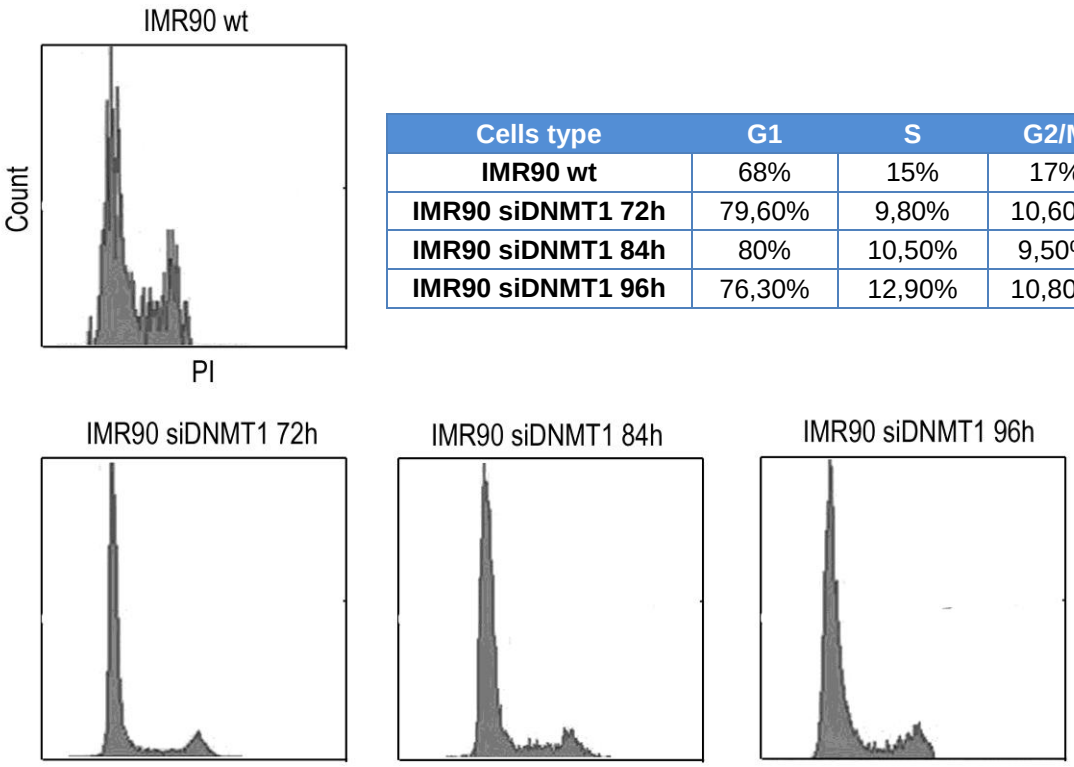



*Figure 17. A) Pictures and histogram showing the effects on proliferation of siRNAs targeting DNMT1 at 72 and 144 hours in IMR90 and HCT116 cells. Human fibroblasts slowed down proliferation in comparison to control cells at 72 h, however they resumed proliferation at 144h. HCT116 cells growth was less affected by DNMT1 silencing. B) Cytofluorimetric profiles of IMR90 and HCT116 cells pulsed with BrdU for 1h, and then stained with anti-BrdU antibody FITC-conjugated and propidium iodide. After DNMT1 silencing both IMR90 and HCT116 cells show delayed S-phase entry. In particular IMR90 arrested in G1/S.*

In order to investigate further on the cell cycle arrest induced in IMR90 siDNMT1 cells and understand at what time cells restart to proliferated between the 72 and 144 hours, I performed a cytofluorimetry analysis staining cell DNA with propidium iodide at 72, 84 and 96 hours post transfection. These experi-

ments showed that cells exit G1 phase between 84 and 96 hours post transfection, since the number of G1 cells is slightly reduced.

These results suggest that the cell cycle arrest induced by DNMT1 depletion is not transient.




| Cells type | G1 | S | G2/M |
|---|---|---|---|
| **IMR90 wt** | 68% | 15% | 17% |
| **IMR90 siDNMT1 72h** | 79,60% | 9,80% | 10,60% |
| **IMR90 siDNMT1 84h** | 80% | 10,50% | 9,50% |
| **IMR90 siDNMT1 96h** | 76,30% | 12,90% | 10,80% |

*Figure 18. Cytofluorimetric profiles of IMR90 wt and DNMT1 silenced for 72, 84 and 96 hours stained with propidium iodide (PI). IMR90 cells restart to enter S phase between 84 and 96 hours post siDNMT1 transfection.*

## *Dnmt1 depletion induces a global DNA demethylation.*

One potential role of the G1-arrest triggered by Dnmt1 depletion is to protect the epigenome from global loss of DNA methylation. To address this question I investigate if DNMT1 silencing causes DNA demethylation.

I used the tumor HCT116 cell line for the study of DNA methylation. Using anti-5-methylcytosine antibody I compared the state of methylation of HCT116 cells treated for 72 hours with siDNMT1 and not treated. HCT116 cells treated with siDNMT1 and its control were stained with the “capture antibody” and subsequently the “detection antibody” (secondary antibody) and were quantified through an ELISA-like reaction. HCT116 siDNMT1 cells resulted demethylated (1,2% of methylated DNA vs 5,65% of control) (table 1).

| Sample | Adsorbance | % Methylated DNA |
|---|---|---|
| HCT116 wt | 0,46 | 5% |
| HCT siDNMT1 | 0,28 | 1,20% |

*Table 1. Level of global DNA methylation in HCT116-wt and HCT116-siDNMT1 cells. DNA extracted from HCT116-siDNMT1 cells appeared demethylated in comparison to that extracted from HCT116 cells.*

The G1 arrest following DNMT1 silencing in IMR90 fibroblasts possibly protects cells from genome wide loss of methylation.

Next, I addressed the question of whether this difference in the kinetics of global DNA methylation between siDNMT1 treated and untreated cells is also observed when specific genes are examined. I focused on the hypermethylated promoter of CHFR gene in HCT116 cells [91] and I determined the pattern of its methylation after siDNMT1 treatment. The methylation pattern was studied by Methylation Specific PCR (MSP). This technique consists in a previous treat-

ment of genomic DNA with sodium bisulfite that change the unmethylated cytosines in uracyl that in turn becomes a thymine inducing point mutations in DNA sequence. By using two different sets of primers, designed on the methylated and unmethylated sequence, it is possible to distinguish the methylation status of a particular sequence. First, I tested the efficiency and specificity of the technique using two different cell lines that are known to have the CHFR gene promoter hypermethylated (HCT116) or unmethylated (SW480) in both strands of DNA [91]. MSP revealed the specificity of both sets of primers since they allow the amplification only of the sequence for CHFR unmethylated or methylated promoter (fig. 19). Moreover, to be sure that CHFR promoter could undergo demethylation I performed some treatments with 5-aza-deoxycitydine, a demethylating agent, used at different concentrations (1, 2 and 5µM) (fig. 19).

The result of this analysis showed no significative difference of methylation state between HCT siDNMT1 and the untreated cells since CHFR promoter remained fully methylated 72 hours after siDNMT1 transfection (fig. 19).

I supposed that the residual protein present in HCT116 cells treated with siDNMT1 for 72 hours might be the cause of the maintenance of CHFR promoter methylation. In order to obtain more silencing of Dnmt1 protein in HCT116 cells, I performed two rounds of 80nM siDNMT1 transfection separated by 48 hours of rest (HCT116 siDNMT1$^{(2t)}$). Unexpectedly I did not observe demethylation on CHFR gene promoter (fig. 19).

My results excluded the possibility that CHFR promoter methylation depends only on Dnmt1 protein activity. Thus I hypothesized that this methylation occurs

in collaboration with others DNA methyl-transferases, such as DNMT3b, even if Real Time RT-PCR did not reveal any increase of its transcription.

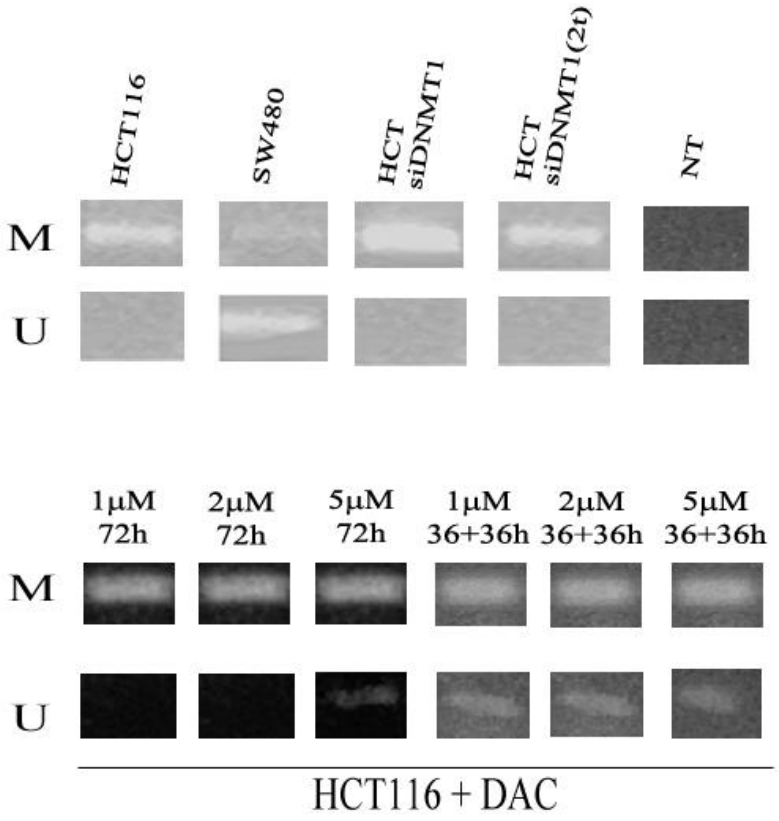



*Figure 19. Methylation Specific PCR (MSP) amplification shows methylation status of CHFR gene promoter in human cell lines. SW480 cells were used as a positive control of unmethylated status of the CHFR gene promoter. HCT116 cells treated with one and two rounds (2t) of siDNMT1 did not show any modification in methylation of CHFR gene promoter. HCT116 cells were treated with 5-aza-deoxycitydine (DAC) (1, 2, 5μM) for 72 hours continuatively or by adding fresh DAC at 36 hours in order to induce CHFR gene promoter demethylation.*

*IMR90 cells sense early DNMT1 decrease by activating TP53 and p21$^{waf1}$.*

I reasoned that DNMT1 downregulation might be targeting a pathway similar to that responding to DNA replication stress, leading to inhibition of firing origins of DNA replication and arrest of DNA synthesis. I therefore examined the effect of DNMT1 silencing on the expression of the most important genes involved in G1 (p21$^{waf1}$ and TP53) and G2/M (BubR1, MAD2 and AuroraA) cell cycle check-points.

