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-
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1 INTRODUCTION

During the last years, considerable efforts in genetic research yielded many up to then unknown observations that can be summarized in one idea: *epigenetics*. This term refers to changes in gene expression without affection of the DNA sequence of an organism. (BIRD, 2007) The major characteristic of epigenetic events is that they can be passed on not only from cell to cell but also from organism to organism, i. e. the changes are heritable. (JABLONKA & LAMB, 1989) Such events may have considerable influence on the differentiation of cells, the phenotypical development of an organism and eventually the evolutionary progress of a population or species.

The meaning of epigenetics has not always been consistent. In 1957 Conrad H. Waddington defined epigenetics as the formation of a phenotype due to environmental influences on a certain genotype. (WADDINGTON, 1957) In the 1970s this theory was expanded by Holliday and Pugh who suggested that chemical DNA modification in form of cytosine methylation was the molecular background of Waddington's hypothesis. (HOLIDAY & PUGH, 1975) Later, by the end of the 20th century, Riggs and colleagues established the current definition of epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence". (RUSSO & al., 1996) This definition clearly points out that various mechanisms are at work which do not lead to mutational changes of DNA, but still have an impact on whether and when genes are expressed. More definitions have been created, all giving respect to observations that many constituents of the cell nucleus seem to influence gene expression. (BIRD, 2007)

The involved mechanisms are quite numerous (FRASER & BICKMORE, 2007; ALTUCCI & STUNNENBERG, 2009), ranging from influences on nucleobases, e. g. the methylation of cytosine, the chemical modification of histones (FAN & al., 2008), the structure of chromatin

(BERGER, 2007) and the transcription of non-coding types of RNA. (GUIL & ESTELLER, 2009) The variety of possibilities is high. For example there are about 60 known modifications of histones. Methylation, acetylation, phosphorylation and ubiquitinylation are only some of them. Histones are globular proteins around which the DNA is wrapped, forming a structure similar to a pearl chain. One histone 'carries' 146 base pairs of double-stranded DNA and such a unit is called nucleosome. Histones are normally octameres consisting of one dimer of each of the subtypes H2a, H2b, H3 and H4, respectively. The histone spheres have a 'tail' whose lysine and arginine residues are typically subject to modification, whereas the kind of modification and the position of a certain residue can be correlated with the status of a gene, i. e. activation or repression of expression. Acetylation of lysine, methylation of arginine and phosphorylation of serine and threonine are generally linked to activation, in contrast to methylation of lysine that can be linked to activation and repression, depending on the position of lysine. (BERNSTEIN, 2007; KOUZARIDES, 2007)

Histone modification is connected to the state of chromatin, meaning that epigenetic properties can be attributed to the latter. Chromatin is the series of nucleosomes that eventually makes up a chromosome. In general, acetylation of histones leads to a loose and more 'open' structure termed euchromatin. In contrast, other modifications and deacetylation lead to a packed structure termed heterochromatin. Euchromatin contains the majority of genes and unique DNA sequences, because its structure makes it accessible for the enzymical transcription machinery. The nucleosomes of heterochromatin, in contrast, are highly condensed, making it more difficult for the transcription machinery to gain access. Consequently, few genes and many repetitive DNA sequences can be found in heterochromatic regions. During mitosis and meiosis euchromatic structures are located at the chromosomal arms, whereas heterochromatic structures are located at the centromere and the telomeres of a chromosome. (GREWAL & ELGIN, 2007)

According to modern definitions, the long known cellular mechanism of alternative splicing could also be described as epigenetic, even though it is

generally not included in the current literature on the topic. Alternative splicing means that expendable sequences of incomplete messenger RNA (so-called introns) need not always be excised in the same way. This leads to a different protein version, even though no changes ever occurred in the associated gene. (KARP, 2007) Thus alternative splicing might be included into the series of epigenetic mechanisms. The RNA interference pathway by which micro RNA (miRNA) is used to regulate and eliminate cellular transcripts or foreign RNA, respectively (KARP, 2007; GUIL & ESTELLER, 2009), would also fall into this category.

One of the most important epigenetic factors is the methylation of DNA, especially the nucleobase cytosine. Such modification is known to be potentially inheritable and can have remarkable consequences for the phenotype and health of an organism, depending on the location of the particular nucleotide. (BIRD, 2002) This phenomenon’s potential molecular mechanisms and the methodology of cytosine methylation analysis will be the topic of the following work.

2 GENERAL ASPECTS OF DNA METHYLATION

Another definition of epigenetics by Bird resumes this phenomenon as “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”. (BIRD, 2007) More likely than the former definitions, this one pronounces the biological significance of epigenetic processes. Nevertheless it does not differentiate between DNA methylation and other single epigenetic phenomena of distinct nature which can be related by biochemical processes in a given cell or organism.

DNA methylation is a feature of a number of multi-cellular eukaryotic organisms. Mammalian genomes exhibit rather specialised functions of DNA methylation, such as ‘genomic imprinting’ (IDERAABDULLAH & al., 2008) and ‘metastable epialleles’. (DOLINOY & al., 2007) Both phenomena will be discussed in the according chapters. Moreover, the enzymatic pathways for establishing methylation marks and removing them show broad variations, especially between the animal and the plant kingdom. (GOLL & BESTOR, 2005) Here as well, the major differences will be pointed out in the according chapters.

The quantitative range of DNA methylation is dramatically diversified. In the DNA sequence of the solitary cell organism Saccharomyces (yeast) or the nematode Caenorhabditis elegans, both serving as popular models for genetic and metabolic studies, no methylation has been detected and neither has any according enzymatic activity been identified. (HENDRICH & TWEEDIE, 2003) Another multi-cellular model organism, the fruit fly Drosophila melanogaster, exhibits low levels of methylation and an enzyme similar to the known methylating enzymes has been discovered. (LYKO & al., 2000) On the other hand, plant and vertebrate animal organisms possess the highest quantity of methylation marks and the highest degree of diversification within their respective genomes. (BIRD, 2002)

To date the only nucleotide known to be able to accept a methyl group is cytosine. By the enzymatic addition of a methyl group to the carbon atom of position 5 of the pyrimidine ring, 5-methyl cytosine (5mC) is created. (GOLL & BESTOR, 2005) For the chemical mechanism of this conversion, see the according chapter. Cytosine can be located within various nucleotide contexts. The most common is the 5' addition of guanine. A frequent occurrence of this dinucleotide sequence in a certain DNA sequence is commonly referred to as CpG island. Other sequence contexts are CpT, e. g. present in D. melanogaster genomes, and CpNpG, a common trinucleotide sequence in plants, whereas N stands for an arbitrary nucleotide. (BIRD, 2002)

The pattern of cytosine methylation varies distinctly among organisms as well. In general, non-vertebrate organisms exhibit mosaic methylation patterns, as was observed in the sea squirt Ciona intestinalis. (SIMMEN & al., 1999) A mosaic pattern means a series of highly methylated DNA sequences that take respective turns with unmethylated sequences. In contrast, cytosine methylation in vertebrate genomes is spread across the DNA sequence. (COLOT & ROSSIGNOL, 1999) Especially in mammalian organisms there even exists a temporal variation of methylation marks, which is a crucial factor for the development of a respective organism during all phases of its life. Early embryonal development in mice, serving as a model organism for mammals, is associated with a higher decrease of methylation marks than other animals. (KAFRI & al., 1992) Details will be discussed in the according chapters.

