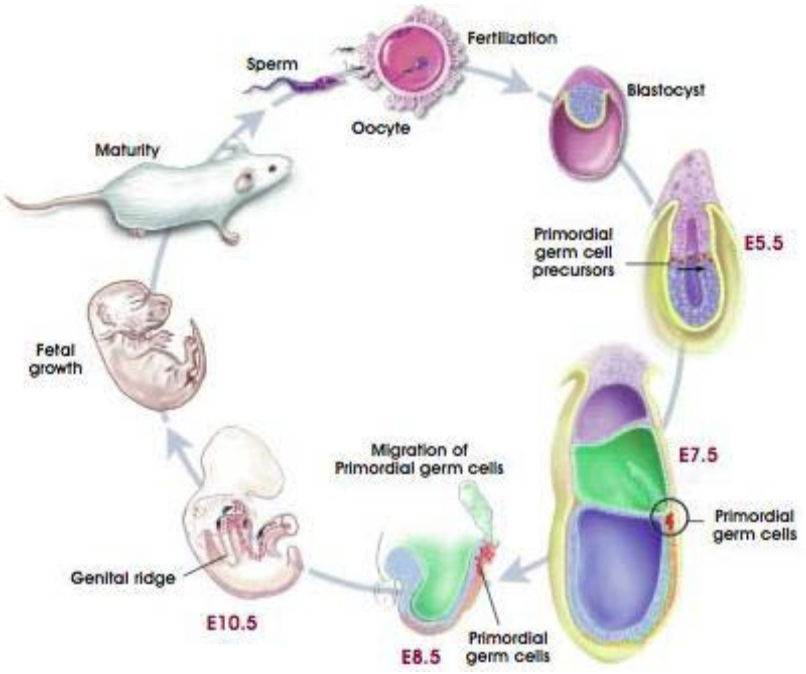
ARISTEA
MARIA
MAGARAKI

DNA METHYLATION IN EARLY MAMMALIAN DEVELOPMENT



Supervised by Dr. Petra de Graaf |Physiological Chemistry, UMC

DNA METHYLATION DURING EARLY MAMMALIAN DEVELOPMENT

Master student: Aristea Maria Magaraki

Student number: 3294420

Master's programme: Cancer Genomics and Developmental Biology

Current address: Kriekenpitplein 102

3583 EH, UCU

Utrecht, The Netherlands

Master thesis

Period: September 2009 – November 2009

Physiological Chemistry,

University Medical Centre Utrecht (UMC Utrecht)

Group leader: Prof.dr. H.T.M. Timmers

Supervisor: Dr Petra de Graaf

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First of all I would like to thank Prof. Timmers for giving me the opportunity to accomplish this thesis in his group.

My deepest thanks to my supervisor Dr Petra de Graaf for giving me more than enough time to complete this thesis, for her help, advise and remarks. I really appreciated her constant patience, especially in this period of my life.

Of course, I cannot forget my family and friends for being there, for their support and patience.

Lastly, I hope all the best for my friend Sofia. I hope you will wake up soon…

SUMMARY

DNA methylation – a DNA modification taking place predominately at CpG dinucleotides – shows a very dynamic behavior during early mammalian embryonic development. Soon after fertilization the parental genomes undergo demethylation with only a few methylated sequences escaping it, for example imprints. Later in development a methylation wave will establish new methylation marks to the forming embryo, important for its survival. In addition, sequences which escape demethylation at the first place, will eventually become demethylated in the forming Primordial Germ Cells (PGCs), so that new sex – specific epigenetic marks will be established during gametogenesis. DNA methylation, together with other modifications like histone modifications, are of a crucial importance during embryonic development and stem cell differentiation, since aberrant methylation patterns are linked with lethality, diseases and cancer.

A GENERAL ASPECT OF DNA METHYLATION

DNA methylation is a non – random, DNA chemical modification which occurs predominately at CpG dinucleotides. For the accomplishment of this modification methionine reacts with ATP to form S – Adenosyl Methionine (SAM), which is the methyl - group donor. The methyl – group is then added at the 5' position of cytosine residues, a reaction which is catalyzed by a conserved group of enzymes called DNA nucleotide methyltransferases (DNMTs), producing this way 5 – methylcytosine and S – adenosylhomocystine (SAH) (*Attwood, Yung et al. 2002*) (Fig. 1)

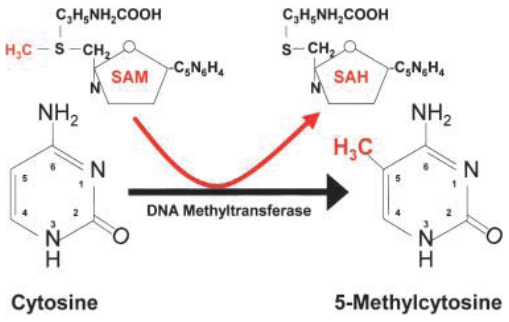


Fig. 1: Conversion of cytosine to 5 – methylcytosine is catalysed by a DNA methytransferase, which transfers a methyl group from adenosylmethionine (SAM) to the 5' – position of cytosine.

In general, DNA methylation is a typical epigenetic and heritable event and during mammalian development it appears as a very dynamic process with developmental periods of genomewide reprogramming of methylation patterns in vivo *(Reik, Dean et al. 2001*). Importantly, this modification does not alter the sequence information that DNA carries, but influences transcription by suppressing it and is of a great importance during many processes such as embryonic mammalian development, cell differentiation, genomic imprinting and transposon control (*Bird 2002*).

DNA methyltransferases in eukaryotes are grouped in four distinct families - Dnmt1, Dnmt2, Dnmt3 and Chromomethylase family - (Fig. 2) according to sequence similarities of the C – terminal catalytic domains (*Goll and Bestor 2005*). The Chromomethylase family is solely found in plants, while the rest of the families are met, among other eukaryotes, in mammals. A schematic representation of these enzymes showing their catalytic and regulatory domains is given in figure 3.

Epigenetics and epigenome:

The term 'epigenetics' has adopted multiple definitions through the years. Nowadays, 'epigenetics' is commonly defined as the field which studies heritable changes in genome function, that occur without any change in DNA sequence. The term 'epigenome' refers to epigenetic patterns that distinguish or are variable between cell types and these patterns include DNA methylation, histone modifications and chromatin – associated proteins (*Jirtle 2007*).

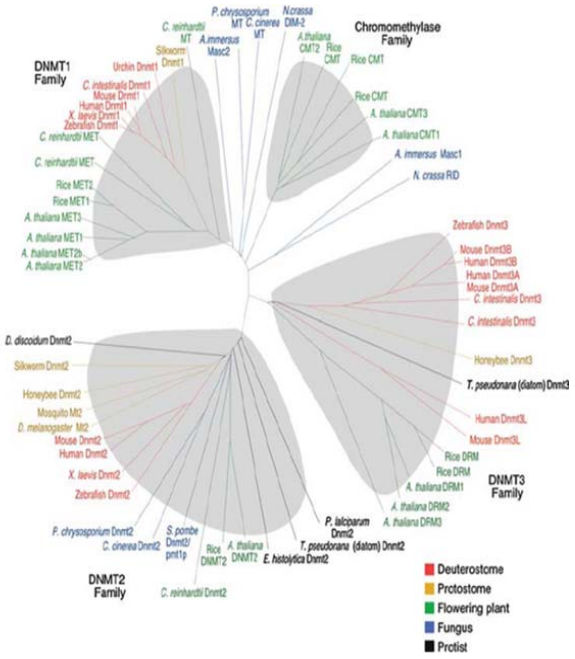


Fig. 2: Families of DNA Methyltransferases in Eukaryotes. The Chromomethylase family is solely found in plants, while the rest of the families are met among other eukayrotes, in mammals
Figure taken by reference *(Bestor 2000)*.

a) <u>*Dnmt1*</u>: it is the first DNA methyltransferase to be identified and it contains a C – terminal domain related to the bacterial restriction methyltransferases, and a N – terminal domain with a number of motifs, which provide to the enzyme its catalytic activity (*Bestor 2000*). Dnmt1 is expressed ubiquitously and it is linked with the maintenance of DNA methylation, since it shows a high preference for hemimethylated substrates (*Yoder, Soman et al. 1997*). In other words, during DNA replication, the generating daughter strand does not contain initially the corresponding parental methylation marks. These will be added by Dnmt1, so that eventually the daughter cells will be identical to the parental cell and this is why this enzyme is termed as 'maintenance methyltransferase', since it contributes to the maintenance of methylation patterns during cell division.