Real time RT-PCR revealed a strong increase of p21$^{waf1}$ transcript in IMR90 siDNMT1 cells in comparison with control cells, and a slight increase in TP53 transcript (fig. 20 A). These results were accompanied by correspondent protein increase assessed by Western blot analysis. Moreover, I noticed that after 144 hours from single DNMT1 silencing, when IMR90 cells restart to proliferate, TP53 protein level is reduced in respect of cells at 72 hours (fig. 20 B, C).

On the contrary I did not observe increase of p21$^{waf1}$ and TP53 in proliferating HCT116 siDNMT1 cells (fig. 20 A, B).

As a confirmation of the G1 arrest of IMR90 siDNMT1, transcriptional levels of BubR1 and Aurora A, involved in mitosis, were very low (fig. 20 A). On the contrary MAD2 levels were higher than in control cells (fig. 20 A), even if MAD2 is involved in mitosis too. This result could be a side effect of DNMT1 silencing probably correlated with MAD2 gene promoter demethylation.

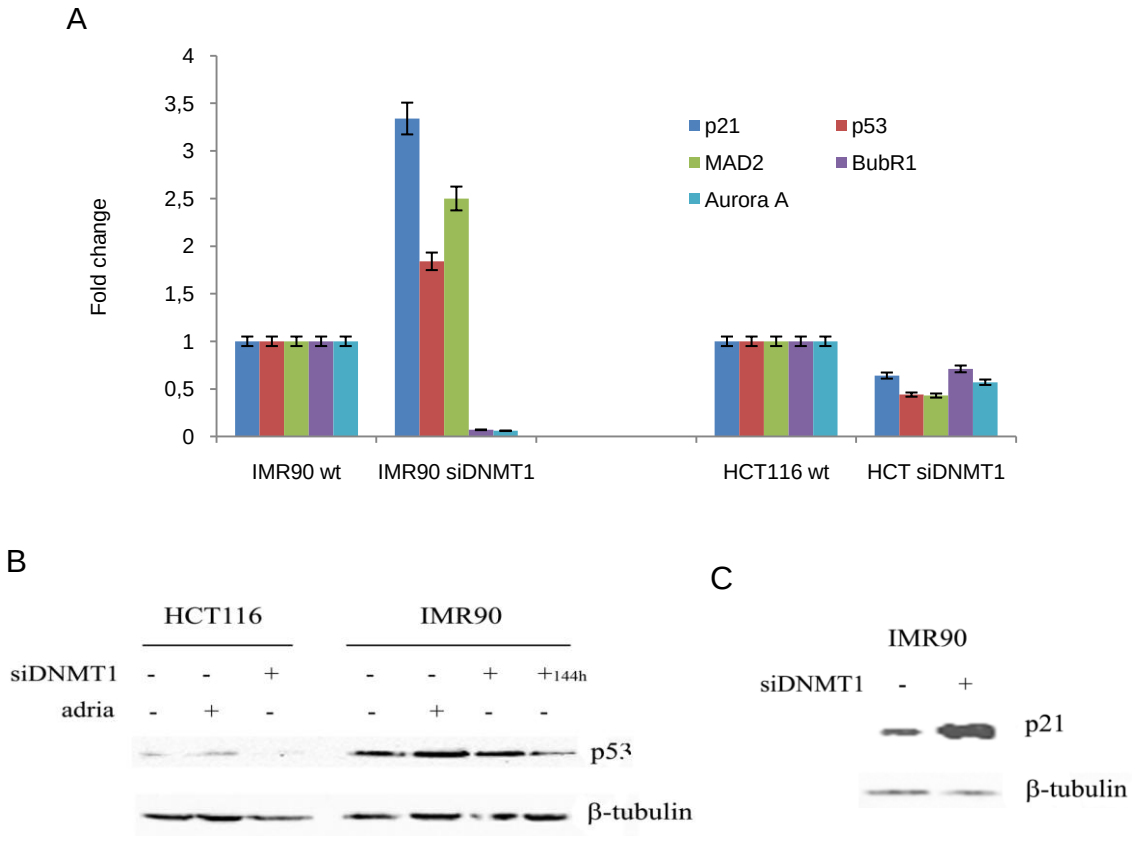



*Figure 20. A) Real time RT-PCR and (B,C) WB analysis revealed a strong increase of p21$^{waf1}$ and TTP53 proteins level in IMR9O cells and not in HCT116 cells. At 144 hours from siDNMT1 transfection TTP53 level came back to basal level. Cells treated with adriamicyn to induce DNA damage were used to assess the functionality of the TTP53 pathway.*

These results agreed with TP53 involvement in the cell cycle arrest triggered by DNMT1 downregulation.

*IMR90 cells sense Dnmt1 decrease and activates a TP53-controlled pathway.*

To understand if TP53 is really involved in cell cycle arrest triggered by DNMT1 downregulation, I performed a double siRNAs transfection with siDNMT1 and siTP53 together in IMR90 cells. Real Time RT-PCR and Western blot analysis confirmed DNMT1 and TP53 decrease in respect to control cells (fig. 21 A, B). 72 hours post-transfection I found a number of cells in culture comparable to control, suggesting an almost unchanged cell proliferation (fig. 21 C). Biparametric cytofluorimetric assay showed also that siDNMT1/siTP53 cells underwent a normal cell cycle recovering from the G1 arrest of IMR90 siDNMT1 cells (fig. 21 C).

Moreover, Western blot analysis showed that in double silenced cells $p21^{waf1}$ is not incremented (fig. 21 B).

These findings suggest that TP53 reduction allowed cell cycle progression of Dnmt1 depleted IMR90 cells bypassing the G1 arrest and in turn that TP53 is involved in the pathway that induced the G1 arrest in IMR90 siDNMT1 cells.

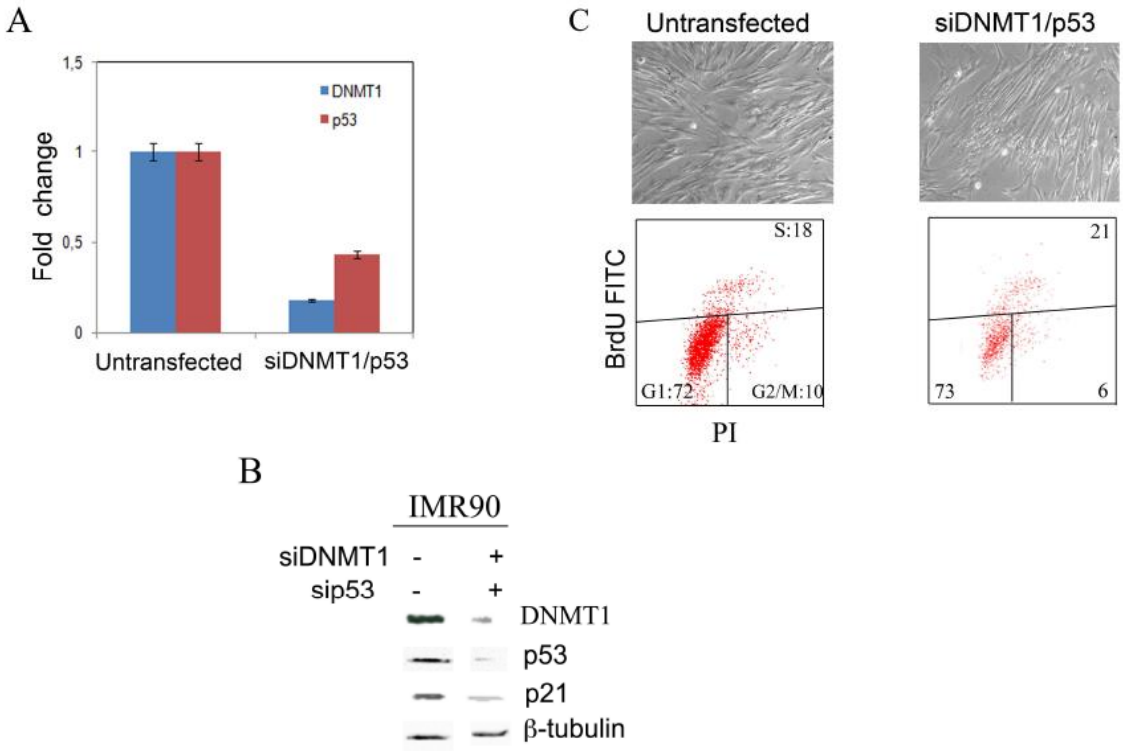



*Figure 21. A) Real time RT-PCR and (B) WB detection of TTP53 and Dnmt1 levels in IMR90-siDNMT1/TP53 cells and in IMR90-wt cells. C) Top panels: IMR90-wt and MR90-siDNMT1/TP53 cells pictures at 72 hours post-transfection showing that the cell density/dish in IMR90-siDNMT1/TP53 cells is comparable to that of IMR90-wt cells. Bottom panels: cytofluori-metric profiles showing no significative differences in cell cycle distribution of wt and IMR90-siDNMT1/TP53 cells.*

## *DNMT1 depletion does not induce DNA damage.*

In order to understand the pathway that activates/stabilizes TP53 after DNMT1 depletion in IMR90, I evaluated the activation of those pathways that are known to induce a G1 arrest involving TP53 (fig. 22).

To this aim I checked if DNMT1 depletion could induce DNA damage. The fact that HCT116 respond to adriamicyn, a drug that induce DNA double strand

breaks, and not to DNMT1 deregulation activating TP53 (fig. 20B) was already a first suggestion that DNMT1 downregulation did not induce DNA damage.