CpG islands are mostly located within gene promoters. This suggests a role for cytosine methylation in gene expression. Indeed, numerous experiments have shown that heavily methylated CpG islands within the promoter region of a given gene are associated with a repressed state of this gene. (BIRD & WOLFFE, 1999) Moreover, CpG island methylation appears to play a general role in genomic stability. In somatic human cells around 70-80% of all CpG islands located across the DNA sequence are affected by cytosine methylation. The variety of methylation marks applies to genes, as well as repetitive or satellite sequences and transposable elements. The latter observations lead to the assumption that methylation is not only a way to

suppress gene activity, but moreover to stabilize the genome by repressing all potentially hazardous DNA sequences. (BIRD, 1995; YODER & al., 1997A) In summary, cytosine methylation is both a path to the differentiated state of a cell and a mechanism to keep the genome in a stable and employable state.

Probably the most striking observation in connection with DNA methylation is induced by the definition of Riggs, but excluded in the definition of Bird. This is the potential heritability of changes in gene activity or repression by epigenetic mechanisms, not only from cell to cell, but from one organism to a succeeding one. Whereas not much is known about properties of heritability of histone modification, this has particularly been shown for methylation marks. (BIRD, 2007) Moreover, the germ lines of an organism can be affected without phenotypical changes, whereas phenotypical changes can become visible in offspring generations. The model organism of the Agouti mouse has revealed striking evidence in this regard. (MORGAN & al., 1999) The exact context will be discussed in the according chapter. Such notice has not been taken for other epigenetic phenomena, making the methylation of cytosine a promising candidate for the research on influences of lifestyle, diet and environment on the physiological and pathological development of humans and animals and potential impacts on their respective progeny.

3 MOLECULAR MECHANISMS OF DNA METHYLATION

3.1 Cytosine methylation

Basically, there are two different versions of DNA methylation. First, there is maintenance methylation by which a newly synthesized DNA strand becomes methylated according to the already methylated original strand. This way of methylation typically occurs in the course of DNA synthesis before cell division. Next to maintenance methylation there is de novo methylation by which methyl-groups are added to DNA strands with low levels of or without methylation. (CHEN & LI, 2004) This procedure is characteristic for embryonic stem cells in which gene expression is programmed by epigenetic processes. (NG & al., 2008)

The enzymes that carry out the transfer of methyl groups in many eukaryotes are called DNA methyl transferases (DNMTs). There are several isoforms of these enzymes with different functions (CHEN & LI, 2004) as will be explained in the following chapters. Apart from DNMTs, there are several enzymes in non-mammalian organisms with a similar function but marked differences in their structure. One example is the group of chromomethylases which are typical for the plant kingdom. (ZILBERMANN, 2008)

DNMTs are divided into three families. There is the DNMT1 family which appears to be deeply connected with the DNMT3 family. Representatives of the DNMT2 family can be found in organisms with DNMT1 and DNMT3 homologues, but there are several organisms containing only DNMT2 homologues. The general function of these enzymes and their relatives is the catalysis of the transfer of a methyl-group to cytosine residues on the DNA strand. Favoured targets are CpG islands in regions outside of genes or in gene

promoters. However, DNMTs show remarkable differences in functional details, structure, appearance and evolutionary history. (BESTOR, 2000)

Altogether there are 10 motifs equal to all known eukaryotic methyltransferases that are shared by prokaryotic enzymes of the same type (see Fig. 1). A structure motif of a protein is a region inside this protein whose sequence and three dimensional structure can also be found in other proteins with the same assumed function. The degree of variation is very small, which can be interpreted as an indicator of evolutionary conservation of an essential biological function. These motifs proved valuable for the identification of methyltransferases of higher organisms. A so-called target recognition domain (TRD) is located between the motifs VIII and IX. (TRAUTNER, 1988) It connects the enzyme to the major groove of the DNA double strand. Therefore motif IX organises the TRD and motif VIII serves as a DNA backbone and makes contact with the target cytosine. Motif VI contains the amino acid for the protonation of the N atom at position 3 of the cytosine (see below) and motif IV contains the catalytic region for covalent binding of a methyl group to the C5 position of cytosine (see below). Eventually the motifs I and X bind the co-enzyme S-adenosyl-L-methionine (SAM). SAM serves as the methyl group donor and the methionine part is reduced to homocysteine by the methylation reaction. (WILKE & al., 1988; CHENG & al., 1993; CAO & al., 2000)

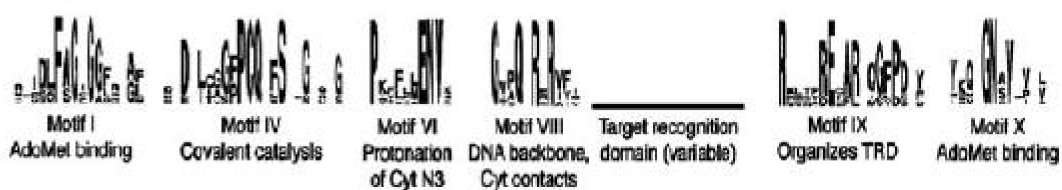

Fig. 1: DNMT motifs

Common structural motifs of methyl-group transferring enzymes (GOLL & BESTOR, 2005)

By clarifying the molecular mechanisms of DNMT catalysis researchers were confronted with some difficulties. The reactivity of the C5 position in cytosine is very low, thus a basic covalent addition of a methyl group could not be assumed. Next to that, the amino acid structure around the catalytic centre is incompatible with the DNA double strand, so a solution for the steric issue had to be found as well. The latter problem was solved by the observation that the enzyme breaks the hydrogen bonds between the cytosine and the opposite thymidine and turns it around so that it fits into the catalytic centre (see Fig. 2).

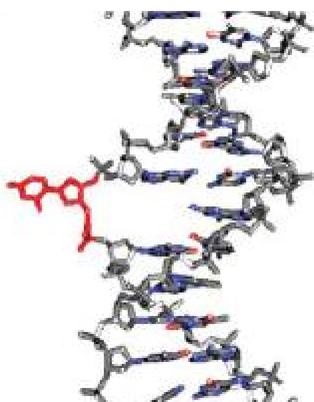

Fig. 2: Cytosine eversion

Eversion of cytosine to be accessible to the methylation enzyme (GOLL & BESTOR, 2005)

The next step is a covalent bond between the C6 position of cytosine and the thiol group of cysteine. Following this step, the N atom at position 3 becomes protonated by a carboxyl group at the catalytic centre, forming an enamine structure between the positions C4 and C5. Before SAM comes into play, the protonation at position 3 is reversed and the double bond of the enamine moves back between positions 3 and 4. In the course of this procedure SAM provides a methyl group that is covalently added to the C5 position. The N at position 3 becomes protonated again and the H atom at the newly methylated position 5 is removed, which again moves the double bond between positions 4 and 5. Eventually the original double bonds between positions 3 and 4, as well as 5 and 6 are restored by deprotonation of position 3 and removal of the

methyl transferase's cysteine by beta-elimination (see Fig. 3). The newly synthesised 5-methyl-cytosine (5mC) can now serve its role as an epigenetic marker for whatever purpose. (CHENG & ROBERTS, 2001)

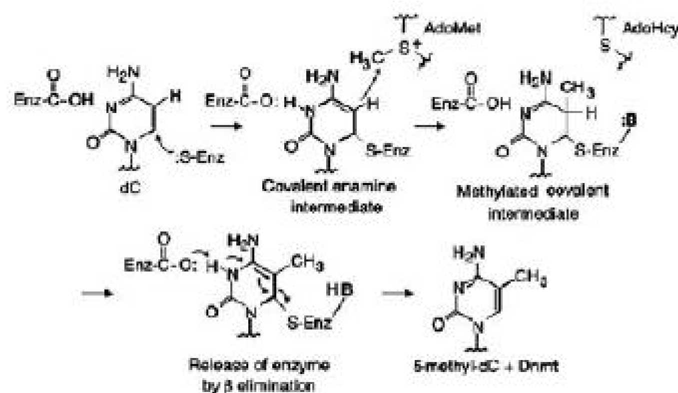

Fig. 3: Enzymatic methyl transfer

Reaction steps of the transfer of a methyl-group from cofactor to nucleotide by the methylation enzyme (GOLL & BESTOR, 2005)