Nevertheless, Dnmt1 is not only connected to the maintenance of DNA methylation patterns, but also with the establishment of new, through the *de novo* DNA methylation mechanism, since a co – operation is implicated between Dnmt1 and the *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b *(Kim, Ni et al. 2002*). Lastly, the Dnmt1 family includes Dnmt1 splice variants – members, which are expressed in specific tissues or at specific stages of development. One example is Dnmt1o, which is solely met in oocytes, and it is shorter in comparison to Dnmt1. This variant will be eventually replaced by the full length enzyme when the embryo, which arises after fertilization, will be implanted to the uterine wall *(Bestor 2000*).

b) <u>*Dnmt2*</u>: Another DNA methyltransferase family found in mammals includes Dnmt2 and its variants. Dnmt2 is expressed ubiquitously and

contains all the motifs, but lacks the N – terminal domain. Despite the fact that the catalytic domains are conserved to this enzyme, studies have failed to reveal its function and its methylation substrate and additionally Dnmt2 mutants do not show any methylation or phenotypical abnormalities (*Bestor 2000; Goll and Bestor 2005; Tost 2008*), appointing this way this family as the most enigmatic in terms of function. Nonetheless, it has been suggested that Dnmt2 may serve a function as a RNA and not as a DNA methyltransferase, specifically methylating $tRNA_{Asp}$, but still the role of this modification remains elusive (*Goll, Kirpekar et al. 2006*).

c) <u>*Dnmt3*</u>: the last DNA methyltransferase family expressed in mammals is Dnmt3. The mouse (and the human) Dnmt3 family consists of two catalytically active members (and their variants), Dnmt3a and Dnmt3b, and of a regulatory member, with no catalytic activity, Dnmt3l.

These methyltransferases have been implicated with the *de novo* methylation of DNA in embryonic stem cells, germ cells and early mammalian development, since if they become inactivated, *de novo* DNA methylation is disrupted (*Okano, Bell et al. 1999*). New marks of DNA methylation are set, when Dnmt3l intermediates and links Dnmt3a and Dnmt3b to their CpG targets. However, this family is not only substantial for establishing *de novo* methylation marks, but also for maintaining imprints in specific genomic loci.

<u>Imprint:</u> An epigenetic modification, which restricts the expression of a gene to one of the two parental chromosomes

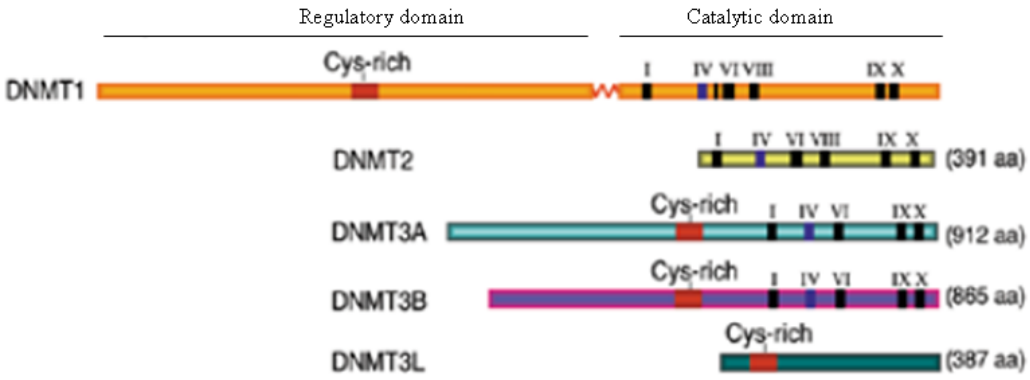


Fig. 2: A schematic representation of the mammalian DNA methyltransferases. The catalytic domain is conserved among catalytically active DNA methyltransferases, and includes a number of motifs. Dnmt2 lacks the N – terminal regulatory domain. Figure taken by reference (*Allis, Jenuwein et al. 2007*).

LINKING DNA METHYLATION AND CHROMATIN STRUCTURE

DNA methylation is not the only modification which controls gene expression. Chromatin structure can be regulated by histone modifications, which relate both with active and repressed chromatin.

Activating marks are the acetylation of histone 3 and 4 N – terminal tails and the methylation of lysine 4 of histone 3 (H3K4). On the other hand, histone de - acetylation and methylation of lysine 9 or lysine 27 of histone 3 (H3K9 and H3K27 respectively) are connected to inactive chromatin, thus silent genes.

Many efforts have been made in order to reveal if histone modifications dictate DNA methylation patterns or if DNA methylation is actually responsible for modification of histones to occur. Data suggest that both ways are possible, hence proposing a bidirectional relationship between DNA methylation and histone modifications.

A remarkable example of how histone modifications dictate DNA methylation was demonstrated by *Ooi et. al.,* who showed that Dnmt3l recognizes unmodified histone H3K4 tails and induces *de novo* DNA methylation by recruitment or activation of Dnmt3a2, a Dnmt3a isoform. This implies that epigenetic chromatin modifications repress DNA methyltransferases from catalyzing their function in early development (*Ooi, Qiu et al. 2007*).

In a similar manner, DNA methylation patterns may induct the recruitment of deacetylases or histone methyltransferases, the last being necessary for the methylation of specific lysines linked with inactive chromatin. For example, Methylation Binding Proteins (MBPs), a five member family, recognize and bind methylated CpGs. These MBPs subsequently bind repressors and histone deacetylases, which will mediate repression of transcription (*Miranda and Jones 2007*).

The above indicate that in many cases DNA methylation and histone modifications are interconnected. However, both types of modification can exist independently of each other.

DNA METHYLATION DURING MAMMALIAN EMBRYONIC DEVELOPMENT

In mammals fertilization occurs in the oviduct, where sperm encounters and fuses with the oocyte. As a result the oocyte's nucleus completes meiosis II and the two parental nuclei fuse to form the diploid zygotic nucleus. Thereafter, the zygote cleaves, passing through characteristic

stages of early embryonic development (Fig. 4).

of the PGC's formation by the second wave of DNA demethylation

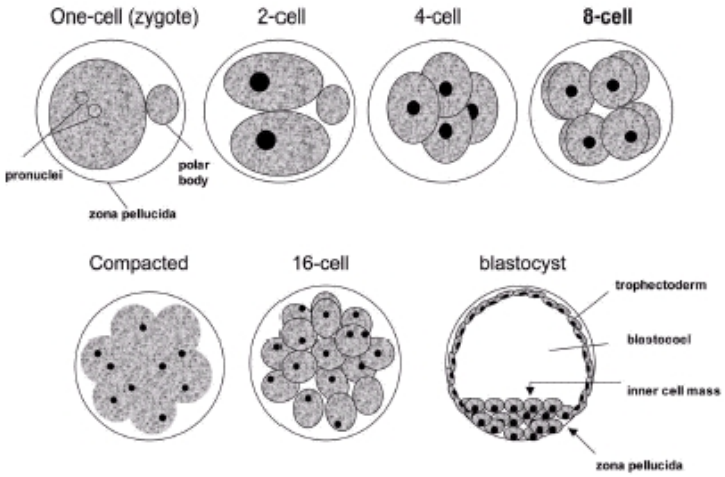


Fig. 4 : After fertilisation, the zygote starts to cleave into blastomeres, but without visible growth of the embryo. After a few divisions, the embryo undergoes compaction to become a morula, a compact smooth sperical structure. The number of the blastomeres, which undergo compaction* differs between mammalian species, as well as the timing between stages. After compaction, the presumptive trophectoderm cells form the outer layer of the embryo, and a fluid – filled cavity is also formed on the one side of the embryo, while on the opposite side we have the formation of the Inner Cell Mass (ICM), which will give rise to the individual. The zona pellucida, a membrane which protects the embryo, is removed at the time of implantation of the blastocyst to the uterus wall. Figure taken by reference (*Mor 2006*)

****Compaction:*** the phenomenon where the blastomeres of the embryo increase their surface - contact to each other.

The parental chromosomes are characterized by DNA modifications, which will be reprogrammed during development so that the genome of the newly formed embryo can become totipotent.

This reprogramming involves two waves of DNA demethylation during the mammalian development, one targeting the parental genomes, which are demethylated in separate time periods, thus creating epigenetic asymmetry, and one targeting the Primordial Germ Cells (PGC's), the precursors of the germ cells. New DNA methylation patterns of the genome are then re – established as the embryo grows. Notably, parental imprints, where DNA methylation is a major player, escape the first wave of DNA demethylation. However, they will be erased on the onset and new, sex – dependent imprints, will be established, in order to discriminate the fates of PGC's from the somatic cells.

To begin with, the dynamics of DNA methylation will be described, firstly at the fertilization stage, followed by the two – cell stage embryo onwards until the blastocyst stage. It needs to be considered, that these mechanisms are best studied in the mouse, therefore creating difficulties in interspecies comparison attempts.

Totipotent: the ability of a cell to form an entire organism

1. DNA METHYLATION DYNAMICS AT FERTILISATION

Before the formation of the zygotic nucleus, both parental genomes carry modifications on their DNA and chromatin, which will be reprogrammed at

early stages of embryogenesis. The first major alterations upon fertilization are:

i. the replacement of sperm's protamines - proteins which probably mediate packaging of the DNA into the minor sperm head - by histones, whereas
ii. the oocyte completes meiosis II, so that it reaches the same meiotic maturation stage as the sperm.