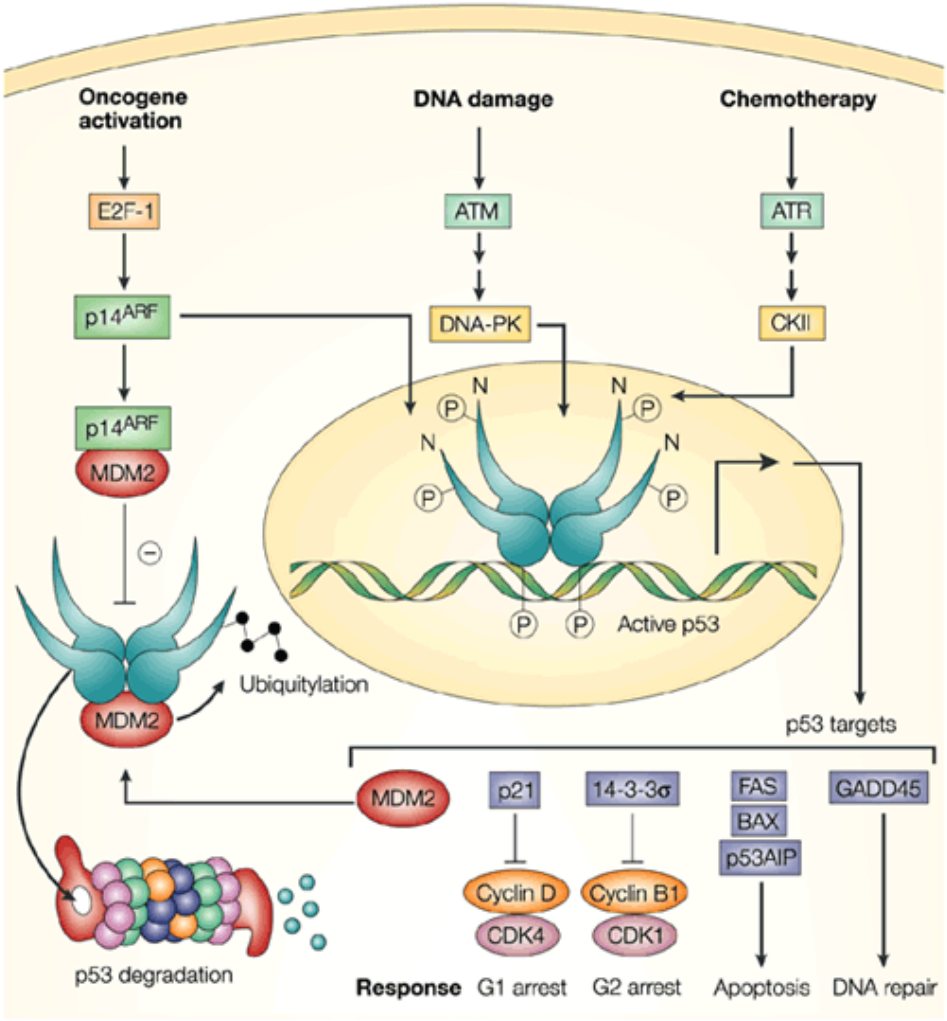



*Figure 22. Scheme of the pathways activating TP53. Cellular stress (oncogenes activation) induces p14ARF, which sequesters MDM2. In addition, DNA damage and chemotherapeutics activate ATM and ATR, which phosphorylate the amino terminus of TP53 to prevent MDM2 binding, and the carboxyl terminus of TP53 respectively to increase sequence-specific DNA binding. These events increase TP53 levels and activate the transcription of TP53 target genes*[92].

However, in order to exclude the induction of DNA damage I monitored the amount of phosphorylated H2A.X protein (γH2AX) by immunofluorescence mi-

croscopy. Phosphorylated H2A.X is a modified histone that accumulates at double-stranded breaks and functions to recruit DNA repair protein. I observed a small difference of γH2AX signals between control (IMR90 wt and HCT116 wt) and transfected cells (IMR90 siDNMT1 and HCT siDNMT1). On the contrary, cells treated with adriamycin were positive to H2A.X antibody (fig. 23).

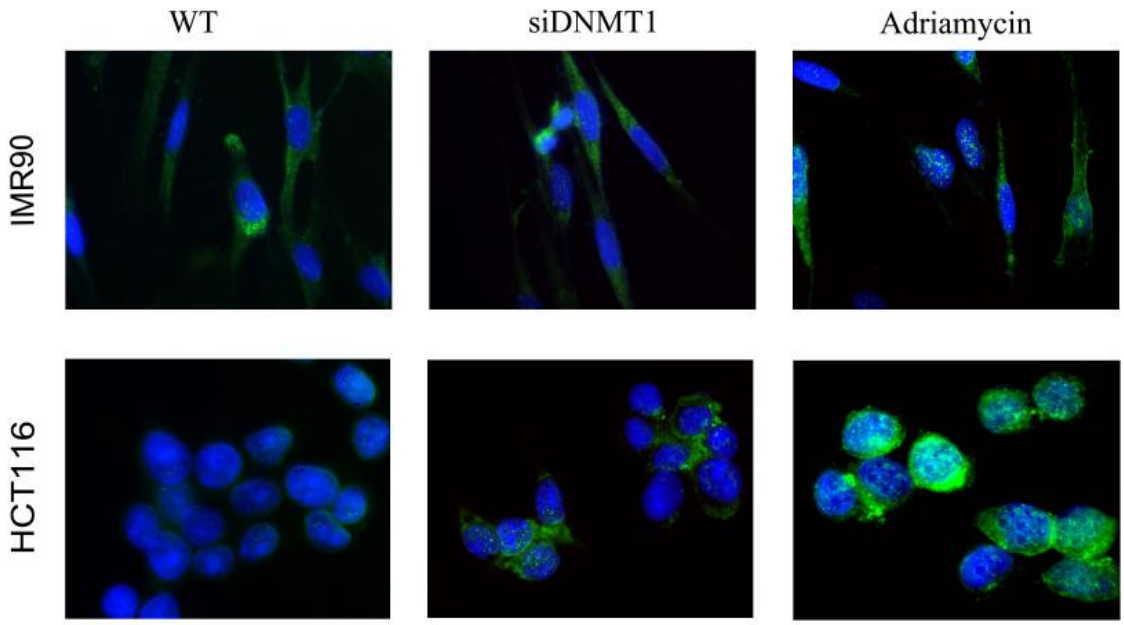



*Figure 23. Immunofluorescence analysis against γH2A.X revealed that DNMT1 silencing did not induce γH2A.X positive foci formation in both IMR90 and HCT116 cells. Cells treated with adriamycin were used as a positive control.*

Moreover, I evaluated also the possible transcriptional activation of DNA repair proteins (RAD51L1, RAD51L2, RAD51L3) by Real time RT-PCR, but I did not observe overexpression of these genes (fig. 24).

Altogether these results showed that the putative pathway mediated by TP53 and triggered by DNMT1 downregulation is not induced by DNA damage.

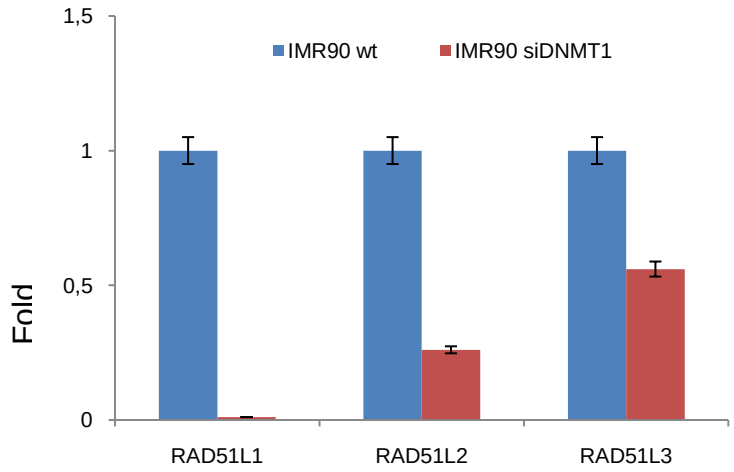



*Figure 24. Real time RT- PCR showing underexpression of RAD51L1-3 in IMR90 siDNMT1 cells.*

## *Double silencing of p14ARF and DNMT1 abrogates G1 arrest in IMR90 cells.*

In order to investigate on the pathway that activates TP53 in IMR90 siDNMT1 cells triggering the G1 arrest, I checked also the involvement of p14ARF, that is known to stabilize TP53 sequestering MDM2 (fig. 22). First, I looked at its transcriptional levels by Real time RT-PCR, but I did not found significative differences in respect to untreated cells.

P14ARF expression remains almost unchanged also at 144 hours post siDNMT1 transfection, but it is underexpressed in siDNMT1/TP53 double silenced cells (fig. 25).

To investigate further on p14ARF potential involvement in TP53 activation after DNMT1 depletion I performed a double siRNAs transfection with siDNMT1 and

sip14ARF in IMR90 cells. 72 hours post-transfection the number of cells in culture

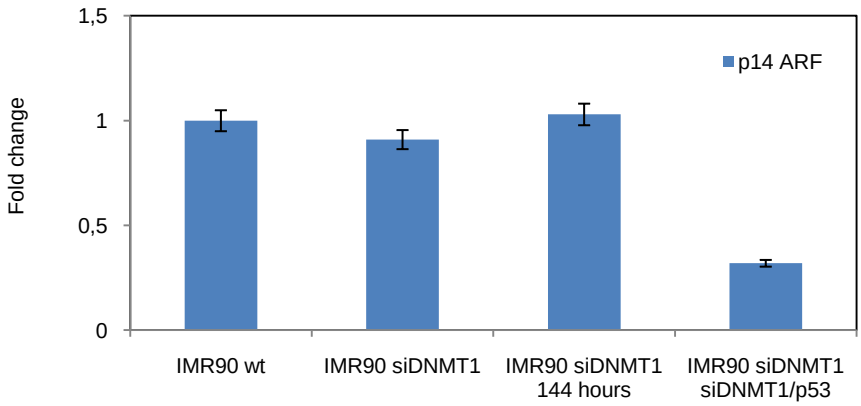



*Figure 25. Real time RT-PCR showing the relative levels of p14ARF transcripts in wt, siDNMT1, siDNMT1 144 hours and siDNMT1/TP53 IMR90 cells. P14ARF levels do not changed after DNMT1 silencing, but are reduced in siDNMT1/TP53 cells.*

was comparable to control, suggesting an almost unchanged cell proliferation (fig. 26).