Remarkably, experiments showed that some organisms, like the plant Arabidopsis thaliana, have the potential of expressing at least 10 different versions of a methyl transferase, however, some invertebrates, like C. elegans or D. melanogaster, show hardly any signs of such an enzyme at all. (GOLL & BESTOR, 2005)

3.1.1 DNMT1

The first identified eukaryotic methyl transferase was DNMT1. (BESTOR & al., 1988) Observations showed that DNMT1 catalyses methylation of both unmethylated DNA double strands and DNA with one already methylated and one newly synthesised unmethylated strand. However, hemimethylated DNA was affected with up to 30-fold efficiency, depending on the substrate sequence. These results led to the conclusion that DNMT1 can be considered a maintenance methylation enzyme. However, the de novo methylation activity of

DNMT1 is greater than that of any other known methyl transferase, therefore it is assumed that its activity in an organism is restricted to maintenance methylation by additional to date unknown factors. Assuming DNMT1 as competent solely for maintaining DNA methylation, two possible activity models have been established. In the first model, DNMT1 is bound to proteins of the replication fork and therefore methylates hemimethylated sites parallel to DNA synthesis. The other model proposes factors independent of the replication process (see below). However, to date both models still lack consistency and will most probably be explicitly modified. (YODER & al., 1997B)

DNMT1 is found in both embryonal and adult mammalian tissues. In a cell DNMT1 is present in the nucleus and in the cytoplasm. During the non-replicative cell phases and after DNA replication the enzyme is located in the cytoplasm at very low concentrations, but it is imported into the cell nucleus before the S-phase. Located on the N-terminal region there is a domain for the import into the nucleus and a domain for making contact with the replication machinery. When a cell returns to normal metabolism after mitosis DNMT1 is degraded and the initiative sequence is again located on the N-terminal region. Moreover, there is at least on domain on the N-terminal region for the interaction with DNMT1-associated proteins, but the exact function is unclear.

The structure of DNMT1 has first been determined from murine erythroleukemia (MEL) cells and the nucleotide sequence corresponding to the amino acid sequence has been compared to cDNA of other organisms. The mouse DNMT1 contains about 1600 amino acids of which 500 belong to the C-terminal region and 1100 to the N-terminal region. Both regions are connected by a domain consisting of alternating glycine and lysine residues. (LEONHARDT & al., 1992)

Comparison with other eukaryotic DNMT1 homologues showed similarities. All known DNMT1 enzymes possess a glycine-lysine-domain that connects the N- and the C-terminal region. The catalytic centre is located on the C-terminal region. DNMT1 contains two bromo-adjacent homology (BAH) domains which are supposed to have an interactive function with other proteins. In addition to these features there is a cysteine-rich domain on the N-terminal

region that can bind Zink ions. This domain is limited to mammalian DNMT1s and its function has yet to be determined. (CALLEBAUT & al., 1999)

Loss of function of DNMT1 has several severe consequences, whereas two of them are quite remarkable. First, loss of function appears to result in slight defects in base mismatch repair, suggesting that DNA methylation could play a role in this phenomenon. (GUO & al., 2004) Second, transposons are activated at an unusually high level. Of all known methyl transferases DNMT1 is the only one in mammals to be demonstrated as a repressor of transposons. (WALSH & BESTOR, 1999) Furthermore, mice embryos and embryonic stem cells can grow normally in their stem cell state without DNMT1, but die due to apoptosis as soon as they are forced to differentiate. This might be because of the inability of maintaining DNA methylation of non-essential genes in differentiated cells and thus aberrant cell metabolism. (LEI & al., 1996) For the connection of DNMT1 expression and the state of DNA methylation in human and experimental tumours, see the according chapter.

The lymphoid specific helicase (Lsh) in mice has been reported a cofactor for maintenance methylation. Mutations of the *Lsh* gene lead to severe phenotypic appearances and early death. In addition, about 50% demethylation occurs, mostly in repeated sequences. Lsh belongs to the protein group of Swi2/Snf2 and several members of this group have been identified as possible cofactors for cytosine methylation in diverse organisms. For example, *ATRX* and *DDM1* encode proteins that act in some way in maintenance methylation in mammals and plants, respectively. Moreover, the proteins methyl-CpG-binding protein 2 (MeCP2) and the kinase cyclin-dependent kinase-like 5 (CDKL5) have been reported to directly bind to DNMT1. Concerning CDKL5, it is assumed that this kinase activates the methyl transferase by active phosphorylation. UHRF1 is another cofactor for the maintenance methylation process. In a current model this protein is stated to bind DNMT1 and transport it to hemimethylated sites. (NAN & al., 1997; GEIMAN & al., 2001; KAMESHITA & al., 2008)

Two special forms of DNMT1 shall be addressed in this chapter. One is the homologue located in oocytes which is therefore named DNMT1o. The N-terminal domain responsible for degradation is missing in DNMT1, thus the

enzymes remains in the cytoplasm for a longer time by binding of the shortened sequence to annexin V. DNMT1o functions during embryo development as well, especially at the 8-cell-stage which has been referred to as a critical time to avoid demethylation. (DOHERTY & al., 2002; CIRIO & al., 2008)

The other version similar to DNMT1 is the plant methyl transferase MET1, first identified in A. thaliana. (FINNEGAN & DENNIS, 1993) The C-terminal region is about 50% equal with other DNMT1 homologues, the N-terminal region about 25%. The preferred targets of MET1 appear to be CpG islands, but not CpNpG islands which are typical for plant genomes. In contrast to organisms in which DNMT1 is normally the only representative of its family, A. thaliana can express three more genes encoding isoforms of MET1. However, MET1 is the major enzyme of this group. Although experiments could not find any methylation activity in vitro, knock-out experiments and phenotypic assessments accounted MET1 for being a homologue of DNMT1. It is considered an enzyme for maintenance methylation, but shows properties of de novo methylation as well. (SAZE, 2008; ZILBERMANN, 2008)

3.1.2 DNMT2

DNMT2 is a puzzling methyl transferring enzyme. Although its prevalence is high among eukaryotes, to date studies failed to identify its exact function. Knock-out experiments on several organisms did not generate any obvious phenotypes, nor could any aberrations in DNA methylation or chromosome structure be detected. Additionally, in vitr*o* experiments provided no or only negligible results concerning methylation of either double- or single-stranded DNA. Structural analyses even bear doubt that the substrate of DNMT2 is double-stranded DNA. However, protein structure and thoroughly existent expression in adult mammalian tissues point out rather straight that the DNMT2 family has a methyl transferase function. It has been shown that DNMT2 catalyzes methylation of CpG, CpA and CpT islands, although whether these sequence contexts are the real substrates has yet to be determined.

The general motifs of all DNMT enzymes have been identified in DNMT2, as well as the presence of the coenzyme SAM or its reduced

counterpart S-adenosyl-L-homocysteine (SAH), respectively. Only the sequence containing the TRD differs from other types of methyltransferring enzymes and additionally, there are no extensions in the N-terminal region.