Just a few hours after fertilisation, even before the zygote starts to cleave, the paternal genome undergoes a massive wave of demethylation. Since, this demethylation is not dependent on replication events, there are implications that enzymes actively remove the methyl groups from the sperm's DNA.

Many questions rise up in conjunction to this phenomenon with the most important concerning how DNA demethylation takes place, since no DNA de - methyltransferases have been identified yet. A number of mechanisms and enzymes have been proposed to be candidates of the active demethylation phenomenon, although all of them met controversy, thus appointing the subject still in debate.

The most dominant 'active DNA demethylation' model involved a DNA repair mechanism, and proposed cytosine deaminases as strong 'DNA – demethyltransferase, candidates. According to this model AID (Activation Induced Deaminase) and APOBEC1 (Apolipoprotein B mRNA editing enzyme catalytic polypeptide 1) – cytosine

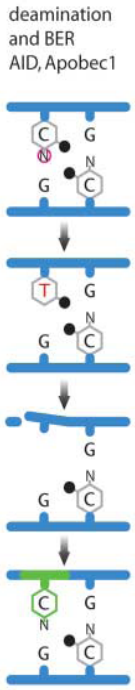


Fig. 5: Deaminases deaminate 5 – methyl – cytosine to thymine (T). The T mismatch is removed by excision and replaced by a cytosine. Figure taken by reference (*Morgan, Santos et al. 2005*).

Box 1 ***BER pathway:*** Base Excision Repair mechanism is one of the pathways that cells follow to repair their DNA in case of damage. This pathway involves DNA glycosylases, enzymes which recognize and hydrolytically remove the altered base from DNA. Hereafter, the position of the removed altered base is recognized by AP – glycosylase, which cleaves the phosphodiester backbone. The created gap is sealed by the activity of DNA polymerase which adds the new nucleotide and DNA ligase which finally seals the strand's nick (*Alberts, Wilson et al. 2008)*.

deaminases, both detected in mouse oocytes – catalyze the deamination of 5 – methyl – cytosine to thymine. The subsequent T:G DNA mismatch is then recognized and repaired through the Base

Excision Repair (BER) pathway (*Gehring, Reik et al. 2009*) (Fig. 5, Box1).

Recent evidence in zebrafish (*Rai, Huggins et al. 2008*), suggest that DNA demethylation indeed occurs through a deamination and BER mechanism, proposing that this could well be the DNA – demethylation mechanism in the animal kingdom. However, the role of the corresponding to the zebrafish enzymes and factors participating in the demethylation mechanism has been questioned in mammals, thus underlining the need of further investigation.

Lastly, other enzymes, which have been proposed to possess a deaminase activity, are Dnmt3a and Dnmt3b. This impressive suggestion supports that the *de novo* methyltransferases hold a dual activity, one of DNA methyltransferase on the one hand and one of DNA demethyltransferase on the other hand. However, the last property requires certain conditions *in vitro*, e.g. limiting concentrations of AdoMet, which are not known yet if they are substantial *in vivo* (*Ooi and Bestor 2008*).

Interestingly new studies revealed that TET1 (Ten – eleven translocation1, methylcytosine dioxygenase) could be a potential DNA demethyltrasferase, since it converts 5 – methylcytosine to 5 – hydroxyl – methylcytosine, the last being present in the mammalian genome (*Tahiliani, Koh et al. 2009*). Yet, many questions wait to be answered such as what is the extent of 5 – methylcytosines which undergo this conversion, if TET1 is present in oocytes and how 5 – hydroxyl – methylcytosine is removed.

Predominately, in most species studies so far, sperm's demethylation takes place at the zygote stage. However, there are exceptions, e.g. sheep, goat and the rabbit, where the paternal DNA is suggested to be demethylated after the zygote starts to cleave (*Hou, Lei et al. 2005; Thurston, Lucas et al. 2007*). It needs to be considered that these controversial results may be due to different experimental approaches and might reflect an artifact, which does not present the real picture of active DNA demethylation in mammals.

2. DNA METHYLATION DYNAMICS FROM THE 2 – CELL STAGE EMBRYO UNTIL THE BLASTOCYST

In contrast to the sperm's genome, which becomes demethylated at the zygote stage, the oocyte's genome escapes active demethylation and initially retains its methylation marks. A possible 'protective mark' against active demethylation could be the existence of di – and trimethylation marks on lysine 9 (H3K9me2, H3K9me3) and lysine 27 (H3K27me2, H3K27m3) of histone 3 in the female pronucleus, marks which seem to be absent in the paternal pronucleus (*Santos, Peters et al. 2005*). Maternal genome demethylation takes place when the zygote starts to cleave, and unlike the paternal, it is replication – dependent and characterized as 'passive'.

During passive demethylation, the methyltransferase responsible for maintaining the methylation patterns, Dnmt1, renders the nucleus and resides the blastomeres' cytoplasm. Therefore, within each DNA replication cycle, the daughter DNA strand does not acquire the corresponding methyl – groups, thus leading to global DNA demethylation per cell division (Fig. 6). Interestingly, according to research data, the timing of

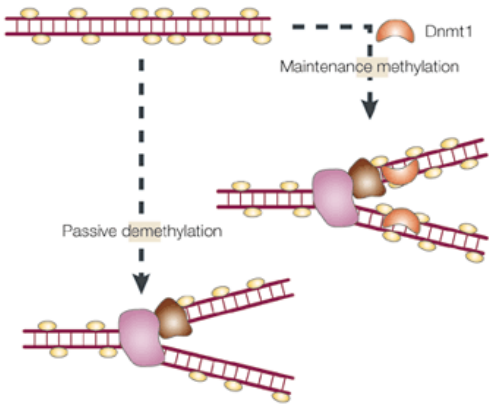


Fig. 6: For maintaining the methylation patterns during replication cycles, Dnmt1 associates with the replication machinery. The methyl groups on the template strand are recognized and new ones are added in the daughter strand. During passive demethylation Dnmt1 renders the nucleus, and maintenance of methylation patterns cannot take place. Figure taken by reference *(Reik and Walter 2001)*

passive demethylation varies between mammalian species, in some initiating during early cleavage events e.g. mouse (2 – cell stage), while in others later e.g. pig (blastocyst stage). These differences may reflect differences in factors, which contribute to the genome's re – organization in every species, or different experimental approaches. However, passive demethylation takes place before implantation in all species examined.

When demethylation of the parental genomes is finally completed, a DNA methylation wave is initiated at the blastocyst stage, where the first differentiation event in the mammalian embryonic development takes place. The trophectoderm, which will give rise to the placenta, expresses the transcription factor Cdx2, while the ICM, which will form the fetus, expresses the

Cdx2:
A transcription factor required for the formation of the placenta in mammals

Oct4:
A transcription factor required for the maintenance of pluripotency, also known as POU5F1

Cdx2 and Oct4 are expressed in all blastomeres in the early embryo; however in the blastocyst stage their expression is restricted to the trophectoderm and ICM respectively (*Wolpert 2007*)

transcription factor Oct4. Coupled with this differentiation event, at least in the mouse, are the asymmetric levels of methylation between these cell lineages, with the ICM being hyper - methylated in comparison to the trophectoderm, the last remaining hypo - methylated. Nevertheless, the opposite seems to be the case in the human, rhesus monkey and rabbit, where the trophectoderm presents higher levels of methylation in comparison to that of the ICM (*Thurston, Lucas et al. 2007*). Surprisingly, in bovine embryos *de novo* methylation initiates from the 16 – cell stage, resulting in high methylation patterns both in the trophectoderm and the ICM (*Dean, Santos et al. 2001*).

It is clearly observed, that even though DNA demethylation and methylation are two conserved events among mammalian species, with demethylation always taking place before implantation, apparently their establishment timings and the *de novo* methylation levels between the trophectoderm and the ICM fluctuate per species.

There could be multiple reasons for these controversies. Firstly, these contrasts could be indeed species – specific, however it cannot be excluded the possibility that results obtained in some cases might not be consistent with the *in vivo* reality, since *in vitro* conditions e.g. embryo manipulation, culture conditions and so on, may influence DNA methylation levels. In addition, experimental approaches, may provide artificial results, therefore the interpretation of such experiments should be critically conducted. Furthermore, differences in methylation levels between the trophectoderm and the ICM between mammalian species might reflect the time of the blastocyst isolation for the experimental conduction. The methylation levels between these two cell lineages could be different in the early or late blastocyst. Also, the high methylation levels in the trophectoderm in some species e.g. human and cow, might reflect their long gestation period. In these species, it could be the case that there is a need for a genetically stable placenta which will be able to support fetal development for a long period. Lastly, the lack of extended studies in other species, rather than the mouse, renders the need of further research so that accurate interspecies comparisons can be carried out.