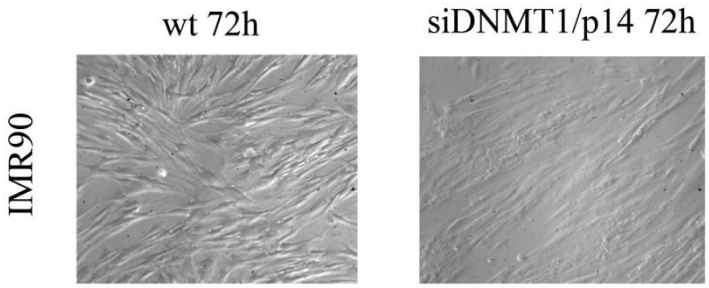


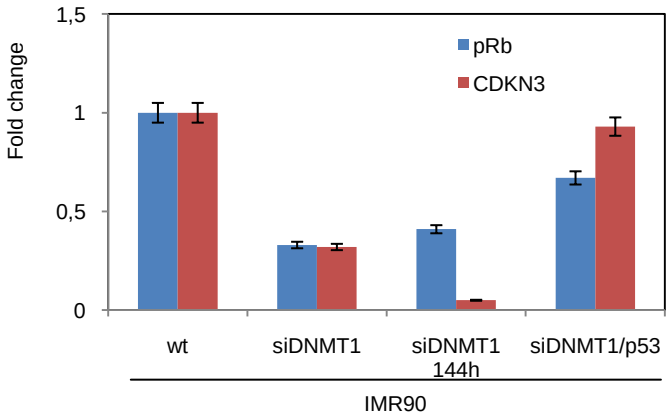

*Figure 26. IMR90-wt and MR90-siDNMT1/p14ARFARF cells pictures at 72 hours post-transfection showing that the cell density/dish in IMR90-siDNMT1/p14ARFARF cells is comparable to that of IMR90-wt cells.*

*DNMT1 post-transcriptional silencing induces pRb reduction*

The retinoblastoma protein, pRb, is a tumor suppressor protein belonging to the pocket protein family that is dysfunctional in many types of cancer. It binds and inhibits transcription factors of the E2F family that can push a cell into S phase. As long as E2F is inactivated, the cell remains stalled in the G1 phase.

To investigate further on the G1 arrest induced by DNMT1 downregulation I looked at pRb expression after DNMT1 downregulation. Real time RT-PCR and Western blot analyses showed pRb decrease in IMR90 cells treated for 72 hours with siRNA targeting DNMT1. PRb protein levels were restored at 144h post-transfection (fig. 27 A, B). Accordingly to these results there was also a decrease of CDKN3 transcript after DNMT1 post-transcriptional silencing (fig. 27 A). CDKN3 inhibits CDK2/cyclinE complex contributing to not phosphorylate pRb and consequently to deactivate E2F. Moreover, in DNMT1/TP53 silenced

A




B

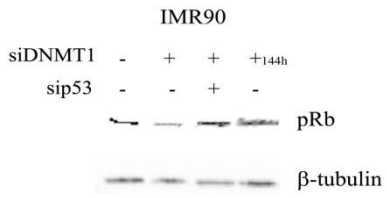



*Figure 27. A) Real time RT-PCR showing the relative transcriptional levels of pRb and CDKN3 in wt, siDNMT1, siDNMT1 144 hours and siDNMT1/TP53 IMR90 cells. B) Western blot analysis showing protein levels of pRb in IMR90 wt, siDNMT1 at 72 and 144 hours and siDNMT1/TP53.*

cells pRb transcriptional levels were reduced in respect of untreated cells, but were higher than in siDNMT1 cells (fig. 27 A). Western blot analysis showed that pRb protein levels were restored when TP53 is silenced with DNMT1 (fig. 27 B).

*Double silencing of DNMT1 and TP53 induced aneuploidy in IMR90 cells*

My results are consistent with the hypothesis of TP53 involvement in the G1 arrest induced by DNMT1 depletion in IMR90. In order to understand if the abrogation of this cell cycle arrest alters cellular ploidy I performed a cytogenetics analysis in siDNMT1/TP53 IMR90 cells. The analysis showed that the number of IMR90-siDNMT1/TP53 metaphases was similar to that of untransfected IMR90 cells (mitotic index 3%) and that the majority of them (70%) were hypodiploid (fig. 28) like the HCT-siDNMT1 cells that did not arrest in G1.

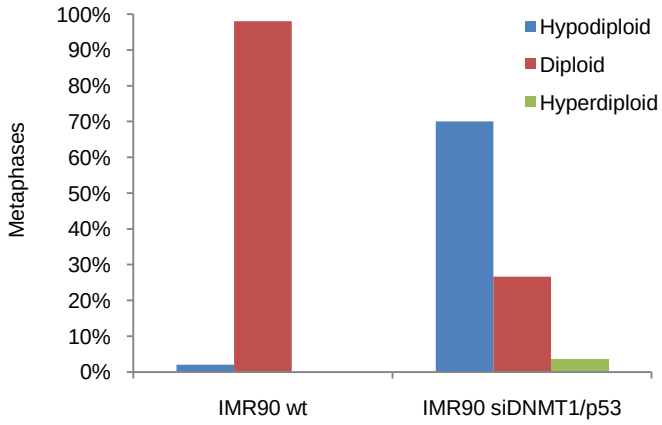



*Figure 28. Histogram showing percentages of both aneuploid and diploid metaphases. IMR90 siDNMT1/TP53 cells acquired hypodiploidy.*

**Evaluation of the effects of DNMT1 overexpression in human cells.**

In order to investigate on the long term consequences of DNA hypermethylation in normal cells I studied the effects of DNMT1 overexpression in IMR90 and MCF10A cells.

*Generation of pRB stably depleted MCF10A cells*

Previous experiments have highlighted that pRb depletion induce DNMT1 over-expression in human cells (IMR90 and HCT116). So the first approach to obtain DNMT1 overexpression was indirect by inducing pRb stable post-transcriptional silencing in MCF10A cells. In order to silence pRb I used retroviral particles containing MSCV-LMP vector (LMP microRNA adapted vector) [93] (fig. 29 A). In this vector is cloned a synthetic microRNA (mir670) that once processed originates antisense RNA directed against a portion of the pRb transcript. Pairing between miRNA and mRNA strands provokes mRNA translation inhibition or degradation. In this way MSCV-LMP induces a stable post-transcriptional silencing of pRb. Retroviral particles were produced by packaging cells (Phoenix) transfected with the MSCV-LMP vector. Transfection efficiency was assessed by fluorescence microscopy since the vector contains GFP (Green Fluorescence Protein) cDNA (fig. 29 B). At 48 hours post transfection culture medium containing retroviral particles was collected, filtered and used to infect MCF10A cells. At 72 hours post infection cells containing retroviral vector were selected in puromycin for a week. Real Time RT-PCR confirmed pRb reduction (90%) (fig. 29 C). Unexpectedly pRb silencing gradually inhibited MCF10A cell growth then inducing a complete cell cycle arrest, assessed by cytofluorimetry (fig. 29

D). In order to verify DNMT1 overexpression I did a real time RT-PCR reaction, that showed, instead, DNMT1 downregulation. In addition to DNMT also others genes such as DNMT3b, MAD2 and Aurora A were downregulated after pRb silencing (fig. 29 C). Probably pRb post-transcriptional silencing induced a strong cellular stress in these cells leading them to metabolic and growth arrest. For these reasons it was not possible to investigate further these cells.

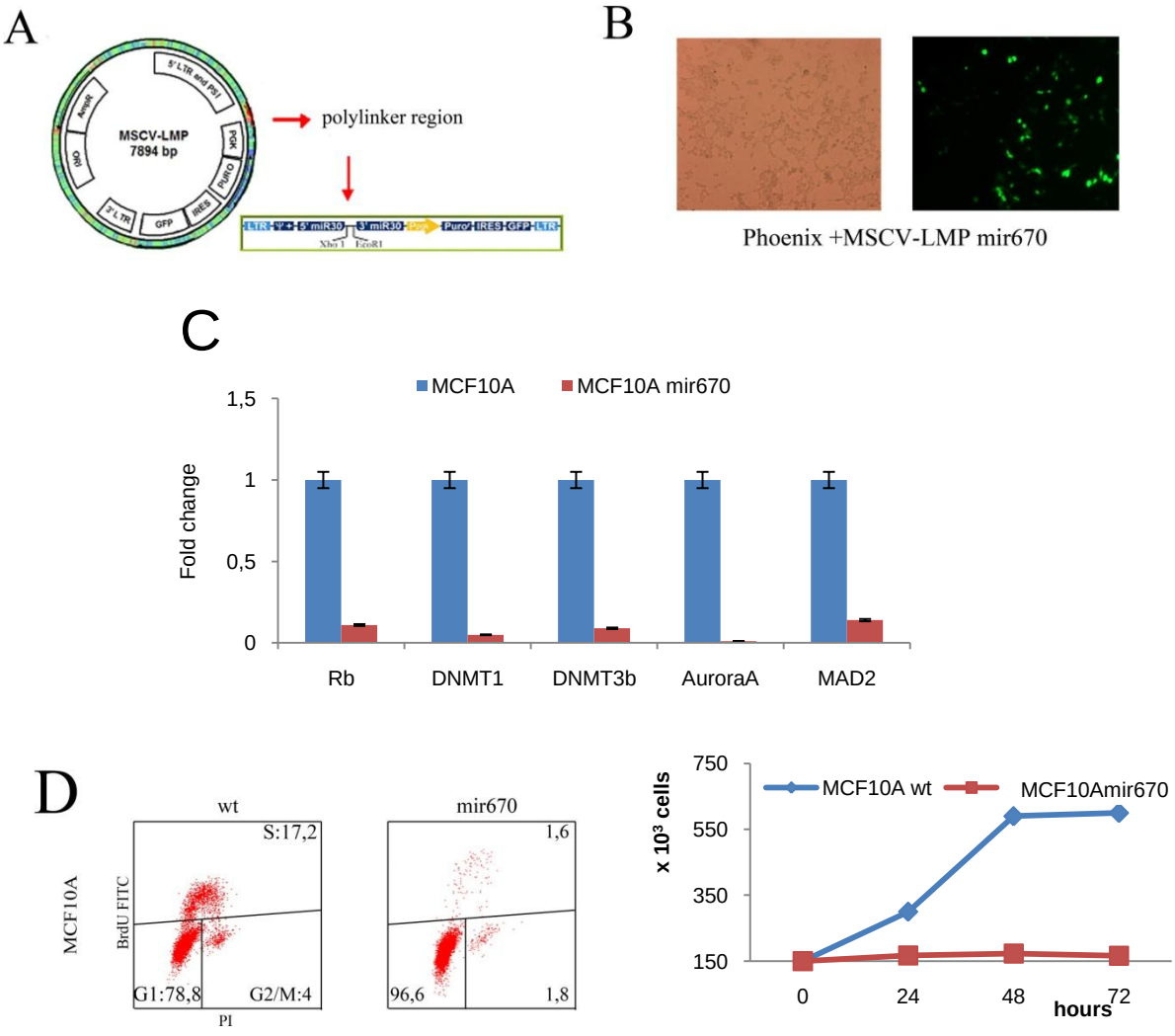



*Figure 29. A) Scheme of MSCV-LMP vector containing mir670. B) Pictures showing Phoenix cells transfected with MSCV-LMP mir670 vector. GFP fluorescence indicates cells that received the plasmid. C) Real time RT-PCR showing transcript levels of Rb, DNMT1, DNMT3b, Aurora A and MAD2 in MCF10A mir670 cells. D) Cytofluorimetric profiles and time course of MCF10A mir670 cells compared to that of untreated cells.*

*Generation of IMR90 cells overexpressing DNMT1*

I used a second strategy to obtain DNMT1 overexpression by transfecting a vector (PCMV HMT) harboring DNMT1 cDNA cloned [94] (fig. 30 A)in IMR90 cells. I did several experiments to transfect this vector in IMR90 cells and every time cells selected in G418 for a week died immediately (fig. 30 B). I supposed that DNMT1 overexpression was lethal for IMR90 cells.