There are some enzymes with methyl transferase activity in bacterial cells as well. The DNMT2 homologues appear to resemble bacterial transferases, like M.Hha1 in E. coli, more than other eukaryotic methyl transferring enzymes. Surprisingly, DNMT2 is present in organisms with low or no signs of cytosine methylation, like yeast or D. melanogaster. (DONG & al., 2001; PONGER & LI, 2005)

3.1.3 DNMT3

The last group of major eukaryotic methyl transferases comprises the DNMT3 enzymes. Apart from some special forms, the two main representatives are DNMT3A and DNMT3B. Both enzymes catalyze methylation of both hemimethylated and unmethylated DNA substrate without measurable differences. However, murine stem cells that cannot express the two enzymes are unable to methylate viral DNA after its integration into the genome. Because of this the DNMT3 enzymes are associated with de novo methylation. Once again, the basic target sites are CpG islands. In germ lines, de novo methylation appears to preferentially operate on repetitive sequences, like transposons or satellite sequences near the centromeres. (OKANO & al., 1999)

Next to the general motifs of DNMTs, mammalian DNMT3A and DNMT3B possess a PWWP domain, the Zink-binding cysteine domain familiar from DNMT1, and several methyl group binding domains on their N-terminal region. (XIE & al., 1999)

An exact functional difference between both methyltransferases has not been identified yet. DNMT3A is necessary for normal imprinting of the *H19* and *Gtl2-Dlk1* alleles in male germ cells, but it is not associated with regular methylation of the *Rasgrf* allele. The provision of genetic imprints seems, however, not to be the only function of DNMT3A. A lack of this enzyme in male mouse germ cells leads to complete demise of these cells, which might probably not be the case, if there were only defects in allele-specific imprints. To date no observations

have been made that DNMT3A function is limited to genetic imprinting or if it also methylates transposons or other repetitive sequences in vivo. (KANEDA & al., 2004)

DNMT3A2, a shortened isoform of DNMT3A, exists in murine prospermatogonia in which most de novo methylation occurs. DNMT3A2 is the product of alternative splicing, whereas the *DNMT3A* gene is transcribed from a promoter-like region between exon 6 and 7 and the resulting protein thus lacks 219 amino acids. It is not known whether DNMT3A2 plays a role in setting allele-specific imprints or has any functional distinctions to the full-length enzyme. (CHEN & al., 2002)

DNMT3B appears to be vital for normal methylation of satellite DNA. Loss of methylation in such sequences, especially near centromeric regions, leads to abnormal chromosome structures. Point mutations in the *DNMT3B* gene lead, among others, to the severe immunodeficiency – centromeric instability – facial abnormalities (ICF) syndrome in humans. DNMT3B-deficient mouse embryos suffer significant lack of satellite sequence methylation and are not viable. Interestingly, this is in contrast to DNMT3A-deficient mice. These animals show severe malformations, like microsomia and aganglionic megacolon, but survive the developmental period. In mouse embryos, DNMT3B is preferentially expressed in the trophoectoderm and later in the inner cell mass, where most de novo methylation occurs. Whether this enzyme plays a role in de novo methylation in germ cells is not known. (KONDO & al., 2000; LORINCZ & al., 2002)

There is an isoform of DNMT3 which is specific for germ cells only, called DNMT3L. This enzyme is closely related to DNMT3A and DNMT3B, although it lacks the characteristic PWWP domain. Furthermore, no methyl transferase activity has been observed, although it is essential for regular methylation patterns. Evolutionary reasons might have led to the loss of a once existing catalytic activity. Instead more variable proteins could have evolved, as seen by the difference of mouse and human homologues. These new proteins could then serve as enhancers of the actual methyl transferring enzymes. In oocytes DNMT3L is associated with gene imprinting, in prospermatogonia it

acts in the methylation of repetitive sequences. Deficiency appears not to be relevant for meiosis in female germ cells, but the more in male germ cells which show severe abnormal cellular formations and die due to apoptosis. These deformations are likely consequences of desiderate transposon inactivation. Deficiency in both sexes show sexual dimorphism in mice and symptoms similar to those associated with DNMT3A deficiency. This, together with an experiment that DNMT3L could stimulate DNMT3A and DNMT3B activity, led to the conclusion that the interaction with the isoform is needed to guarantee normal methylation patterns in allele-specific imprints. (PRADHAN & ESTEVE, 2003; BOURC'HIS & BESTOR, 2004; SUETAKE & al., 2004)

The occurrence of DNMT3 homologues in invertebrates is more variable than in vertebrates. Many examined species contain all three DNMT families, some of them only DNMT2 homologues. In whatever organisms DNMT1 homologues have been detected, it is expected that these organisms also contain at least one DNMT3 homologue.

Plants once again show a specific version of DNMT3, the so-called domains rearranged methyl transferase (DRM). However, this modification has only been observed in flowering plants. There are some similarities, especially the region around the highly conserved catalytic centre. The amino acid sequence of the C-terminal region is 28% equal to mammalian DNMT3 isoforms. However, DRMs together with all known plant methyl transferases do not contain the cysteine accumulation that is characteristic for the N-terminal region of DNMT1 and DNMT3 homologues. The N-terminal region contains an ubiquitin-associated domain which has not been observed in other methyl transferases and whose function is unknown. The motifs I to X are existent, even though the motifs VI to X are located prior to I to V. DRM homologues function in a mechanism called RNA directed methylation. This process appears to be based on the creation of short RNA sequences which recognize and mediate methylation of complementary DNA sequences. More homologues without a definite task have been identified and it is assumed that some of them evolved out of similar evolutionary reasons like DNMT3L in mammals. (CAO & al., 2000; GOLL & BESTOR, 2005)

3.1.4 Chromomethylases

Chromomethylases (CMTs) are another kind of methyl transferases specific for flowering plants. In contrast to conventional enzymes, CMTs catalyse methylation of CpNpG islands. N stands for any nucleotide. A distinguishing property of CMTs is the existence of a chromodomain located between the motifs II and IV. A chromodomain is a region assumed to be an interface between proteins and heterochromatin. (HENIKOFF & COMAI, 1998)

CMTs are generally associated with maintenance methylation, whereas de novo methylation is assumed to take place in the course of RNA directed methylation. (CAO & al., 2003)

Analyses of A. thaliana revealed the three homologues CMT1, CMT2 and CMT3. More homologues have been identified in the genomes of other plants. Their exact function remains unclear and some of the encoding genes seem to be expendable at all. Only CMT3 in A. thaliana was shown to be involved in CpNpG methylation in repetitive sequences lacking CpG islands. *CMT3* knockout plants show decreased CpNpG island methylation and increased transposon activity, but no observable changes in the phenotype have been reported. The first effect is even more significant when *CMT3* and *MET1* are knocked out. (KATO & al., 2003)

There is evidence that histone methylation is involved in CMT function as well. *Kryptonite* codes for an enzyme that catalyzes methylation of the lysine residue 9 in histone 3 (H3K9) and knocking out this gene results in the same amount of demethylation of CpNpG islands. In organisms without methylation of other than CpG sequences, H3K9 appears not to play a role in the regulation of DNA methylation. (JOHNSON & al., 2002)

It is hypothesized that the development of this enzyme class occurred because of transposable gene elements with few or without CpG, but rather CpNpG sequences. Those transposons seem to be preferentially located in plants which had to devise more specific defensive mechanisms. (KATO & al., 2003; ZILBERMANN, 2008)

3.2 Cytosine Demethylation

The issue of hypermethylated DNA regions in various diseases, including cancer (see according chapter), as well as the general erasure of methylation marks in mammalian primordial germ cells raises the question of possible mechanisms of cytosine demethylation. Several theories on mammalian cytosine demethylation are available, whereas most of them are at least entangled in some contradictions. However, all agree with the assumption that DNA demethylation is on the one hand active, and on the other hand occurs via base excision repair. This assumption goes along with the hypothesized mechanism of plant cytosine demethylation which is more clearly elaborated than its mammalian counterpart. In this context, both the plant and recent mammalian models will be addressed. (JIRICNY & MENIGATTI, 2008)

3.2.1 Base excision repair in plants

Knockout experiments revealed two different enzyme classes that operate in plant cells. Demeter, expressed by the gene *Medea*, catalyzes demethylation in germ cells, whereas the repressor of silencing 1 (ROS1) and the Demeter-like enzymes DML2 and DML3 act in somatic cells. The mechanism starts with enzymatic removal of 5mC from deoxyribose, followed by cleavage of the phosphodiester bond by an AP endonuclease with lyase activity. Eventually, dCMP is inserted and the DNA strand is joined. This mechanism differs significantly from the proposed mammalian way of action in that base excision functions without the conversion of 5mC to thymine (see below).