At this point it is important to look at the mechanism used for the establishment of the *de novo* methylation patterns. Responsible for this *de novo* DNA methylation event are the *de novo* methyltransferases Dnmt3a and Dnmt3b. A model for establishing DNA methylation in the developing embryo suggests that before *de novo* methylation takes place,

RNA polymerase II binds on the genome and recruits histone methyltransferases, which will methylate lysine 4 on histone 3 (H3K4). In the absence of these histone methylation marks, Dnmt3l will bind on histone tails and it will recruit the *de novo* methyltransferases, which with their turn will catalyse the methylation of CpG dinucleotides (Cedar and Bergman 2009) and establish the first methylation marks, so that development can proceed further.

IMPRINTED GENES ESCAPE DEMETHYLATION TAKING PLACE BEFORE IMPLANTATION

A hallmark of DNA methylation dynamics during embryonic development is that not all methylated marks are erased when demethylation takes place, while CpG regions, which should be maintained unmethylated escape *de novo* methylation. Sequences with such 'ability' are the imprinted genes and the intracisternal A – type particle (IAP).

IAP: Intracisternal A particle *belongs* to the long terminal repeat (LTR)-type mouse *retrotranspos on* family (*NCBI*)

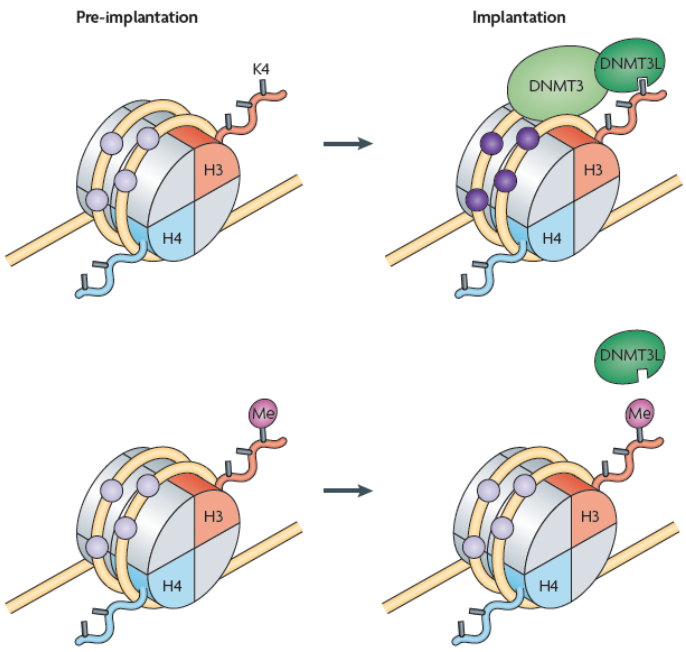


Figure 7: At the time the mammalian blastocyst implants to the uterine wall, a wave of *de novo* DNA methylation is initiated. Methylation of lysine 4 of histone 3 is linked with *de novo* methylation at this stage. Dnmt3l recognizes and binds unmethylated lysines 4 on the histone 3 tail, and thereafter recruits Dnmt3a and Dnmt3b. In the presence of methylated H3K4, Dnmt3l fails to bind on the histone tail, and the underlying DNA region remains unmethylated. Figure taken by reference (*Cedar and Bergman 2009*).

Mammalian cells contain two copies (or alleles) of each gene, one inherited by the mother and one by the father. These two copies are both expressed in the cell. However, there is a small percentage of genes, where mono – allelic expression takes place, depending on their parent origin. These genes are termed as 'imprinted', and are intimated with embryonic development, placenta formation, fetal and postnatal growth and maternal behavior (*Hirasawa, Chiba et al. 2008*).

Imprinted genes are mostly organized in clusters, where a sequence called Imprinting Control Region (ICR) carries the imprint and coordinates the expression of the clustered genes. Which allele will be expressed depends on the imprint marks that the parental genomes will acquire during gametogenesis and the genes which will be imprinted are distinct between two sexes. For example, the allele of the gene which encodes the Insulin – like growth factor -2 (Igf2) inherited by the father is expressed in the early embryo, while the allele from the mother is not. The reciprocal is true for the alleles of the gene encoding the Igf – 2 receptor (Igf2r).

Igf2: member of the insulin family of polypeptide growth factors, which is involved in development and growth

Igf2r: Igf2 receptor (*NCBI*)

An intriguing question which links DNA methylation dynamics during early embryonic development and imprinting is how imprinted genes (and IAPs) and their unmodified alleles resist demethylation and *de novo* methylation respectively.

Cis- and *trans*- factors and signals, have been proposed to have a role in this process, however mostly the role of Dnmt1 and its variants will be described. Dnmt1 is the methyltransferase responsible for maintaining methylation patterns during DNA replication cycles. However, studies in early development show that Dnmt1o, a Dnmt1 isoform expressed in the oocyte, is excluded from the nucleus during the pre – implantation period with exception the 8 – cell stage, where it shows nuclear localization. Is it sufficient though for the imprinting and IAP methylation maintenance the nuclear translocation of Dnmt1o for only one replication cycle? Recent studies support that the somatic Dnmt1 isoform, Dnmt1s, fulfils the requirements for imprinting and IAPs methylation maintenance in the pre – implantation embryo (Figure 8). According to these findings, there are two sources of Dnmt1s; a maternal, which

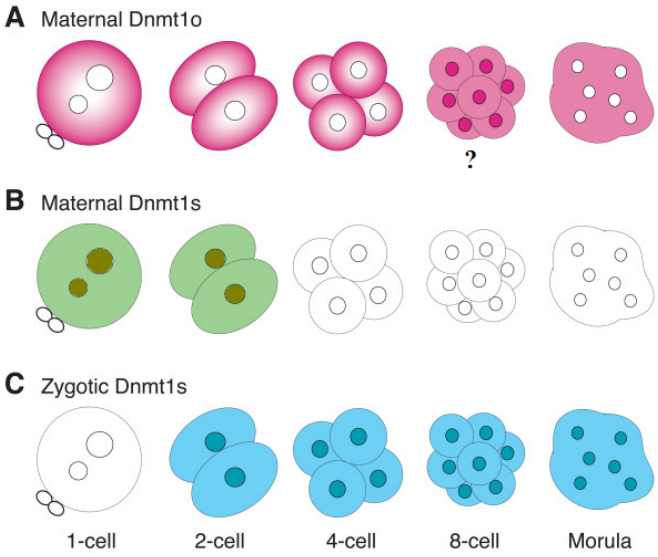


Fig. 8: Distribution of Dnmt1 isoforrms during development for maintaining parental imprints. **(A)** The oocyte Dntm1o isoform is thought to possess a cytoplasmic localization throughout embryonic development, with exception the 8 – cell stage. The question mark indicates that this citation needs further investigation, since recent evidence failed to detect a similar observation. Somatic Dnmt1 isoforms have also been found to contribute to the maintenance of paternal imprints. **(B)** Maternal Dnmt1s possesses a nuclear position during the zygote and the 2 – cell stage. Dnmt1s carries initially an asymmetric localization between the two paternal nuclei. While protamines are being replaced from the paternal genome by histones, maternal Dnmt1s is only present in the maternal pronucleus, providing an extra protecting mechanism against active demethylation. However, maternal Dnmt1s is recruited to the paternal genome during pronuclear maturation. **(C)** At the 2 – cell stage, the embryo's genome is activated, thus zygotic Dnmt1s takes over in maintaining the methylation marks located on ICRs. Figure adopted by reference (*Cirio, Ratnam et al. 2008*)

assures maintaining imprints from the zygote until the two – cell stage, and a zygotic which assumes action from the two – cell stage onwards. Nevertheless, the same studies failed to confirm the localization of Dnmt1o in the nuclei of the eight – cell stage embryo *(Hirasawa, Chiba et al. 2008; Kurihara, Kawamura et al. 2008*), creating discrepancies which should be further investigated.

It is yet obscure how Dnmt1s' specificity for imprinted regions and IAP is established, since at the same time this enzyme does not confer methylation maintenance of demethylating marks. A protein proposed to facilitate inheritance of methylation marks is Np95.

Np95 has been shown to recognize and bind hemi - methylated substrates at replication forks and form complexes with PCNA and Dnmt1, hence promoting methylation inheritance during replication cycles (*Sharif, Muto et al. 2007*) (Fig. 9). Other proteins, which participate in chromatin modifications are also involved in the complexes formed with Np95. It could be the case that the same or a similar mechanism is involved in maintaining imprints and IAP methylation marks by recognizing specific chromatin modifications located on these regions.