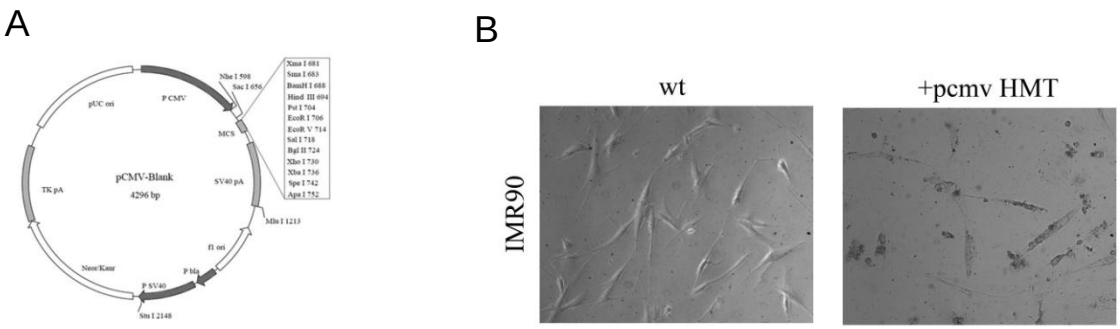



*Figure 30. A) Scheme of PCMV vector. B) Pictures showing IMR90 cells transfected with PCMV-HMT vector in respect to wt cells.*

# Discussion

It is known that DNA methylation, histone methylation and deacetylation combine to bring about DNA hypercondensation typical of centromeric and pericentromeric chromatin [95,96]. Imbalance in cytosine methylation at CpG islands and deregulation of DNA methyl-transferases, in particular DNMT1, are recurrent in human sporadic cancers [46-49] and it seems that they could be involved in the acquisition of chromosomal instability [88] by inducing chromatin decondensation at the centromere region of chromosome. This in turn could result in altered kinetochore formation/assembling and chromosomes missegregation. The aim of my work has been to understand if DNMT1 transient depletion by RNA interference could be implicated in the generation of aneuploidy by altering DNA methylation patterns.

By using two different cell strains, human primary fibroblasts (IMR90) and stable near-diploid tumor cells (HCT116), I determined that Dnmt1 transient depletion had different outcomes related to the genetic context of the cell. The first observed consequence of DNMT1 post-transcriptional silencing was the arrest of cellular proliferation in IMR90 human fibroblasts. On the contrary, only a slight decrease of cell proliferation was observed in HCT116 cells. This difference was confirmed by FACSscan analysis showing IMR90 cells arrested in the G1 phase of the cell cycle and HCT116 cells lightly accumulated in S phase. As a consequence of the proliferation arrest mediated by Dnmt1 transient depletion IMR90 fibroblasts showed very few mitotic cells (mitotic index 0,3%). Untransformed cells activated a G1/S checkpoint preventing S-phase entry, while tumor

cells delayed cell cycle progression extending S-phase duration when DNMT1 was reduced.

DNMT1 usually localizes at replication foci because of its interaction with the proliferating cell nuclear antigen, PCNA [41,97]. It is possible that low levels of DNMT1 lead to a delayed and difficult S phase progression in tumor cells. On the contrary its severe depletion is perceived before entering S phase in order to block cell cycle immediately (G1) in normal cells. Propidium iodide staining revealed also that IMR90 siDNMT1 cells can already exit G1 in less than 24 hours after the arrest, suggesting that the arrest is relieved as soon as DNMT1 is re-expressed .

DNMT1 depletion could also be sensed by cells as a danger signal that induce them to arrest in order to prevent the effect of global DNA demethylation. In fact, using anti 5-methylcytosine antibody to check DNA methylation status, I noticed DNA demethylation in HCT siDNMT1 cells that do not arrest. Unexpectedly, I did not observe any modification of methylation status of CHFR gene promoter found to be hypermethylated after DNMT1 post transcriptional silencing in HCT116 cells, suggesting that DNMT1 is not the only methyl-transferase involved in its hypermethylation status [98]. My results are consistent with the notion that the lack of DNA replication following DNMT1 depletion in normal cells prevents the generation of daughter cells with hypomethylated DNA. Moreover, I noticed that in a genetic contest of cancer cells where DNMT1 is overexpressed cells do not sense its scarce and/or strong decrease as a danger and do not arrest but have only a delayed S phase.

The cellular checkpoint triggered by Dnmt1 depletion seems mediated by TP53 activation because of its overexpression/stabilization in arrested IMR90

cells. TP53 involvement in this response was further directly suggested by the findings that DNMT1 and TP53 double silenced IMR90 cells did not arrest anymore. Moreover, the observation that IMR90 cells restarted to proliferate at 144 hours from siDNMT1 transfection and that TP53 amount returned to basal levels, are additional evidences of the existence of a checkpoint TP53 controlled that responds to Dnmt1 lack.

However, this checkpoint seems different from the classic TP53 pathway activated by DNA damage. In fact, HCT116 cells, that responded correctly to the presence of DNA damage - as showed by adriamycin treatment that induced double strand breaks - by increasing the levels of TP53 and of its effector p21 $^{waf1}$, were unable to halt cellular proliferation in response to transient Dnmt1 depletion. In addition, it was not possible to find DNA damage in IMR90 siDNMT1 cells, at least as revealed by γH2A.X foci, even if DNMT1 depletion was previously associated with γH2A.X foci in T24 cells [99]. I hypothesize that a specific intermediate activating TP53 in response to Dnmt1 depletion in IMR90 cells is lacking in HCT116 cells, thus allowing cell cycle progression of these cells.

Investigating the other classic TP53 pathway that is induced by p14ARF, I discovered that, unless p14ARF transcript level remained almost unchanged in DNMT1 depleted in respect to control cells, it decreased in double DNMT1 and TP53 silenced cells that were not arrested. In addition, double DNMT1 and p14ARF silenced IMR90 cells seem to proliferate normally, reinforcing the idea of the p14ARF involvement in the G1 arrest. The fact that HCT116 cells do not express p14ARF, because one allele is mutated and the other have the promoter region hypermethylated [100], could explain the lack of G1 arrest in these cells. These results suggest that p14ARF could be the intermediate that acti-

vates TP53 triggering the G1 arrest in IMR90 cells and its lack allow cell cycle progression of DNMT1 depleted HCT116 cells.

Generally, in a G1 arrest induced by p21 $^{waf1}$ activation, p21$^{waf1}$ inhibits pRb hyperphosphorylation by Cdk/cyclin complexes. In this way pRb does not release E2F and S-phase genes are not induced. The observed pRb and CDKN3 decrease was unexpected in a contest of p21$^{waf1}$ and TP53 overexpression and G1 arrest. However, it has been reported that cell cycle arrest with p21$^{waf1}$ overexpression is associated with pRb decrease [101]. Moreover, pRb and CDKN3 decrease seems to depend on cell cycle arrest since pRb levels are restored when the putative checkpoint is silenced as observed after 144 hours post siDNMT1 transfection and in siDNMT1/siTP53 cells. These results suggest the existence of a negative feedback which gets cells ready for reentering cell cycle after the arrest (the arrest is transient).

In order to understand DNMT1 correlation with aneuploidy, I checked chromosome numbers in IMR90 and HCT116 cells after DNMT1 silencing. Since IMR90 cells were arrested I could analyse only few metaphase cells (mitotic index 0,3%) that were mainly hyperdiploid. On the other hand mitotic index of HCT116 cells was not compromised by DNMT1 silencing and I observed a high percentage of hypodiploid cells. Probably, the fact that IMR90 cells promptly arrest after DNMT1 silencing prevents the acquisition of an aneuploid phenotype, even if cells that escape the block do. On the contrary, HCT116 cells acquired aneuploid phenotype because they did not arrest in G1.

Moreover, the finding that the centromere of mitotic IMR90 siDNMT1 cells seems to be les stained suggest the presence of decondensed chromatin around centromeric and pericentromeric region. These alterations could be the

cause of aneuploidy generation. Altered chromatin structure around the centromere could compromise the correct kinetochore assembly and/or the correct microtubules attachment to chromosomes. These could result in incorrect segregation of sister chromatids [76,77]. Since I noticed that if cells succeed in entering S phase and continue to proliferate, even in DNMT1 absence (HCT siDNMT1cells), their genome is demethylated, demethylation could involve pericentromeric chromatin leading to decondensation. This, in turn, could affect the correct chromosome segregation generating aneuploidy.

As a confirmation of this hypothesis, an increased number of IMR90 aneuploidy cells was seen only after unscheduled G1/S progression induced by the transient TP53 silencing when DNMT1 is depleted (DNA demethylation).

In this way one potential role for the TP53 induced G1 arrest triggered by DNMT1 reduction could be not only to protect the epigenome from global loss of DNA methylation pattern, but also to prevent the subsequent aneuploidy.

Finally, these findings suggest that primary human cells perceive the Dnmt1 absence that might lead to hypomethylation, profoundly altering the epigenome, as a stress signal and thus activate a TP53 controlled pathway, probably induced by p14ARF, that in turn induces G1 arrest. When this pathway is not working as in tumor cells or it is abrogated by silencing TP53, cells progress incorrectly in the cell cycle with altered DNA methylation pattern (hypomethylation) that might affects also the right chromosomal segregation thus resulting in aneuploidy (fig. 31).