There is another striking difference between plant and mammalian demethylation, namely in connection with genomic imprinting. Whereas genomic imprinting occurs in mammals and other eukaroytes by de novo methylation of excessive alleles, demethylation occurs in plants for one of both transcriptionally inactive alleles.

The purpose of cytosine demethylation in germ cells refers to the expression of genes needed after fertilisation, whereas its counterpart in somatic cells appears to be a protective mechanism against hypermethylation by the according methyltransferases (see according chapter) in case repetitive sequences are located nearby. (OOI & BESTOR, 2008A)

3.2.2 Base excision repair in mammals

The history of suggested cytosine demethylation mechanisms in mammals is more distinct from that of plants.

The first proposal included the activity of a ribozyme or ribozyme-like enzyme. The ribozyme theory was later abandoned. However, an RNA-dependent enzyme was later discovered. This enzyme turned out to be a 5mC glycosylase and was named thymine DNA glycosylase (TDG). Its function in active DNA demethylation was later doubted because of its inefficiency, but it is still part in some theories. (JOST, 1993; CORTÁZAR & al., 2007)

Another protein, called methyl binding domain 2 (MBD2), was discovered soon after the first RNA involving hypothesis. However, the suggested reaction with 5mC turned out to be energetically unfavourable. In vitro experiments with this protein turned out to be irreproducible and furthermore knockout experiments on mice did not reveal altered phenotypes or significantly different methylation patterns from control animals. A related protein, MBD4, however, is still part of theories on mammalian DNA demethylation. (BHATTACHARYA & al., 1999; JIRICNY & MENIGATTI, 2008)

A recent experiment on Xenopus laevis (African clawed frog) oocytes examined the impact of growth arrest and DNA-damage-inducible protein 45 α (Gadd45α) on DNA demethylation. (BARRETO & al., 2007) Gadd45α is a protein that is located inside the cell nucleus and involved in DNA repair, genomic stability and suppression of cell growth. This experiment revealed that Gadd45α apparently causes global cytosine demethylation when overexpressed and global hypermethylation when knocked out. The suggested mechanism includes an endonuclease involved in nucleotide excision repair termed xeroderma pigmentosum complementation group G (XPG). However, the

results are being questioned and couldn't be reproduced in a subsequent experiment by another group. (JIN & al., 2008) The latter results are again confirmed by Gadd45α-knockout experiments on mice which yielded the result that affected animals developed normally and were viable. Moreover no suspicious methylation patterns could be identified. However, other observations showed that Gadd45α-mutant mice suffer from chromosomal instability and have an increased risk of tumour development. Therefore, this protein may still be regarded as a crucial factor for DNA methylation. (OOI & BESTOR, 2008B; JIRICNY & MENIGATTI, 2008)

The current theory of mammalian DNA demethylation basically involves three steps (see Fig. 4). The first one handles the oxidative deamination of 5mC to thymine and is currently a matter of debate concerning the involved enzymes. In the second step, base excision is carried out, however, by what enzymes and cofactors has not been clarified to date as well. The third step, though, does not cater for disputes any more. Similar to the plant mechanism, dCMP is inserted into the base vacancy and the DNA strand is therefore restored again.

Concerning the first step, experiments provided two possibilities of active enzymes. The first experiments have been conducted very recently on genes targeted by the estrogen receptor α (ERα). The hypothesis derived from the results claims that the methyl transferring enzymes DNMT3A and DNMT3B also have demethylating capacities if SAM concentrations are low. In this case, the more stable enamine form (see according chapter) of cytosine or 5mC, respectively, after enzyme binding would destabilize the amino group at position C4 and increase the rate of oxidative deamination. Thus, C would be converted to U and 5mC to T. (KANGASPESKA & al., 2008) The main concern of this hypothesis is that the kinetics of SAM concentrations in vivo is too much a factor of insecurity to rely on experimental results out of cell nuclear conditions.

In the other experiment, likewise conducted only very recently, methylated DNA fragments or plasmids were injected into Danio rerio (zebrafish) embryos (RAI & al., 2008). This contrary hypothesis claims that the deamination step is carried out by either activation-induced deaminase (AID) or apolipoprotein B RNA-editing catalytic component-1 (Apobec-1) or even both of

them. Gadd45α has been reported a cofactor for the deamination step. Here as well concern has been raised, especially pertaining to AID, that both enzymes are efficient only when their respective substrates are single stranded.

The second step might involve either TDG or MBD4, both enzymes with glycosylase activity (see Fig. 4). However, the activity of TDG has already been asserted to be too inefficient to justify further research. Again, Gadd45α seems to play an enhancing role on MBD4 activity. And once again, unsteadiness for MBD4 has been stated. Knock-out results on mice did not produce any observable phenotypical degradation after birth, meaning that other factors are also involved in the glycosylase step. (RAI & al., 2008)

In regard of the potentially drastic consequences of even minor changes in physiological methylation patterns, it may be assumed that there are still more factors involved in DNA demethylation that can quench the drop-out of one of the reported enzymes. (JIRICNY & MENIGATTI, 2008; OOI & BESTOR, 2008B)