Np95: also known UHRF1 (Ubiquitin – like, containing PHD and RING finger domains1). Np95 binds to specific DNA sequences through its SET and RING associated domain and recruits enzymes e.g. Dnmt1 to regulate gene expression and mediate methylation mark's inheritance (*NCBI*)

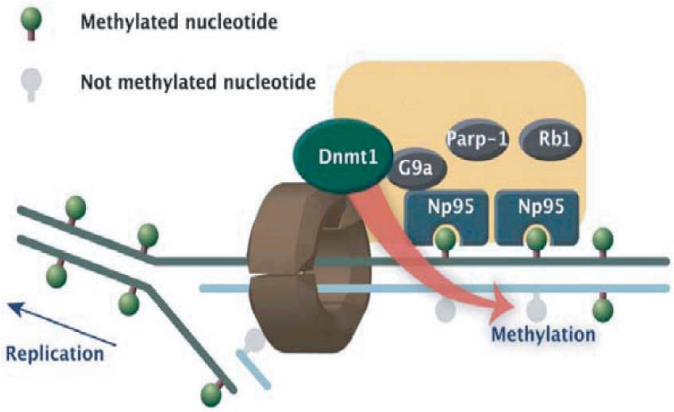


Figure 9: Dnmt1 is recruited to the replication fork by Np95, which recognizes hemi - methylated marks on the replicating DNA. Np95 forms complexes not only with Dnmt1 and PCNA, but with other proteins, which process chromatin modifications. Figure taken by reference (*Sharif, Muto et al. 2007*)

PCNA: Proliferating Cell Nuclear Antigen, a protein which encircles DNA and 'holds' DNA pol. δ to replicating DNA

G9a: a histone methyltransferase responsible for catalyzing and maintaining trimethylation on lysine 9 of histone 3 frequently found in repeats

Parp-1: Poly (ADP – ribose) polymerase 1. It modifies nuclear proteins by poly (ADP – ribosyl)ation and it is involved in the regulation of important cellular processes such as proliferation

Rb-1: Retinoblastoma – 1, negative regulator of cell cycle. It is also involved in maintaining the overall chromatin structure by stabilizing constitutive heterochromatin (*NCBI*)

Except Dnmt1 isoforms, *de novo* methyltransferases seem to possess a role in maintaining methylation marks, albeit that of specific imprinted loci. In particular, Dnmt3a and Dnmt3b facilitate the maintaining of the imprint of the Rasgrf1 paternal locus only, and not that of other loci, suggesting that this could be due to its unusual repetitive nature (*Hirasawa, Chiba et al. 2008*).

A recent opinion though describes that methylation marks are maintained by a more complex mechanism, which includes the collaboration of all DNA methyltransferases. According to the model proposed, *de novo* methyltransferases remain bound on nucleosomes that contain methylated CpG sites. When replication takes place, Dnmt1, with the assistance of Np95, methylates the daughter strand and *de novo* methyltransferases facilitate the process by methylating sites which are missed by Dnmt1 (*Jones and Liang 2009*). Despite how attractive this model might be, many questions remain unanswered, for example why *de novo* methyltransferases remain bound on methylated CpG sites and which factors or chromatin modifications contribute to this binding.

Other factors, rather than DNA methyltransferases, are implicated in the safekeeping of imprints during early embryogenesis. An example is PGC7/Stella protein, which protects maternal imprints against the active demethylation wave, but it does not seem to participate in protection against passive demethylation *(Nakamura, Arai et al. 2007*).

In addition to PGC7/Stella, CpG repeats themselves in specific imprinted loci render also in maintaining methylation during the pre – implantation period (*Tost 2008*).

An important question introduced in the beginning of this chapter was how the unmethylated 'partners' of the imprinted

Rasgrf1: Ras protein specific guanine nucleotide – releasing factor 1. It is primarily expressed in the brain. The main function of the protein encoded is the activation of GTPases in response to signals such as Ca^{+2} flux. Expression takes place from the paternal allele (*NCBI*)

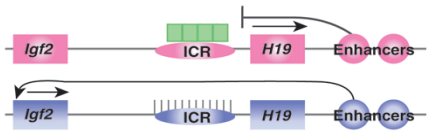
PGC7/Stella: a maternal factor essential for early embryonic development (*Sasaki and Matsui 2008*)

alleles escape *de novo* methylation. The best studied example is the maternal ICR of H19 which contains four binding sites for the CTCF protein (Box 3). The binding of CTCF to its binding sites (insulator) and the integrity of CTCF binding sites as well, are crucial in protecting the maternal H19 ICR against DNA methylation (*Cirio, Ratnam et al. 2008*)

H19: H19 gene expresses a non – coding RNA with a tumor suppressor function. Expression takes place from the maternal allele (*Alberts, Wilson et al. 2008*)

Insulator: a DNA sequence that prevents gene regulatory proteins from influencing distant genes (*Alberts, Wilson et al. 2008*)

Box2 Imprinting is not necessarily linked with gene silencing. CTCF is a protein with 11 zinc fingers, able to recognize and bind sites of unmethylated ICR's (insulators). DNA methylated regions inhibit the binding of CTCF. Binding of CTCF can lead either to gene activation or repression. The example of Igf2 and H19 is described, where those two genes are under the control of a common ICR.



In the case of Igf2 binding of CTCF on the unmethylated maternal ICR (pink) blocks communication between the enhancer and Igf2 promoter, therefore expression of Igf2 occurs solely by the paternal allele (blue), where methylation on ICR blocks binding of CTCF. In the case of H19, binding of CTCF results in the expression of the maternal allele, while the paternal remains silent (*Alberts, Wilson et al. 2008*). (green boxes: CTCF, vertical lines: methylation marks). Figure taken by *Jeong et. al.*, 2004.

Methylation marks on imprinted genes will be eventually erased later in development by a second wave of DNA demethylation, taking place during the formation of germ cells.

DNA METHYLATION DYNAMICS IN MAMMALIAN GERM CELL DEVELOPMENT

At some point of the early mammalian development, a population of cells located at the posterior end of the primitive streak in the extra embryonic mesoderm of the embryo, migrate and reside the presumptive gonads. These cells, the Primordial Germ Cells (PGC), will form the gametes of the fetus, and now imprints, which showed tolerance against parental DNA demethylation process, will be erased. New sex – specific imprints will be established during gametogenesis and the circle of epigenetic modifications in early mammalian development will be completed.

Primitive streak: a structure which forms at the posterior end of the embryo and marks the initiation of gastrulation (*Wolpert 2007*)

Apparently, PGCs will follow a distinct developmental route compared to that of the somatic cells and somatic genes need to be silenced in PGCs; this is accomplished by the combined expression of transcription factors and the establishment of epigenetic modifications. These events are best studied in the mouse, and the description followed will be limited to this mammal.

The first event which will specify which cells in the posterior primitive streak will

Blimp – 1: B – lymphocyte maturation induced protein 1 is a transcriptional regulator, essential for PGC specification (*Sasaki and Matsui 2008*)

become PGC is the expression of Blimp – 1 and Stella. The exact role of Blimp – 1 is currently obscure, however it is thought to mediate suppression of somatic genes in cells that will follow a germ cell fate (*Sasaki and Matsui 2008*) and facilitate the initiation of germ cell program *(Hayashi and Surani 2009*).

As expected, after the precursors of PGCs are established, major epigenetic modifications take place on their genome in order to follow a germ – cell fate. Thereby two phases of epigenetic alterations can be distinguished:

1. One during PGC's migration towards the developing gonads and
2. an additional at the time that PGC's reside the gonads.

During the first phase of reprogramming, the migrating cells lose epigenetic marks, and acquire others. Specifically, they lose dimethylation on lysine 9 of histone 3 (H3K9me2 – repressive mark) and acquire trimethylation on lysine 27 and lysine 4 of histone 3 (H3K27me3 and H3K4me3 – repressive and activation mark respectively). These modifications are accompanied by a low decrease of DNA methylation levels (*Hemberger, Dean et al. 2009*) and upregulation on acetylation marks mainly on lysine 4 of histone 3 (H3K9ac – activation mark) and symmetrical methylation on arginine 3 of histone 4 and H2A ($H4/H2Ar3me_s$ – repressive mark) (*Hajkova, Ancelin et al. 2008*). All these changes occur before PGCs enter the developing gonads and they are PGC – specific, meaning that cells with a somatic fate within the developing embryo do not acquire these modifications and additionally they seem to prepare PGCs to eventually acquire totipotency (*Hajkova, Ancelin et al. 2008*).

At the second phase of reprogramming, when PGCs have entered the genital ridges, extensive epigenetic alterations persist, which are also accompanied with changes on chromatin structure such as loss of the linker histone 1 (H1) (*Hayashi and Surani 2009*). During this phase, imprints will also be erased, however it is still unknown whether DNA demethylation in this case occurs through an active or passive mechanism; active DNA demethylation is favored though, since demethylation of imprints occurs rapidly (*Hajkova, Ancelin et al. 2008*).

Once demethylation in PGCs is completed, remethylation occurs for the establishment of new, sex – specific imprints. This process is accomplished in different time points between the two sexes.