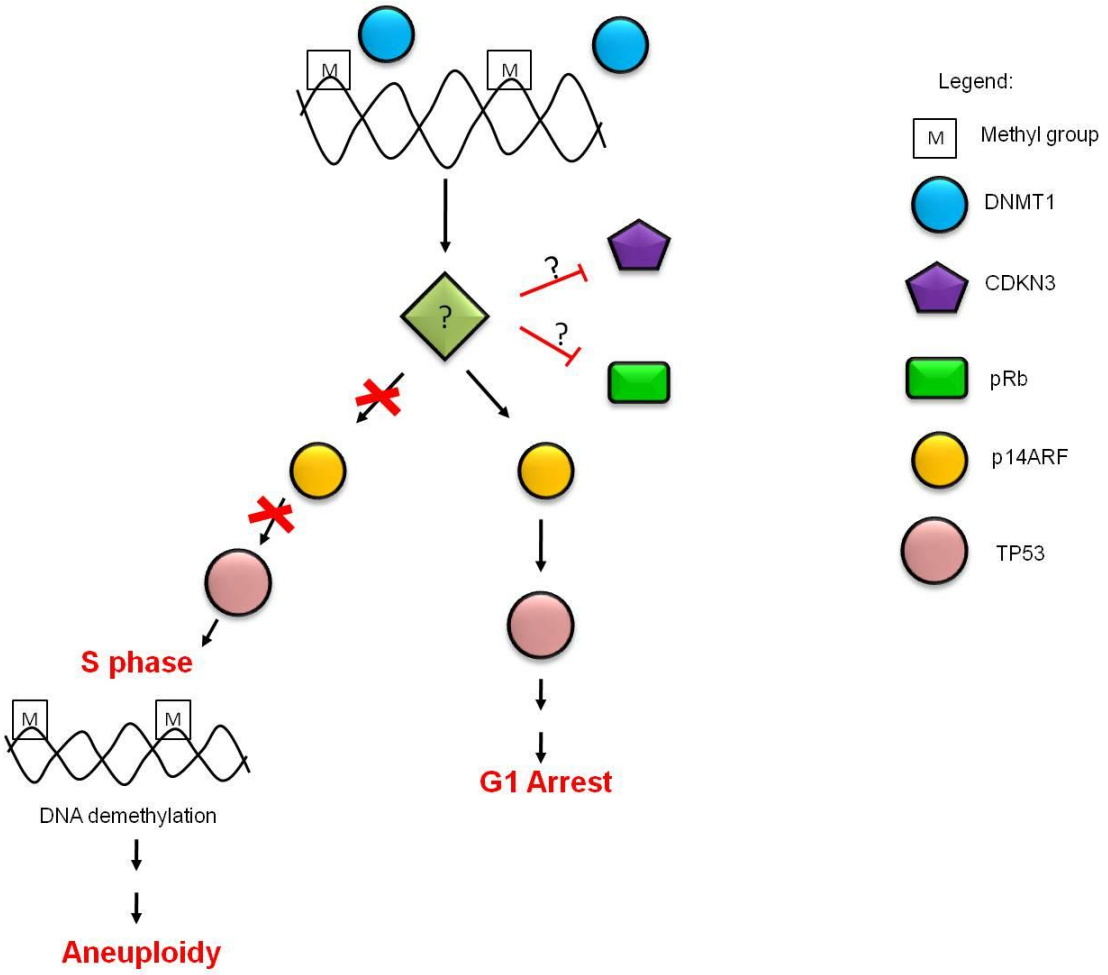



*Figure 31. Speculative model of a putative pathway induced by DNMT1 depletion. Dnmt1 depletion is perceived by a putative "sensor" factor that activates a pathway p14ARF/TP53 controlled triggering G1 arrest in normal cells. Moreover, the putative sensor factor induces pRb and CDKN3 decrease in order to assure a prompt relief from cell cycle arrest. If TP53 or p14ARF are absent or not working properly, cells are unable to sense Dnmt1 depletion and thus progress in the cell cycle incorrectly with wrong DNA methylation patterns triggering aneuploidy.*

# Material and methods

## Cells and cell culture

Human primary fibroblasts (IMR90 passage 12, ATCC) were cultured in EMEM supplemented with: 10% FBS (GIBCO, Invitrogen), 100units/ml penicillin and 0,1 mg/ml streptomycin (Euroclone Ltd UK), 1% NEAA (GIBCO); the human colon cancer cell line HCT116 were cultured in DMEM supplemented with: 10% FBS, 100units/ml penicillin and 0,1 mg/ml streptomycin; MCF10A cells (a generous gift of Prof. G. Stassi, University of Palermo) were cultured in DMEM/F12 (GIBCO, Invitrogen) supplemented with: 10% FBS, 100units/ml penicillin and 0,1 mg/ml streptomycin, 0,5mg/ml Hydrocortisone, 10mg/ml Insulin, 20ng/ml EGF (SIGMA). Cells were cultured in a humidified atmosphere of 4% $CO_2$ in air at 37°C.

## RNA interference

For siRNAs transfection $1{,}5x10^5$ IMR90 cells and $2{,}5x10^5$ HCT116 cells were plated in 6-well dishes and incubated at 37°C. Specific siRNAs duplex were mixed with Lipofectamine2000 Reagent (Invitrogen), according to manufacturer's recommendation and added to the cells. After 6 hours at 37°C, the transfection medium was replaced with fresh medium. To silence genes of interest post-transcriptionally, cells were transfected with siRNAs targeting DNMT1 (siDNMT1: 5'- AUU ACG UAA AGA AGA AUU A dTdT-3') [90] at final concentrations of 60, 80 and 100nM, siRNAs targeting TP53 (si TP53: 5'-GCA UGA ACC

GGA GGC CCC AUtt-3') [102] and p14ARF (sip14: 5'-GAA GAU CAG GUC AUG AUG Att-3') at a final concentration of 60nM. All siRNAs were synthesized by Eurofins-MWG (Germany).

The day of transfection the siRNA and the transfection reagent (Lipofectamine 2000, Invitrogen) were diluted separately in Opti-MEM (Invitrogen) mixed gently and then incubated for 5 minutes at room temperature. After incubation the siRNA and Lipofectamine 2000 (Invitrogen) were mixed gently, allowed to sit 20 minutes at room temperature to allow complex formation, and added to the plates with 2 ml of medium for 72 hours.

Stable pRb post transcriptional silencing in MCF10A cells.

For MSCV-LMP mir670 transfection $3x10^6$ packaging cells (Phoenix) were plated in 100mm dishes and incubated at 37°C. 8µg of plasmid DNA was mixed with Lipofectamine2000 Reagent (Invitrogen), according to manufacturer's recommendation and added to the cells. During 72 hours retroviral particles were produced by cells and released in the medium. At 72 hour the medium was filtered and added to MCF10A cells with 4µg/ml Polybrene. After 5 hours from infection the medium was changed and plates incubated at 37°C. At 72 hours cells were selected with puromicyn 1µg/ml for a week.

PCMV-HMT transfection in IMR90 cells.

For PCVM-HMT plasmid (a gentle gift from Paula Vertino, Emory University, Atlanta) transfection $5x10^5$ IMR90 cells were plated in 100mm dishes and incu-

bated at 37°C. 8µg of plasmid DNA were mixed with Lipofectamine2000 Reagent (Invitrogen), according to manufacturer's recommendation and added to the cells. At 72 hour cells were selected with G418 100µg/ml for a week.

Real time RT-PCR

Primers to be used in real time RT-PCR experiments were designed with Primer Express software (Applied Biosystems) choosing amplicons of approximately 70-100 bp. The selected sequences were tested against public databases (BLAST) to confirm the identity of the genes. Total RNA was extracted from cells by using the RNAeasy mini kit (GE) or the "All prep DNA/RNA kit" (Qiagen) according to the manufacture's instruction. RNA was reverse-transcribed in a final volume of 100 µL using the High Capacity c-DNA Archive kit (Applied Biosystems) for 10 minutes at 25° C and 2 hours at 37°C. For each sample 2 µL of cDNA, corresponding to 100 ng of reverse transcribed RNA, was analyzed by Real time RT-PCR (95°C for 15 sec, 60°C for 60 sec repeated for 40 cycles), in quadruplicate, using the ABI PRISM 7300 instrument (Applied Biosystems). Real Time RT-PCR was done in a final volume of 20µl comprising 1x Master Mix SYBR Green (Applied Biosystems) and 0,3µM of forward and reverse primers for: DNMT1 (Fwr: 5'-GCACCTCATTTGCCGAATACA-3'; Rev: 5'-TCTCCTGCATCAGCCCAAATA-3'), TP53 (Frw: 5'-TTCGACATAGTGTGGTGGTGC-3'; Rev: 5'-AGTCAGAGCCAACCTCAGGC-3'), $p21^{waf1}$ (Frw: 5'-CTGGAGACTCTCAGGGTCGA-3', Rev: 5'-CGGATTAGGGCTTCCTCTTG-3'), GAPDH (Frw: 5'-CTCATGACCACAGTCCATGCC-3', Rev: 5'- GCCATCCACAGTCTTCTGGGT-3'), pRb (Frw: 5'-GCAGTATGCTTCCACCAGGC-3', Rev: 5'-AAGGGCTTCGAGGAATGTGAG-3'), MAD2l1 (Frw: 5'-

CTCATTCGGCATCAACAGCA- 3', Rev: 5'- TCAGATGGATATATGCCACGCT-3'), AuroraA human (Frw: 5'-TTTTGTAGGTCTCTTGGTATGTG-3', Rev: 5'-GCTGGAGAGCTTAAAATTGCAG-3'), DNMT3b (Frw: 5'-CAGACGTGTCCAACATGGGC-3', Rev: 5'-GCCTTCAGGAATCACACCTC-3'), CDKN3 (Frw: 5'-CATAGCCAGCTGCTGTGA-3', Rev: 5'-CCCGGATCCTCTTAGGTCTC-3'), p14ARF (Frw: 5'-TGATGCTACTGAGGAGCCAGC-3', Rev: 5'-AGGGCCTTTCCTACCTGGCTC-3'), BubR1 (Frw: 5'-TACACTGGAAATGACCCTCTGGAT-3', Rev: 5'-TATAATATCGTTTTTCTCCTTGTAGTGCTT-3'). Data were analyzed by averaging quadruplicates Ct (cycle threshold). Levels of RNA expression were determined by using the SDS software version (Applied Biosystems) according to the 2-ΔΔct method. Levels of RNA expression of selected genes were normalized to the internal control GAPDH.

Western Blotting.

Protein concentration was measured using the Bio-RadProtein Assay (Bio-Rad Laboratories). Proteins (40µg) were separated by 10% SDS-PAGE containing 0,1% SDS and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science) by electroblotting. The membranes were sequentially incubated with goat anti-DNMT1, rabbit anti-$p21^{waf1}$, rabbit anti-pRb (Santa Cruz, CA), mouse anti-TP53 (ABCAM) as primary antibodies, and HRP-conjugated mouse, rabbit (ABCAM) and goat (Santa Cruz, CA) IgG as secondary antibodies. The target protein was detected with enhanced chemiluminescence Western blotting detection reagents (PIERCE). I used also mouse anti-β-tubulin (SIGMA, Italy)

as internal control of loading. Membranes were stained by Ponceau-Red to confirm equivalent loading of total protein in all lanes.