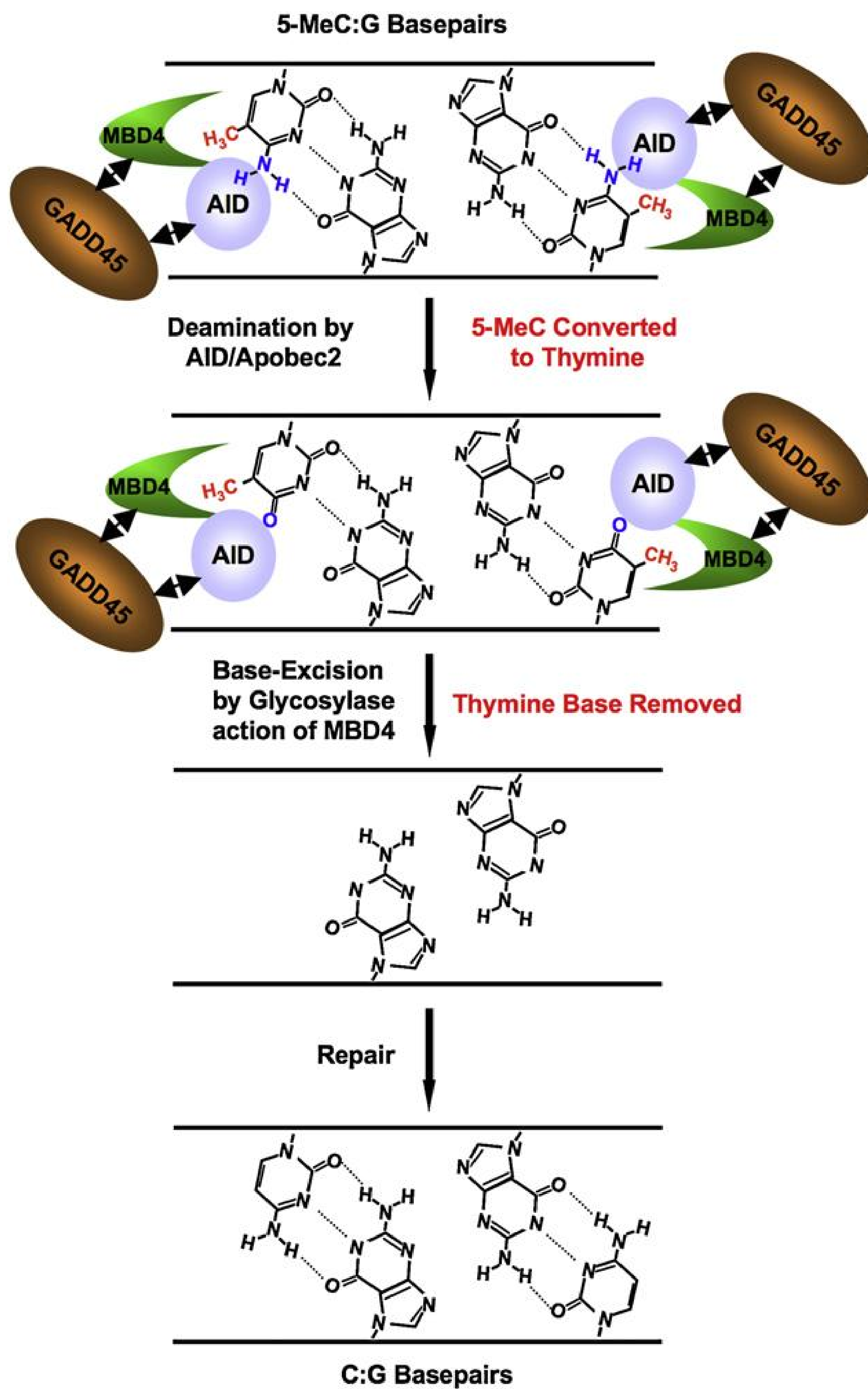

Fig. 4: Demethylation scheme

Reaction steps of the enzymatic cytosine excision, including cofactors; for explanations, see text (RAI & al., 2008)

4 METHYL DONATORS

4.1 SAM

S-Adenosyl-L-Methionine (SAM) (see Fig. 5) is, among other biochemical pathways, the cofactor for eukaryotic methyl transferases. For carrying out its effect, it is located at a specific cofactor-binding domain of the respective methyl transferring enzyme (see according chapter).

SAM is synthesized by methionine adenosyl transferase which connects adenosyl triphosphate (ATP) to the amino acid methionine (see below). After the addition of the adenosyl moiety of ATP to the thiole group of methionine, the methyl group that is bound to the same thiole group becomes chemically more reactive and can serve as the donor methyl group for various substrates including cytosine.

After delivering the methyl group to the according substrate, the resulting product S-adenosyl-L-homocysteine (SAH) is hydrolysed by S-adenosyl homocysteine hydrolase. Homocysteine is again oxidized to methionine by homocysteine methyl transferase and various methyl donators. Together with ATP SAM is regenerated. (ELMADFA & LEITZMANN, 2004)

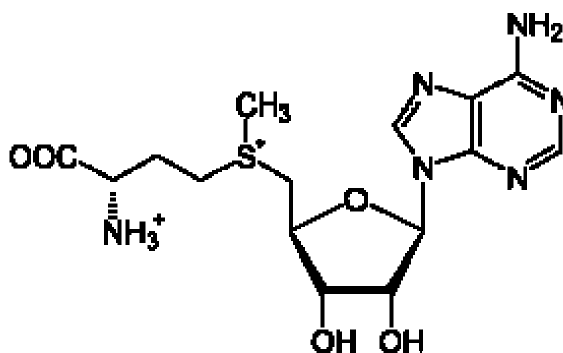

Fig. 5: SAM

Chemical structure of S-Adenosyl-L-Methionine (SAM)

4.2 Methionine

Methionine (see Fig. 6) is one of eight essential amino acids, meaning the human organism cannot synthesize it by himself and has to take it up via nutrition. Considerable amounts are found in meat and fish, eggs, plant seeds, some nuts and grain products. It contains a thiol group at position 5 with a methyl group instead of an H atom. The transferability of this methyl group makes methionine an important cofactor in various methylation processes.

Methionine can be synthesized only by plants and microorganisms. In various steps, aspartate is converted to homoserine to which succinate is added. Then succinate is removed and exchanged by cysteine, forming cystathionine. The latter is converted to homocysteine. Eventually, a methyl group from e. g. methyl tetrahydrofolate (MTHF) (see below) or cobalamin (see below), respectively, is transferred to homocysteine, completing the biosynthesis of methionine. Mammals and other animals are capable only of the last step.

Methionine deficiency leads to fatty liver in rats and other symptoms. (ELMADFA & LEITZMANN, 2004)

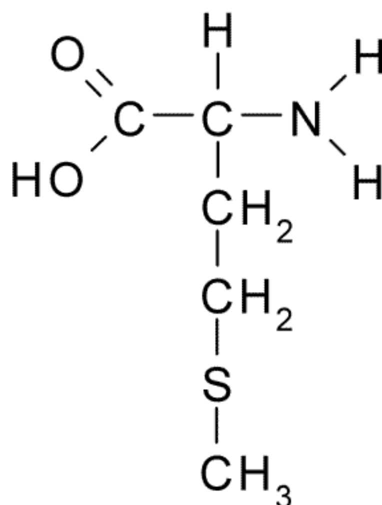

Fig. 6: Methionine

Chemical structure of L-Methionine

4.3 Folic acid

Folate is a vitamin and essential cofactor in biochemical processes that require the transfer of methyl groups. Considerable amounts of folate are commonly found in green vegetables, some fruits, yeast, liver, kidneys and eggs. Intestinal microorganisms contribute to the folate supply in humans to a certain degree.

The correct name is pteroyl mono- or polyglutamate. Folate consists of a pteridine ring, a p-amino-benzoic acid ring and one or up to eight glutamate moieties. The biologically active form is tetrahydrofolate (THF), whereas the positions 5, 6, 7 and 8 contain an H-atom and the double bonds between these positions are resolved (see Fig. 7). The enzymes capable of this reduction are 7,8-dihydrofolate reductase and 5,6,7,8-tetrahydrofolate reductase. A one-carbon group can be added to the positions 5 and 10 by 5,10-MTHF synthase, forming 5,10-methylene tetrahydrofolate (5,10-MTHF). The methylene group can be reduced to a methyl group on position 5 by 5,10-MTHF reductase, whereas the result is 5-methyl tetrahydrofolate (5-MTHF). Both, 5,10-MTHF and 5-MTHF, can serve as methyl donators for various biochemical pathways, including methionine and SAM regeneration (see above). Methyl donators for ‘activating’ one-carbon THF are serine, glycine, histidine and formiate.

Lack of folate leads to elevated homocysteine levels, indicating that it is associated with methionine regeneration. Moreover, since THF is involved in purine and DNA synthesis, cell division is slowed down. The consequence becomes visible by anemia, because erythrocytes are among the cell types with the fastest reproduction rate. Other deficiency symptoms include general signs of exhaustion, depression, behavioral disorders and especially neural tube defects in case of deficiency during pregnancy. In other fast replicating tissues, e. g. intestinal tissues, lack of folate seems to be involved in tumourigenesis as well. (ELMADFA & LEITZMANN, 2004; BOLLHEIMER & al., 2005)