In the male germline, *de novo* methylation initiates at the prospermatogonia stage and continues until birth (*Kato, Kaneda et al. 2007*). For the accomplishment of

Prospermatogonia: PGCs, which after their migration to the male developing gonads enter mitotic arrest, and will resume mitosis after birth, in order spermatogenesis to take place (*Maronpot 1999*)

imprinting establishment in the male germline essential is the role of Dnmt3l and *de novo* methyltransferases *(Kaneda, Okano et al. 2004; Sasaki and Matsui 2008*). The mechanism proposed for *de novo* methylation here is similar to that taking place in the ICM at the blastocyst stage, with Dnmt3l recognizing and binding unmenthylated lysine 4 of histone 3 (H3K4) and subsequently recruiting and activating Dntm3a and Dntm3b, in order the last to catalyse methylation of their CpG targets. However, with a minor difference; Dnmt3a is sufficient for the establishment of paternal imprints and Dnmt3b is required only for methylation of the Rasgrf1 locus *(Kato, Kaneda et al. 2007*).

Follicles: PGCs, which after their migration to the female developing gonads become oogonia and get surrounde by somatic cells for protection and nutrition (*Maronpot 1999*)

In the female germline, *de novo* methylation takes place after birth, where follicles are arrested in prophase of meiosis I, and continues throughout the follicular maturation. Again, Dnmt3l and Dnmt3a, but not Dnmt3b, are essential for establishing maternal imprints *(Kaneda, Okano et al. 2004*).

An interesting case, which cannot be overlooked during the above described events, is that of retrotransposons. Interestingly, transposon sequences such as IAPs, do not undergo complete demethylation during the second phase of PGCs reprogramming, rather they lose partially their methylation marks (*Lees-Murdock and Walsh 2008)*.

Remethylation of transposons takes place at the same time as imprinting establishment in the germlines, and this probably comprises a protection mechanism against their deleterious effects, since these elements can affect genome's integrity by copying and introducing themselves back to the genome.

Methylation of transposons is best studied in the male germline. The roles of Dnmt3l and Dnmt3a are essential, while the extra activity of Dnmt3b is needed in specific cases, such as for remethylation of IAPs (*Kato, Kaneda et al. 2007*). Furthermore, recently it has been shown that Mili and Miwi2, members of the PIWI subfamily of the Argonaute family (Box 3), have a role in *de novo* methylation of retrotransposons; however the mechanism underlying this function is yet unclear (*Aravin, Sachidanandam et al. 2008*).

Box 3 The Argonaute proteins interact with small RNAs and participate in many processes such as in the RNAi mechanism, transposon silencing and control, chromatin modification and silencing *(Peters and Meister 2007; Hock and Meister 2008*)

One of the Argonaute subfamilies, the PIWI subfamily, is found exclusively in animals. Piwi proteins are thought to be solely expressed in germ cells, they interact with pi – RNAs (a novel class of small RNAs), and together participate in numerous functions, such as transposon control, germline maintenance and *de novo* methylation in the germ cells *(Houwing, Kamminga et al. 2007; Klattenhoff and Theurkauf 2008; Kuramochi-Miyagawa, Watanabe et al. 2008)*

How transposons are *de novo* methylated in the female germline is still obscure. A candidate, the LSH helicase, is implicated to have a role, since oocytes from Lsh$^{-/-}$ mutant mice show demethylation of transposable elements (*De La Fuente, Baumann et al. 2006*), something which is not the case neither in Dnmt3l$^{-/-}$ (*Sasaki and Matsui 2008)* nor PIWI $^{-/-}$ (*Kuramochi-Miyagawa, Kimura et al. 2004*) female mutants, where oogenesis proceeds normally.

Despite the fact, that many factors have been identified to be crucial in DNA methylation in the germlines, the exact mechanisms underlying this event remain elusive. Moreover, it is intriguing that the same factors e.g. PIWI proteins, seem to have distinct roles in DNA methylation in the two sexes. It is possible, that there is a kind of redundancy in females so that methylation of retrotransposons can still arise, when PIWI proteins are absent, matter which should be further investigated.

LSH: Lymphoid Specific Helicase. It is involved in replication, repair and transcription regulation, but also in methylation of transposable elements and it is crucial for normal development (*NCBI*)

X CHROMOSOME INACTIVATION

Placental mammals have evolved a mechanism for accomplishing genomic dosage compensation between the two sexes by inactivating one of the two sex chromosomes in female individuals. Like DNA methylation patterns, X chromosome inactivation shows a very dynamic behavior during early embryonic development (Figure 11).

X inactivation occurs as early as the two to four cell stage embryo through the expression of *Xist*. Specifically, the paternal X chromosome (X_p) transcribes *Xist*, a non – coding RNA, which will coat the *Xp* in *cis* and inactivate it. Concomitant to that is the establishment of repressive histone modifications e.g. H3K27me3, which will assure gene silencing (*Senner and Brockdorff 2009*).

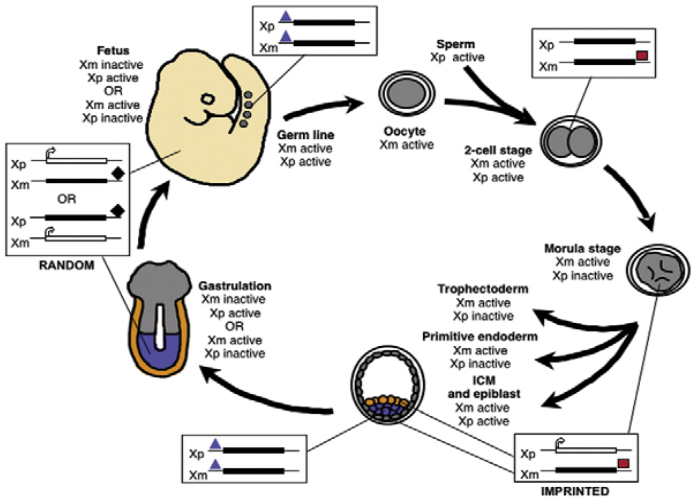


Figure 11: X chromosome dynamics during mammalian development. The X_p remains inactive through *Xist* expression, while the X_m active, until the blastocyst stage. An imprint on the Xm (red box) restricts expression of the *Xist* only from the Xp. This state of the X chromosomes will be maintained in extra embryonic lineages, while in the ICM the X_p will be reactivated and the X_m imprint erased. Sebsequently random X chromosome inactivation (black diamonds) will follow. During the migration of PGCs to the presumptive gonads, the randomly inactivated X allele will be reactivated. Figure taken by reference (*Senner and Brockdorff 2009*).

The blue diamonds indicate one of the ways that *Xist* expression can be regulated. At this stage pluripotency transcription factors such as Nanog, are implicated in suppressing *Xist,* and maintaining the X alleles active (*Senner and Brockdorff 2009)*

Dosage compensation: A mechanism to equalize the dosage of X chromosome gene products between males (XY) and females (XX), since Y chromosome is smaller and contains less genes than the X chromosome (*Alberts, Wilson et al. 2008*)

While the X_p gets inactivated, the maternal X chromosome (X_m) remains active by expressing *Tsix*, a non - coding RNA of antisense to *Xist* orientation.

Both X_p and X_m will remain inactivated and activated respectively throughout development but solely in lineages which will form extra – embryonic tissues, such as the trophectoderm. This phenomenon is speculated to be due to the existence of an imprint on the parental X chromosomes, which is responsible for maintaining X_m in an active and the X_p in an inactive state. The nature of this imprint is unknown, however it is thought to be erased in the cells of the ICM which will form the embryo, thus acquiring two active X chromosomes (*Payer and Lee 2008*).

As embryogenesis proceeds and cells enter a differentiation pathway, random X chromosome inactivation will subsequently occur. In the last step, which coincides with the migration of PGCs to the female genital ridges, the X chromosome silenced in the previous step will be reactivated through downregulation of *Xist* expression and loss of trimethylation on lysine 27 of histone 3 (*Chuva de Sousa Lopes, Hayashi et al. 2008*), in order oogenesis to proceed normally.

It seems that whether X chromosome inactivation will occur, depends on the expression of *Xist*, which is regulated by both *Tsix* dependent and independent mechanisms.

DNA methylation is suggested to have a dispensable role in maintaining random X inactivation maintainance in the embryo, since if Dnmt1 is depleted X reactivation occurs (*Payer and Lee 2008*). In addition to that, DNA methylation is also important for assuring *Xist* silencing in the active X chromosome (*Payer and Lee 2008*).

X inactivation is an example of long term repression in females and numerous factors and modifications are implicated to have a role in this event. Despite the fact that DNA methylation does not seem to have the primal role in this repression, it confers an extra level of gene silencing.

PLURIPOTENCY IN EMBRYONIC STEM CELLS

Embryonic stem cells can be isolated from the morula stage and from the ICM of mammalian blastocysts. These cells hold two features:

1. they are pluripotent, thus they have the ability to differentiate into many different somatic cell types; pluripotency *in vivo* is a very transient step, and

2. they can self – renew and be kept indefinitely *in vitro* under the appropriate culture conditions.

Epiblast: a group of cells within the blastocyst, that gives rise to the embryo proper. It develops from cells of the inner cell mass (*Alberts, Wilson et al. 2008*)

Nevertheless, cells which can be kept *in vitro* in a pluripotent state, derive also from PGCs (Embryonic Germ Cells - EG) and from the epiblast of embryos (Epiblast Stem Cells – EpiSCs).