## Cell cycle analysis

Asynchronously growing cells were treated with 80nM siDNMT1 alone or together with 60nM siTP53 for 72 hours and released into complete medium with Bromodeoxyuridine (BrdU) 0,2µg/ml for one hour. DNA content was determined using Propidium Iodide (PI) staining by treating cells with PBS solution containing 4µg/ml of PI and 40µg/ml RNase. Analysis of BrdU labelled cells was conducted as described previously and samples were analyzed on a FACSCanto (Becton Dickinson). Experiments were repeated at least twice, 10000 events were analyzed by FACSDiva software.

## Cytogenetical analysis

Cells were treated with 0,2µg/ml colcemid (Demecolcine, SIGMA, Italy) for 16 hours. Cells were harvested by trypsinization, swollen in 75mM KCl at 37°C, fixed with 3:1 methanol/acetic acid (3:1 v/v), and dropped onto clean, ice-cold glass microscope slides. The slides were air-dried and stained with 3% GIEMSA in phosphate-buffered saline for 10 minutes. Chromosome numbers were evaluated using Zeiss Axioskop microscope under a 100X objective.

## Immunofluorescence microscopy

Phosphorylated H2A.X immunostaining: cells, both IMR90 and MCF10A, were grown on glass coverslips after siRNAs transfection. Coverslips were fixed with methanol at -20°C, permeabilized with 0.01% Triton X (Sigma, St. Louis, MO) and blocked with 0.1% BSA, both at room temperature. Then, coverslips were incubated with phosphorylated H2A.X rabbit polyclonal antibody (1:100 in PBS-BSA 0.1%, Upstate) overnight at 4°C, washed in PBS and incubated with a FITC-conjugated rabbit anti-mouse (Sigma, diluted 1:100 in PBS-BSA 0,1%) for 1 hour at 37°C.

To detect centrosomes, $4 \times 10^4$ cells were grown on glass coverslips, fixed in methanol at -20°C, permeabilized with 0.01% Triton X (Sigma) and blocked with 0.1% BSA both at room temperature. Then, coverslips were incubated with a mouse monoclonal antibody against $\gamma$-tubulin (Sigma, diluted 1:250 in PBS-BSA 0,1%) overnight at 4°C, washed in PBS and incubated with a FITC-conjugated goat anti-mouse (Sigma, diluted 1:100 in PBS-BSA 0,1%) for 1 hour at 37°C.

Nuclei were visualized with 1µg/ml of 4',6-Diamidino-2-phenylindole (DAPI) and examined on a Zeiss Axioskop microscope equipped for fluorescence, images were captured with a CCD digital camera (AxioCam, Zeiss).

## Methylation Specific PCR (MSP)

Genomic DNA was extracted from cells by using the "All prep DNA/RNA kit" (Qiagen) according to the manufacture's instruction. DNA bisulfite modification was performed with "Epitect Bisulfite kit" (Qiagen) according to the manufac-

ture's instruction. Up to 1µg of genomic DNA was diluted to a total volume of 140 µl with water, bisulfite mix and DNA protect buffer, and sodium bisulfite treatment was carried out for 5 hours alternating denaturation at 99°C and incubation at 60°. Bisulfite- treated DNA samples were then purified with the "epitect column", and desulfonated before precipitation in 20µl of "EB buffer". 100ng of bisulfite-treated DNA was used for MSP. MSP primers were chosen to recognize bisulfite-induced modifications of unmethylated cytosines. The primers sequences have been published previously for CHFR (19) (MCHFR For: 5'-GTAATGTTTTTTGATAGCGGC-3', Rev: 5'- AATCCCCCTTCGCCG-3'; UCHFR For: 5'- GGTTGTAATGTTTTTTGATAGTGGT-3', Rev: 5'-CAAATCCCCCTTCACCA-3'). The MSP utilized a 50µl reaction volume, 1,5U/µl Taq Gold, 1x Taq Gold Buffer, 0,2mM dNTPs, 1,5mM $MgCl_2$, 0,5 µM Primers and 100ng of DNA template, and included 45 cycles at the following annealing temperatures: MCHFR, 58°C; UCHFR, 50°C. DNA isolated from SW480 cells served as a negative methylation control. MSP products were analyzed on 1,8% agarose gel electrophoresis, and were determined to have methylation if a visible band was observed in the methylation reaction.

Global DNA methylation analysis

"Methylamp Global DNA methylation quantification kit" (Epigentek) was used to quantify global DNA methylation. 200ng of genomic DNA extracted from cell samples was immobilized in the strip well specifically treated to have high affinity to DNA for 2 hours at 37°C. The methylated fraction of DNA can be recognized by sequential incubation with 5-methylcytosine antibody for 45 minutes at

37°C, and with secondary antibody for 60 minutes at room temperature. A colorimetric reaction allowed methylated DNA quantification at 450nM with microplate reader.  A methylated DNA was used as positive control.

# References


1 Johnson, D. G. & Schneider-Broussard, R. Role of E2F in cell cycle control and cancer. *Front Biosci* **3**, 447-448 (1998).

2 Hengst, L. & Reed, S. I. Inhibitors of the Cip/Kip family. *Curr Top Microbiol Immunol* **227**, 25-41 (1998).

3 Carnero, A. & Hannon, G. J. The INK4 family of CDK inhibitors. *Curr Top Microbiol Immunol* **227**, 43-55 (1998).

4 Nyberg, K. A., Michelson, R. J., Putnam, C. W. & Weinert, T. A. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu Rev Genet* **36**, 617-656 (2002).

5 McGowan, C. H. & Russell, P. The DNA damage response: sensing and signaling. *Curr Opin Cell Biol* **16**, 629-633 (2004).

6 McGlynn, P. & Lloyd, R. G. Recombinational repair and restart of damaged replication forks. *Nat Rev Mol Cell Biol* **3**, 859-870 (2002).

7 Zhou, B. B. & Elledge, S. J. The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439 (2000).

8 Osborn, A. J., Elledge, S. J. & Zou, L. Checking on the fork: the DNA-replication stress-response pathway. *Trends Cell Biol* **12**, 509-516 (2002).

9 Boddy, M. N. & Russell, P. DNA replication checkpoint. *Curr Biol* **11**, 953-956 (2001).

10 Boddy, M. N., Furnari, B., Mondesert, O. & Russell, P. Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* **280**, 909-912 (1998).

11 Musacchio, A. & Salmon, E. D. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**, 379-393 (2007).

12 Waddington, C. H. The epigenotype. *Endeavour* **1**, 18–20 (1942).

13 Li, E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* **3**, 662-673 (2002).

14 Qiu, J. Epigenetics: Unfinished symphony. *Nature* **11 May 2006**, 143-145 (2006).

15 Mitra, D., Parnell, E. J., Landon, J. W., Yu, Y. & Stillman, D. J. SWI7SNF binding to the HO promoter requires histone acethylation and stimulates TATA-binding protein recruitment. *Molecular Cell Biology* **26**, 4095-4110 (2006).

16 Fry, C. J. & Peterson, C. L. Unlocking the gates to gene expression. *Science* **295**, 1874-1848 (2002).

17 Ducasse, M. & Brown, M. A. Epigenetic aberrations and cancer *Molecular Cancer*, 60 (2006).

18 Bottomley, M. J. Structures of protein domains that create or recognize histone modifications. *EMBO reports* **5**, 464-469 (2004).

19 Baker, E. K. & El-Osta, A. The rise of DNA methylation and the importance of chromatin on multidrug resistance in cancer *Experimental Cell Research* **290**, 177-194 (2003).

20 Zhang, Y. & Reinberg, D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails *Genes & Development* **15**, 2343-2360 (2001).

21 Lachner, M. & Jenuwein, T. The many faces of hisotne lysine methylation *Current Opinion in Cell Biology* **14**, 286-298 (2002).

22 Li, E., Bestor, T. H. & Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915-926 (1992).

23 Luczak, M. W. & Jagodzinski, P. P. The role of DNA methylation in cancer development. *Folia Histochemica et Cytobiologica* **44**, 143-154 (2006).

24 Kass, S. U., Pruss, D. & Wolffe, A. P. How does DNA methylation repress transcription? *Trends Genet.* **13**, 444-449 (1997).

25 Jones, P. L. *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Letters to Nature Genetics* **19 June 1998**, 187-191 (1998).

26 Hansen, R. S. *et al.* The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc Natl Acad Sci USA* **96**, 14412-14417 (1999).

27 Zaitseva, I., Zaitsev, S., Alenina, N., Bader, M. & Kricokharchenko, A. Dynamics of DNA demethylation in early mouse and rat embryos developed in vivo and in vitro. *Mol Reprod Dev* **74**, 1255-1261 (2007).