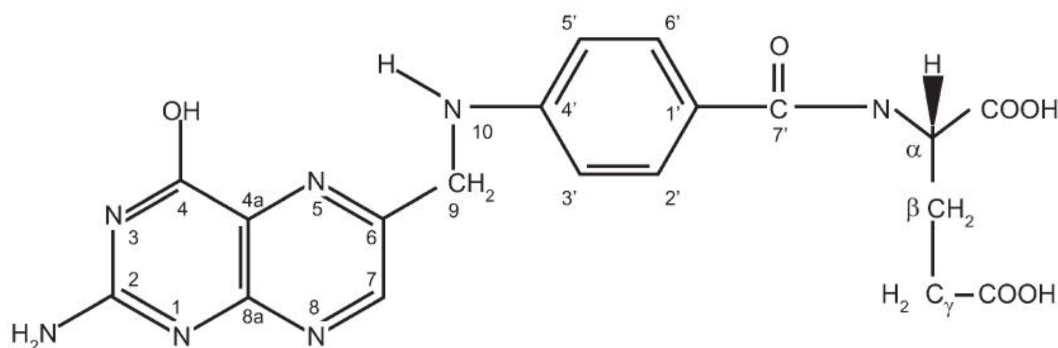

Fig. 7: Folate

Chemical structure of non-hydrated folate with the designated positions for hydrogenation (HART & al., 2006)

4.4 Cobalamin

The second vitamin playing a noteworthy role in methyl metabolism is vitamin B12 or cobalamin (see Fig. 8). Cobalamin is a macromolecule with several chemical functions. The striking structure element is a corrin-ring with a covalently bound cobalt ion in the centre. This cobalt ion additionally connects the 5,6-dimethylbenzimidazol-ribonucleotide to the corrin ring and also binds the functionally characteristic moiety. Among others, this can be a methyl group or a 5'-deoxyadenosyl group, both providing co-enzyme function in the human organism.

Cobalamin can be synthesized only by microorganisms. Hence, nutritional sources are mostly yeast, eggs, meat and dairy products.

5'-deoxyadenosyl-cobalamin plays a role in the degradation of odd-numbered fatty acids and some amino acids by serving as a co-enzyme for methylmalonyl CoA-mutase. Methyl-cobalamin is the physiological methyl donator for the regeneration of methionine by methionine synthase. The connection with THF is that the methyl group from 5-MTHF is transferred to cobalamin, providing cobalamin with its methyl donating function and, on the other hand, empowering THF to travel across cell membranes.

Lack of cobalamin, due to low nutritional amounts or intestinal malabsorption, leads to elevated homocysteine levels, megaloblastose and anemia, degeneration of several regions of the spinal cord and other general symptoms. The consequences of deficiency are rather similar to those of folate deficiency, since the methyl donating function of cobalamin affects rapidly replicating cells as well. (ELMADFA & LEITZMANN, 2004)

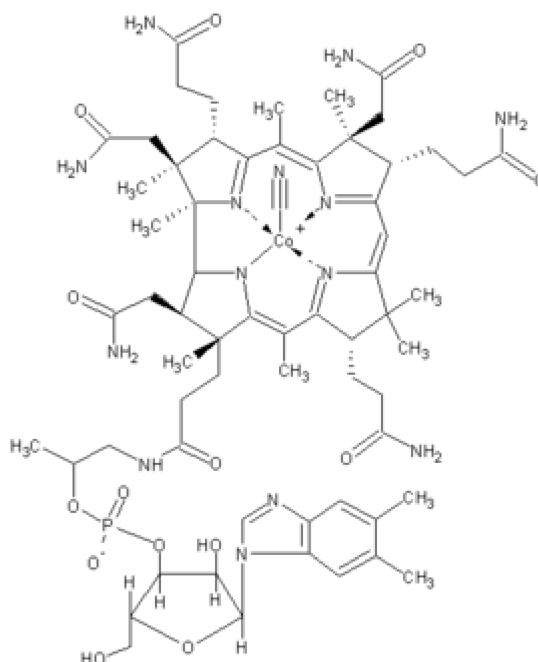

Fig. 8: Cobalamin

Chemical structure of Vitamin B12 / Cobalamin

4.5 Choline

Another notable methyl donator in human nutrition is choline, or N,N,N-trimethyl ethanolamine, respectively (see Fig. 10). The main biological functions next to providing methyl groups are the formation of phospholipids for increased cell membrane flexibility and the synthesis of the neurotransmitter acetylcholine. Although choline has once been classified as a B-vitamin, it is no longer regarded as an essential nutrient, because it can be synthesized by decarboxylation of serine. Among the foods containing considerable amounts of

choline are liver, meat, peanuts, whole grain products, potatoes, coffee and some fruits and vegetables.

Choline itself does not serve as a methyl donator. It rather has to be oxidized to betaine, or N,N,N-trimethyl glycine, respectively, by the mitochondria of liver and kidney cells. The enzyme betaine-homocysteine-methyltransferase constitutes an alternative pathway for methionine regeneration next to MTHF (see above).

To date no well-defined symptoms for choline deficiency have been described, however, evidence suggests that it might lead to growth arrest, infertility, disruption of liver function, muscle weakness and probably even cancer due to aberrations in DNA methylation in animals. Potential symptoms in humans range from neural tube defects to fatty liver, hepatosteatosis and muscle cell damage. (ELMADFA & LEITZMANN, 2004; ZEISEL, 2008)

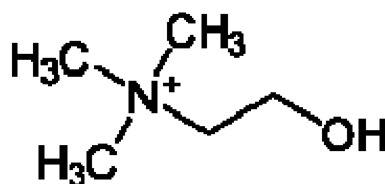

Fig. 9: Choline

Chemical structure of N,N,N-trimethyl ethanolamine / choline

5 COMPLEX PHENOMENA FEATURING CYTOSINE METHYLATION

5.1 Genomic imprinting

Genomic imprinting is a phenomenon that involves DNA methylation. Hereby one allele of a gene is subject to shutdown, whereas the other allele is regularly expressed. For most genes both alleles are expressed. However, in some cases there needs to be an expression of either the maternal or the paternal allele to warrant normal cell metabolism. Recent estimations for mammalian genes go up to 600 genes susceptible to imprinting of which more than 80 have been identified so far and of which about one third are shared by humans and mice. (LUEDI & al., 2005) Those genes are functionally haploid, because only one copy can be expressed in a cell. The pattern of gene imprinting varies between species, but also between different tissues of the same organism. Thus, imprinting plays a role in adult tissue metabolism and embryonic development, as well as in certain diseases like cancer and inborn disorders. The latter cases are characterized by an abnormal expression pattern of a specific gene due to imprinting of both alleles or loss of imprinting (LOI) on both alleles. (DOLINOY & al., 2007; KINOSHITA & al., 2008)

The first hypothesis for gene imprinting was created in the 1980s after the observation that diploid cells derived from two male and two female pronuclei, respectively, had not shown regular growth. (BARTON & al., 1984)

One theory behind the function of gene imprinting claims that there is an evolutionary reason. That is the growth control of embryonic tissue. (HAIG & GRAHAM, 1991) This theory is based on the imprint status of two genes. The first is the gene encoding IGF-2, the other is the gene encoding the receptor for mannose-6-phosphate (M6P) and IGF-2. IGF-2 is an essential growth factor for embryonic development, but it is also produced in adult tissues. Normally, the

expression is monoallelic, with the exception of the adult liver, where expression is biallelic. The receptor binds both M6P and IGF-2, but is not located on the cell surface, but rather on the surface of cellular compartments for degradation of proteins. M6P/IGF-2 binds and transports IGF-2 and phosphomannosylated glycoproteins to the lysosomes, where they are dismantled. The *IGF-2* gene is imprinted and thus repressed on the maternal allele, whereas in connection with the *M6P/IGF-2R* gene the paternal allele is affected by imprinting. These observations support the idea that the paternal alleles of a gene are responsible for growth and the maternal alleles seek to suppress or control growth. This status is typical for humans and remains in most tissues, except for the liver whose cells express the *IGF-2* gene on both alleles. In contrast, rats and mice express both alleles in most tissues. Interestingly, in many experimental and human tumour cells the *IGF-2* gene is overexpressed because of LOI. This overexpression might be one explanation for the uncontrolled growth of tumour cells and for the higher susceptibility of rodents to cancer in comparison to humans. (BARLOW & al., 1991; JIRTLE, 1999)