The above two features imply that embryonic stem cells should retain their 'stemness – pluripotency' in the one hand, but also have the ability to reorganize their genome, when they are to follow a differentiation pathway, on the other hand. Hence, the chromatin of embryonic stem cells appears to be in a de - condensed state appointing it this way accessible to transcription factors and modifying enzymes and complexes which will promote expression of genes responsible for retaining pluripotency, while silence others which will be activated upon differentiation. Support to this 'open – chromatin state' is the dynamic binding – unbinding capacity to the chromatin of proteins related to its structure, such as HP1, reflecting this way the plasticity of chromatin's organization in ES Cells *(Meshorer and Misteli 2006; Allis, Jenuwein et al. 2007)* (Figure 12). Chd1 is thought to be responsible for the loose chromatin state in embryonic stem cells,

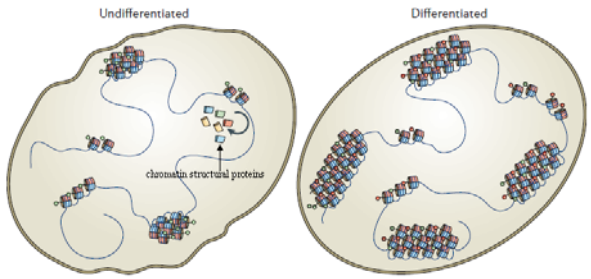


Figure 12: In undifferentiated cells chromatin is decondensed and proteins related with the chromatin structure show an unstable binding capacity to the chromatin. In differentiated cells on the other hand, chromatin appears more condensed and the chromatin structural proteins are stably bound. Figure taken by reference (*Meshorer and Misteli 2006*).

by yet an unknown mechanism (*Gaspar-Maia, Alajem et al. 2009*).

The ability of stem cells to reorganize their genome when differentiation takes place and retaining pluripotency, is not dependent on DNA methylation *per se*, but this is rather a multi – factor process, including transcription factors and epigenetic modifications on histones. Numerous transcription factors expressed in stem cells are key regulators of pluripotency, with the most important ones including Oct3/4, Sox2 and Nanog. While stem cells remain in an undifferentiated state, the promoters of these transcription factors are hypomethylated, thus allowing high levels of their expression (*Fouse, Shen et al. 2008*); these transcription factors can act on different targets. Firstly, independent of each other or by forming heterodimers or complexes, these transcription factors form a network,

Chd1: chromodomain helicase DNA binding protein 1. It contains an ATPAase SNF2 – like helicase domain and chromo domains and it can alter gene expression possibly by modifying the structure of the chromatin (*NCBI*)

which activates genes required for survival and proliferation of stem cells, repress others related with differentiation and at the same time positively regulate their own expression, thus creating a positive feedback loop for maintaining pluripotency (*Hemberger, Dean et al. 2009*). Secondly, these factors are believed to control transcription of epigenetic modifiers – with activating or repressive activity -. The expression or repression of epigenetic regulators is important to prevent embryonic stem cell differentiation; these regulators with their turn have a role in regulating transcription of pluripotency transcription factors; for example JMJD2C mediates activation of Nanog by reversing H3K9m3 repressive marks, thus promoting its expression as long as the cell remains in an undifferentiated state (*Hemberger, Dean et al. 2009*).

JMJD2C: member of Junonji domain 2 family. It contains a JmjC domain, a JmjN domain, two PHD – type zinc fingers and two Tudor domains. It functions as a trimethylation – specific demethylase (*NCBI*)

Another feature suggested in maintaining pluripotency in embryonic stem cells is the so called 'bivalency of chromatin'. Genome – wide analyses suggest that genes, which will be activated or repressed upon differentiation are held in a 'stand – by' mode by carrying both repressive and activating marks. These genes will be activated or permanently switched off upon differentiation with the removal of the corresponding mark. However, it needs to be stressed out, that these bivalent marks have not been proven to co – exist on the same histone tail, or even on the same nucleosome, therefore creating questions if indeed they have a role in maintaining pluripotency (*Herz, Nakanishi et al. 2009*).

Many genes have also been found to be repressed in embryonic stem cells, and to be devoid of bivalent marks. These genes are switched off by DNA methylation, proposing this epigenetic modification as an extra mechanism for retaining pluripotency and ensuring appropriate gene expression (*Fouse, Shen et al. 2008*).

DNA METHYLATION AND CANCER

From what described so far, DNA methylation plays a major role in mammalian development and in regulation of gene expression. Aberrant DNA methylation is linked though with diseases, such as cancer and early studies suggested that in cancer cells only tumor suppressor genes undergo DNA methylation, thus appointing these genes silent.

However, genome wide and microarrays analyses revealed that not only tumor suppressor genes are abnormally methylated in cancer, but methylation marks are found on CpG islands of genes not related to cell growth or tumorigenesis (*Keshet, Schlesinger et al. 2006*). These

genes in normal cells are also silent by chromatin modifications, but lack DNA methylation on CpG islands (*Schlesinger, Straussman et al. 2007*).

De novo methylation in cancer cells is suggested to be instructed by the presence of trimethylation on lysine 27 of histone 3 (H3K27me3 – repressive mark, Figure 13) (*Schlesinger, Straussman et al. 2007*). This modification is catalysed by Ezh2, a PRC2 complex component, which is also found to interact with DNA methyltrasferases to control gene expression (*Vire, Brenner et al. 2006*). However, this strategy is also used by healthy cells in order to set genes transcriptionally silent.

What triggers then *de novo* methylation of CpG islands in cancer? In cancer cells expression of Polycomb group components and DNA methyltransferases has been found to be upregulated. This might lead to the binding of *de novo* DNA methyltransferases in alternative substrates, thus promoting CpG island *de novo* methylation *(Schlesinger, Straussman et al. 2007*).

In addition, CpG islands of genes in normal cells remain unmethylated despite the presence of the H3K27m3 mark, hence creating the possibility of the existence of factors, which either protect CpG islands from methylation, or promote methylation of CpG islands in cancerous cells (*Schlesinger, Straussman et al. 2007*).

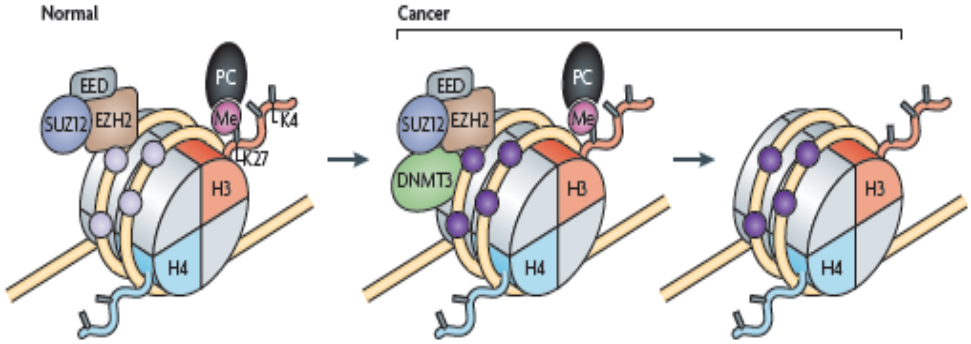


Figure 13: Genes in normal cells can be silenced by trimethylation of lysine 27 of histone 3 (H3K27m3), a reaction catalysed by Ezh2 member of the PRC2. H3K27me3 is an anchoring target for PRC1, which contains the chromodomain protein PC and it is necessary for stabilizing the silencing. These genes do not contain any methylation marks on their CpG islands (light purple circles). In cancer cells, probably due to abnormal regulation of Polycomb proteins or DNA methyltransferases, Ezh2 interacts with *de novo* methyltransferases, which catalyse methylation of CpG islands (dark purple circles). After methylation takes place, some genes lose PRC1 and PRC2, but remained silent due to DNA methylation (Cox, Chao et al. 2000; Cedar and Bergman 2009). Figure taken by reference (*Cedar and Bergman 2009*).

PRC2 Polycomb Repressive Complex 2. It methylates lysine 4 and lysine 9 of histone 3, leading to the transcriptional repression of the affected gene. The minimum components required for its methyltransferase activity are the Eed, Ezh2 and Suz12 (*NCBI*)

CONCLUSIONS

From the above described information it is clear that DNA methylation together with histone modifications are crucial players in developmental processes, which will eventually lead to a healthy individual. For the accomplishment of this goal the integrity of epigenetic modifiers, as well as of factors which regulate their activity, is of a great importance, since mutations that affect them can lead to lethality and serious diseases. For example, genetic disruption of Dnmt1, as well as homozygous Dnmt3b knock out, result in embryonic lethality in mouse embryos and disruption of the Dnmt3l gene, leads to loss of germ cells and paternal DNA methylation imprints in male mice (*Tost 2008*). Numerous other examples prove the importance of epigenetic modifiers and their regulators in development.