28 Lund, A. H. & van Lohuizen, M. Epigenetics and cancer. *Genes & Development* **18**, 2315-2335 (2004).
29 Hermann, A., Goyal, R. & Jeltsch, A. The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. *J Biol Chem* **279**, 48350-48359 (2004).
30 Bestor, T. H. The DNA methyltransferases of mammals. *Hum Mol Genet.* **9**, 2395-2402 (2000).
31 Jair, K. W. *et al.* De novo CpG island methylation in human cancer cells *Cancer Reseach* **66**, 682-692 (2006).
32 Fatemi, M., Hermann, A., Gowher, H. & Jeltsch, A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *Eur J Biochem.* **269**, 4981-4984 (2002).
33 Smale, S. T. The establishment and maintenance of lymphocyte identity through gene silencing. *Nature Immunology*, 607-615 (2003).
34 Margot, J. B. *et al.* Structure and function of the mouse DNA methyltransferase gene: Dnmt1 shows a tripartite structure. *J Mol Biol.* **297**, 293-300 (2000).
35 Araujo, F. D. *et al.* The DNMT1 target recognition domain resides in the N terminus. *J Biol Chem* **276**, 6930-6936 (2001).
36 Pradhan, S. & Esteve, P. O. Mammalian DNA (cytosine-5) methyltransferases and their expression. *Clin Immunol.* **109**, 6-16 (2003).
37 Rountree, M. R., Bachman, K. E. & Baylin, S. B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature Genetics* **25**, 269-277 (2000).
38 Suetake, I., Hayata, D. & Tajima, S. The amino-terminus of mouse DNA methyltransferase 1 forms an independent domain and binds to DNA with the sequence involving PCNA binding motif. *J Biochem.* **140**, 763-776 (2006).
39 Pradhan, S. & Kim, G. D. The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity *EMBO Journal* **21**, 779-788 (2002).
40 Robertson, A. K. *et al.* DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Letters to Nature Genetics* **25 july 2000**, 338-342 (2000).
41 Easwaran, H. P., Schermelleh, L., Leonhardt, H. & Cardoso, M. C. Replication-independent chromaitn loading of Dnmt1 during G2 and M phases. *EMBO Journal*, 1181-1186 (2004).
42 McCabe, M. T., Davis, J. N. & Day, M. L. Regulation of DNA methyltransferase 1 by Rb/E2F1 pathway. *Cancer Reseach* (2005).
43 Campbell, P. M. & Szyf, M. Human DNA methyltransferase gene DNMT1 is regulated by the APC pathway. *Carcinogenesis* **24**, 17-24 (2003).
44 Tan, H. H. & Porter, A. G. p21(WAF1) negatively regulates DNMT1 expression in mammalian cells. *Biochem Biophys Res Commun.* **382**, 171-176 (2009).
45 Kim, J. K., Estève, P. O., Jacobsen, S. E. & Pradhan, S. UHRF1 binds G9a and partecipates in p21 transcriptional regulation in mammalian cells. *Nucleic Acids Research* **37**, 493-505 (2008).
46 De Marzo, A. M. *et al.* Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis *Cancer Reseach* **59**, 3855-3860 (1999).
47 Shukla, V. *et al.* BRCA1 affects global DNA methylation through regulation of DNMT1. *Cell Res.* **20**, 1201-1215 (2010).
48 Lin, R. K. *et al.* Dysregulation of p53/Sp1 control leads to DNA methyltransferase-1 overexpression in lung cancer. *Cancer Reseach* **70**, 5807-5817 (2010).
49 Qu, Y. *et al.* Overexpression of DNA methyltransferases 1, 3a, and 3b significantly correlates with retinoblastoma tumorigenesis. *Am J Clin Pathol.* **134**, 826-834 (2010).
50 Sawan, C., Vaissière, T., Murr, R. & Herceg, Z. Epigenetic drivers and genetic passengers on the road to cancer. *Mutation Research*, 1-13 (2008).
51 Feinberg, A. P. & Tycko, B. The hystory of cancer epigenetics. *Nature Rev. Cancer*, 1-11 (2004).
52 Costello, J. F. & Plass, C. Methylation matters. *J Med Genet.*, 285-303 (2001).
53 Abbaszadegan, M. R. *et al.* Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the fi rst report from Iran. *World J Gastroenterol* **13**, 1528-1533 (2007).

54 Chim, C. S., Wong, A. S. Y. & Kwong, Y. L. Epigenetic Inactivation of the CIP/KIP Cell-Cycle Control Pathway in Acute Leukemias. *Am J Hematology.*, 282-287 (2005).
55 Graff, J. R. *et al.* E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Reseach*, 5195-5199 (1995).
56 Yanagisawa, Y. *et al.* Methylation of the hMLH1 promoter in familial gastric cancer with microsatellite instability. *Int J Cancer*, 50-53 (2000).
57 Wei, M. *et al.* BRCA1 Promoter Methylation in Sporadic Breast Cancer Is Associated with Reduced BRCA1 Copy Number and Chromosome 17 Aneusomy. *Cancer Reseach* **65**, 10692-10699 (2005).
58 Robertson, K. D. DNA methylation and human disease. *Nature rev. Genetics*, 597-610 (2005).
59 Chan, A. O. *et al.* CpG island methylation in aberrant crypt foci of the colorectum. *Am J Clin Pathol.* **160**, 1823-1830 (2002).
60 Rodriguez, J. *et al.* Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Reseach* **66**, 8462-8468 (2006).
61 De Smet, C. & Loriot, A. DNA hypometylation in cancer. *Epigenetics* **5**, 206-213 (2010).
62 Ehrlich, M. DNA methylation in cancer: too much, but also too little. *Oncogene*, 5400-5413 (2002).
63 Feinberg, A. P., Ohlsson, R. & Henilcoff, S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet*, 21-33 (2006).
64 Wild, L. & Flanagan, J. M. Genome-wide hypomethylation iin cancer may be a passive consequence of transformation. *Biochimica et Biophysica Acta*, doi:doi:10.1016/j.bbcan.2010.03.003 (2010).
65 Weaver, B. A. & Cleveland, D. W. Does aneuploidy cause cancer? *Curr Opin Cell Biol* **18**, 658-667 (2006).
66 Williams, B. R. *et al.* Aneuploidy affects proliferation and spontaneous immortalization in mammalian cells. *Science*, 703-709 (2008).
67 Weaver, B. A. & Cleveland, D. W. The aneuploidy paradox in cell growth and tumorigenesis. *Cancer Cell*, 431-433 (2008).
68 Torres, E. M., Williams, B. R. & Amon, A. Aneuploidy: cells losing their balance. *Genetics*, 737-746 (2008).
69 Yuen, K. W. & Desai, A. The wages of CIN. *J Cell Biol.* **180**, 665-672 (2008).
70 Holland, A. J. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol* **10**, 478-487 (Cleveland, D. W.).
71 Lu, L. Y. & Yu, X. The balance of Polo-like kinase 1 in tumorigenesis. *Cell Div.*, 4 (2009).
72 Herrera, L. A., Prada, D., Andonegui, M. A. & Duenas-Gonzàles, A. The epigenetic origin of aneuploidy. *Current Genomics*, 43-50 (2008).
73 Carrol, C. W. & Straight, A. F. Centromere formation: from epigenetics to self-assembly. *Trends Cell Biol* **16** (2006).
74 Stimpson, K. M. & Sullivan, B. A. Epigenomics of centromere assembly and function. *Current Opinion in Cell Biology*, 772-780 (2010).
75 Durand-Dubief, M. & Ekwall, K. Heterochromatin tells CENP-A where to go. *BioEssays*, 526-529 (2008).
76 Folco, H. D., Pidoux, A. L., Urano, T. & Allshire, R. C. Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. *Science* **319**, 94-97 (2008).
77 Gieni, R. S., Chan, G. K. T. & Hendzel, M. J. Epigenetics regulate centromere formation and kinetochore function. *J Cell Biochem.*, 2027-2039 (2008).
78 Tuck-Muller, C. M. *et al.* DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenet Cell Genet.*, 121-128 (2000).
79 Wong, N. C. *et al.* Permissive Transcriptional Activity at the Centromere through Pockets of DNA Hypomethylation *PLOS Genetics*, 172-186 (2006).
80 Lengauer, C., Kinzler, K. W. & Vogelstein, B. Genetic instabilities in human cancers. *Nature* **396**, 643-649 (1998).
81 Lingle, W. L. *et al.* Centrosome amplification drives chromosomal instability in breast tumor development. *Proc Natl Acad Sci U S A* **99**, 1978-1983 (2002).
82 Pihan, G. A. & Doxsey, S. J. The mitotic machinery as a source of genetic instability in cancer. *Semin Cancer Biol* **9**, 289-302 (1999).

83 Fukasawa, K. Centrosome amplification, chromosome instability and cancer development. *Cancer Lett* **230**, 6-19 (2005).
84 Lentini, L., Pipitone, L. & Di Leonardo, A. Functional inactivation of pRB results in aneuploid mammalian cells after release from a mitotic block. *Neoplasia* **4**, 380-387 (2002).
85 Hernando, E. *et al.* Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature* **430**, 797-802 (2004).
86 Iovino, F., Lentini, L., Amato, A. & Di Leonardo, A. RB acute loss induces centrosome amplification and aneuploidy in murine primary fibroblasts. *Mol Cancer* **5**, 38 (2006).
87 Lentini, L., Iovino, F., Amato, A. & Di Leonardo, A. Centrosome amplification induced by hydroxyurea leads to aneuploidy in pRB deficient human and mouse fibroblasts. *Cancer Lett* **238**, 153-160 (2006).
88 Karpf, A. R. & Matsui, S. Genetic Disruption of Cytosine DNA Methyltransferase Enzymes Induces Chromosomal Instability in Human Cancer Cells. *Cancer Reseach*, 863-865 (2005).
89 Hervouet, E., Lalier, L., Debien, E., Cheray, M. & Geairon, A. Disruption of Dnmt1/PCNA/UHRF1 Interactions Promotes Tumorigenesis from Human and Mice Glial Cells. *Plos one* **5**, doi:doi:10.1371/journal.pone.0011333 (2010).
90 James, S. R., Link, P. A. & Karpf, A. R. Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3bCG-X antigen gene regulation by DNMT1 and DNMT3b. *Oncogene*, 6975-6985 (2006).
91 Brandes, J. C., van Engeland, M., Wouters, K. A. D., Weijenberg, M. P. & Herman, J. C. CHFR promoter hypermethylation in colon cancer correlates with the microsatellite instability phenotype. *Carcinogenesis* **26**, 1152-1156 (2005).
92 Bullock, A. N. & Fersht, A. R. Rescuing the function of mutant p53. *Nature Rev. Cancer*, 68-76 (2001).
93 Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R. & Ibarra, I. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nature Genetics*, 1289-1295 (2005).
94 Vertino, P. M., Yen, R. W., Gao, J. & Baylin, S. B. De Novo Methylation of CpG Island Sequences in Human Fibroblasts Overexpressing DNA (Cytosine-5-)-Methyltransferase. *Mol Cell Biol.* **16**, 4555-4565 (1996).
95 Stimpson, K. M. & Sullivan, B. A. Epigenomics of centromere assembly and function. *Curr Opin Cell Biol* **22**, 772-780, doi:S0955-0674(10)00102-X [pii] 10.1016/j.ceb.2010.07.002 (2010).
96 Gieni, R. S., Chan, G. K. & Hendzel, M. J. Epigenetics regulate centromere formation and kinetochore function. *J Cell Biochem* **104**, 2027-2039, doi:10.1002/jcb.21767 (2008).
97 Leonhardt, H., Page, A. W., Weier, H. U. & Bestor, T. H. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, 865-873 (1992).
98 Toyota, M. *et al.* Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci USA* **100**, 7818-7823 (2003).
99 Unterberger, A., Andrews, S. D., Weaver, I. C. G. & Szyf, M. DNA Methyltransferase 1 Knockdown Activates a Replication Stress Checkpoint. *Mol Cell Biol.* **26**, 7575-7586 (2006).
100 Burri, N. *et al.* Methylation silencing and mutations of the p14ARF and p16INK4a genes in colon cancer. *Lab Invest.* **82**, 217-229 (2001).
101 Broude, E. V. *et al.* $p21^{waf1/cip1/sdi1}$ mediates retinoblastoma protein degradation. *Oncogene*, 6954-6958 (2007).
102 Frame, F. M., Rogoff, H. A., Pickering, M. T., Cress, W. D. & Kowalik, T. F. E2F1 induces MRN foci formation and a cell cycle checkpoint response in human fibroblastsE2F1 induces MRN foci and a cell cycle checkpoint. *Oncogene*, 3258-3266 (2006).

# Index