Despite the fact that much progress has been made in the field of epigenetics with the use of advanced technologies, many questions remain to be answered: what are the biochemical mechanisms underlying the heritable maintenance of DNA methylation per cell division? is TET1 a DNA demethyltransferase? and if yes, what is its physiological role? Answering these and a plethora (of course!) of other questions will be crucial for understanding the epigenome, for establishing treatment in pathological conditions such as cancer and for advancing in other fields, such as that of cloning and induced pluripotent stem cells.

REFERENCES

Alberts, B., J. H. Wilson, et al. (2008). Molecular biology of the cell. New York, Garland Science.

Allis, C. D., T. Jenuwein, et al. (2007). Epigenetics. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

Aravin, A. A., R. Sachidanandam, et al. (2008). "A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice." Mol Cell **31**(6): 785-99.

Attwood, J. T., R. L. Yung, et al. (2002). "DNA methylation and the regulation of gene transcription." Cell Mol Life Sci **59**(2): 241-57.

Bestor, T. H. (2000). "The DNA methyltransferases of mammals." Hum Mol Genet **9**(16): 2395-402.

Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev **16**(1): 6-21.

Cedar, H. and Y. Bergman (2009). "Linking DNA methylation and histone modification: patterns and paradigms." Nat Rev Genet **10**(5): 295-304.

Chuva de Sousa Lopes, S. M., K. Hayashi, et al. (2008). "X chromosome activity in mouse XX primordial germ cells." PLoS Genet **4**(2): e30.

Cirio, M. C., S. Ratnam, et al. (2008). "Preimplantation expression of the somatic form of Dnmt1 suggests a role in the inheritance of genomic imprints." BMC Dev Biol **8**: 9.

Cox, D. N., A. Chao, et al. (2000). "piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells." Development **127**(3): 503-14.

De La Fuente, R., C. Baumann, et al. (2006). "Lsh is required for meiotic chromosome synapsis and retrotransposon silencing in female germ cells." Nat Cell Biol **8**(12): 1448-54.

Dean, W., F. Santos, et al. (2001). "Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos." Proc Natl Acad Sci U S A **98**(24): 13734-8.

Fouse, S. D., Y. Shen, et al. (2008). "Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation." Cell Stem Cell **2**(2): 160-9.

Gaspar-Maia, A., A. Alajem, et al. (2009). "Chd1 regulates open chromatin and pluripotency of embryonic stem cells." Nature **460**(7257): 863-8.

Gehring, M., W. Reik, et al. (2009). "DNA demethylation by DNA repair." Trends Genet **25**(2): 82-90.

Goll, M. G. and T. H. Bestor (2005). "Eukaryotic cytosine methyltransferases." Annu Rev Biochem **74**: 481-514.

Goll, M. G., F. Kirpekar, et al. (2006). "Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2." Science **311**(5759): 395-8.

Hajkova, P., K. Ancelin, et al. (2008). "Chromatin dynamics during epigenetic reprogramming in the mouse germ line." Nature **452**(7189): 877-81.

Hayashi, K. and M. A. Surani (2009). "Resetting the epigenome beyond pluripotency in the germline." Cell Stem Cell **4**(6): 493-8.

Hemberger, M., W. Dean, et al. (2009). "Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal." Nat Rev Mol Cell Biol **10**(8): 526-37.

Herz, H. M., S. Nakanishi, et al. (2009). "The curious case of bivalent marks." Dev Cell **17**(3): 301-3.

Hirasawa, R., H. Chiba, et al. (2008). "Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development." Genes Dev **22**(12): 1607-16.

Hock, J. and G. Meister (2008). "The Argonaute protein family." Genome Biol **9**(2): 210.

Hou, J., T. H. Lei, et al. (2005). "DNA methylation patterns in in vitro-fertilised goat zygotes." Reprod Fertil Dev **17**(8): 809-13.

Houwing, S., L. M. Kamminga, et al. (2007). "A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish." Cell **129**(1): 69-82.

Jirtle, R. L. a. M. K. S. (2007). "Environmental epigenomics and disease susceptibility." Nat Genet **8**(4): 253-262.

Jones, P. A. and G. Liang (2009). "Rethinking how DNA methylation patterns are maintained." Nat Rev Genet **10**(11): 805-11.

Kaneda, M., M. Okano, et al. (2004). "Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting." Nature **429**(6994): 900-3.

Kato, Y., M. Kaneda, et al. (2007). "Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse." Hum Mol Genet **16**(19): 2272-80.

Keshet, I., Y. Schlesinger, et al. (2006). "Evidence for an instructive mechanism of de novo methylation in cancer cells." Nat Genet **38**(2): 149-53.

Kim, G. D., J. Ni, et al. (2002). "Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases." EMBO J **21**(15): 4183-95.

Klattenhoff, C. and W. Theurkauf (2008). "Biogenesis and germline functions of piRNAs." Development **135**(1): 3-9.

Kuramochi-Miyagawa, S., T. Kimura, et al. (2004). "Mili, a mammalian member of piwi family gene, is essential for spermatogenesis." Development **131**(4): 839-49.

Kuramochi-Miyagawa, S., T. Watanabe, et al. (2008). "DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes." Genes Dev **22**(7): 908-17.

Kurihara, Y., Y. Kawamura, et al. (2008). "Maintenance of genomic methylation patterns during preimplantation development requires the somatic form of DNA methyltransferase 1." Dev Biol **313**(1): 335-46.

Lees-Murdock, D. J. and C. P. Walsh (2008). "DNA methylation reprogramming in the germ line." Adv Exp Med Biol **626**: 1-15.

Maronpot, R. (1999). "The atlas of mouse development."

Meshorer, E. and T. Misteli (2006). "Chromatin in pluripotent embryonic stem cells and differentiation." Nat Rev Mol Cell Biol **7**(7): 540-6.

Miranda, T. B. and P. A. Jones (2007). "DNA methylation: the nuts and bolts of repression." J Cell Physiol **213**(2): 384-90.

Mor, G. (2006). Immunology of pregnancy. Georgetown, Tex.
New York, Landes Bioscience/Eurekah.com ;
Springer Science+Business Media.

Morgan, H. D., F. Santos, et al. (2005). "Epigenetic reprogramming in mammals." Hum Mol Genet **14 Spec No 1**: R47-58.

Nakamura, T., Y. Arai, et al. (2007). "PGC7/Stella protects against DNA demethylation in early embryogenesis." Nat Cell Biol **9**(1): 64-71.

NCBI. from http://www.ncbi.nlm.nih.gov/.

Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**(3): 247-57.

Ooi, S. K. and T. H. Bestor (2008). "The colorful history of active DNA demethylation." Cell **133**(7): 1145-8.

Ooi, S. K., C. Qiu, et al. (2007). "DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA." Nature **448**(7154): 714-7.

Payer, B. and J. T. Lee (2008). "X chromosome dosage compensation: how mammals keep the balance." Annu Rev Genet **42**: 733-72.

Peters, L. and G. Meister (2007). "Argonaute proteins: mediators of RNA silencing." Mol Cell **26**(5): 611-23.

Rai, K., I. J. Huggins, et al. (2008). "DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45." Cell **135**(7): 1201-12.

Reik, W., W. Dean, et al. (2001). "Epigenetic reprogramming in mammalian development." Science **293**(5532): 1089-93.

Reik, W. and J. Walter (2001). "Genomic imprinting: parental influence on the genome." Nat Rev Genet **2**(1): 21-32.

Santos, F., A. H. Peters, et al. (2005). "Dynamic chromatin modifications characterise the first cell cycle in mouse embryos." Dev Biol **280**(1): 225-36.

Sasaki, H. and Y. Matsui (2008). "Epigenetic events in mammalian germ-cell development: reprogramming and beyond." Nat Rev Genet **9**(2): 129-40.

Schlesinger, Y., R. Straussman, et al. (2007). "Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer." Nat Genet **39**(2): 232-6.

Senner, C. E. and N. Brockdorff (2009). "Xist gene regulation at the onset of X inactivation." Curr Opin Genet Dev **19**(2): 122-6.

Sharif, J., M. Muto, et al. (2007). "The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA." Nature **450**(7171): 908-12.

Tahiliani, M., K. P. Koh, et al. (2009). "Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1." Science **324**(5929): 930-5.

Thurston, A., E. S. Lucas, et al. (2007). "Region-specific DNA methylation in the preimplantation embryo as a target for genomic plasticity." Theriogenology **68 Suppl 1**: S98-106.

Tost, J. (2008). Epigenetics. Norfolk, UK, Caister Academic Press.

Vire, E., C. Brenner, et al. (2006). "The Polycomb group protein EZH2 directly controls DNA methylation." Nature **439**(7078): 871-4.

Wolpert, L. (2007). Principles of development. Oxford ; New York, Oxford University Press.

Yoder, J. A., N. S. Soman, et al. (1997). "DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe." J Mol Biol **270**(3): 385-95.