Another theory suggests that imprinting protects a cell from noxious DNA sequences, like transposons or viral DNA. (SUZUKI & al., 2007)

5.1.1 Molecular control of genomic imprinting

The exact molecular mechanisms are not known to date. One important observation, however, is the dissimilar handling of imprinted genes in male and female gametes. During gametogenesis, or more exactly during the development of primordial germ cells, DNA methylation marks are erased in both sexes, but in female gametes these deletions are reversed, though this seems not to be the case in male gametes. The point of a new establishment of imprinting marks is not exactly known, although evidence suggests that it occurs after the fusion of male and female gametes during the early phases of embryonal development. (PULFORD & al., 1999)

In male primordial germ cells, some exceptions have been highlighted. It is known that next to several repetitive sequences, like the intracisternal A particle (IAP), imprinted genes are not affected. The protein Stella which is

expressed at high levels in primordial germ cells appears to somehow protect methylated CpG islands from active demethylation (see according chapter). It is hypothesized that Stella is transported to the according methylated regions and protects them from the demethylation machinery. After fertilisation the marks of imprinting can be undone, depending on the type of cell. At this stage as well, Stella play a role in protecting methylated CpG islands from losing their methylation marks. (NAKAMURA & al., 2007)

Imprinted genes usually appear in clusters that are regulated by imprinting control regions (ICR). (VERONA & al., 2003) The activity of ICRs themselves is regulated by DNA and histone modifications. Mutations and aberrant methylation can lead to LOI of genes within the cluster. The result that eventually leads to disease is either the loss of gene expression on one of the alleles or biallelic, therefore causing overexpression of genes that code for growth factors or oncogenes.

This model can be exemplified by the *H19/IGF-2* cluster (THORVALDSEN & al., 1998) which contains an ICR between the two genes (see Fig. 10). The function of *IGF-2* has been mentioned above and the function of *H19* is the expression of non coding RNA with oncogenic attributes. Both genes are important during embryonic development and are downregulated in differentiated tissues. The DNA sequence of the cluster is different between human and mouse which most of the experimental data are derived from, but the functionality is rather similar, thus it is transferred from mouse models to human. At the 5' end of the cluster, following *H19*, resides an enhancer region which interacts with the promotor of either gene, depending on the methylation status of the ICR. Normally the ICR of the allele delivered by the male germ cell is methylated causing the enhancer region to interact with the *IGF-2* promoter. In contrast, the ICR of the allele from the female germ cell is unmethylated and here the enhancer region interacts with the *H19* promoter. Experimental data showed that an insulator called CCCTC-binding factor (CTCF) can bind to the unmethylated ICR. An insulator is a protein that binds to functional DNA sequences or also to chromatin proteins and therefore inhibits their capacities. A remarkable difference between the mouse and human ICR of this cluster is

the number of CTCF-binding sites. While the mouse ICR contains four such sites, seven have been identified in the human ICR. (IDERABDULLAH & al., 2008) The consequences of deletions within the human ICR are explained below.

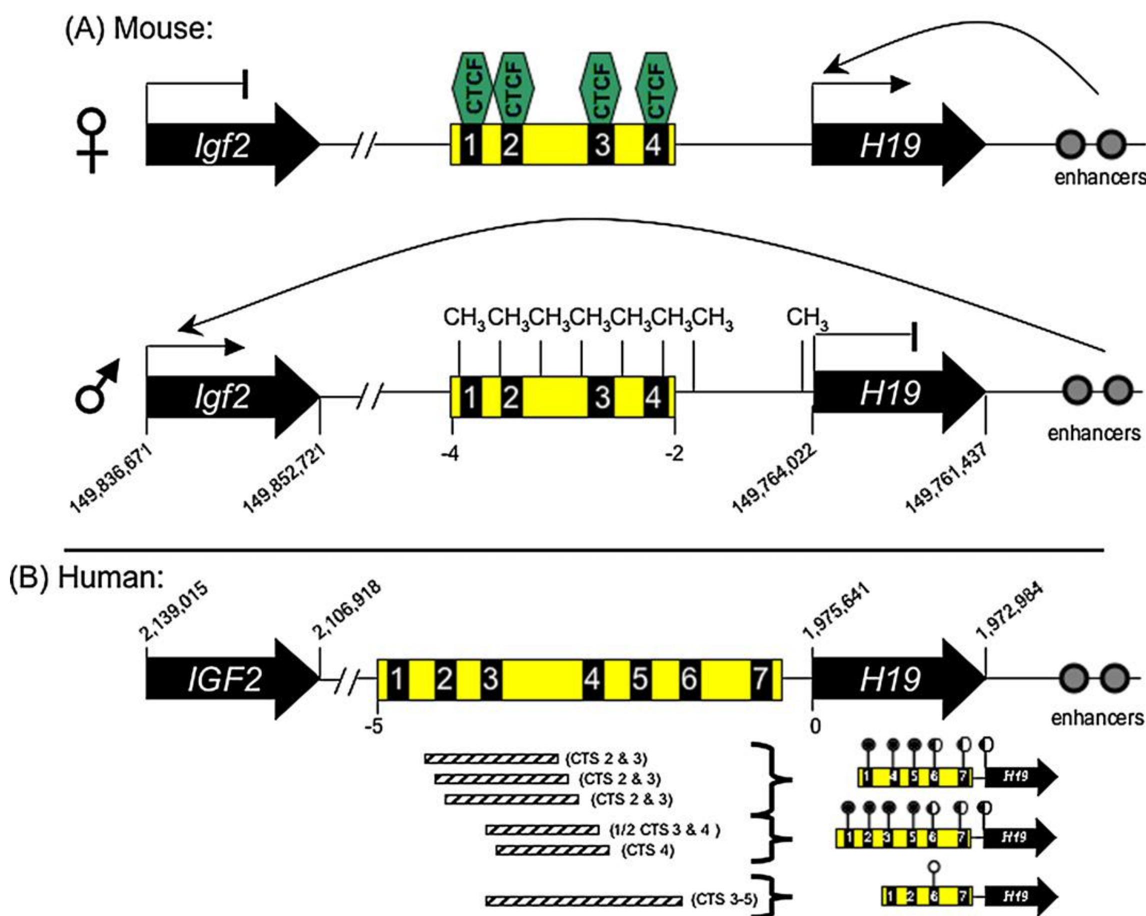

Fig. 10: H19/IGF-2 *imprinting*

Schematic overview on the impact of different methylation states in the ICR of the H19/IGF-2 *cluster; for explanations, see text (IDEERABDULLAH & al., 2008)*

5.1.2 Consequences of aberrant imprints

The molecular mechanisms of these events have, however, not been exactly clarified. On the paternal allele there appears to be physical interaction between the enhancer and the *IGF-2* promoter. It is assumed that on the

maternal allele there is some change of chromosomal conformation as well, but experimental data are contradicting. Next to several types of tumours two inborn diseases are related to genetic or epigenetic irregularities of the *H19/IGF-2* cluster. These are the Beckwith-Wiedemann-Syndrome (BWS) and the Silver-Russel-Syndrome (SRS). BWS includes several severe birth defects and affected children have an increased risk of childhood cancer. SRS is a dwarfism growth disorder with a number of additional conditions. Because of the mentioned oncogenic features of the *H19* transcript and the function as a growth factor of IGF-2 it seems reasonable that defective imprinting of the cluster leads to either undergrowth in case of loss of expression or overgrowth disorders in case of biallelic overexpression. The exact mechanisms have not been identified yet. However, LOI caused by microdeletions or aberrant methylation of the ICR seems to be involved. The ICR of BWS patients is hypermethylated, whereas the one of SRS patients is hypomethylated, both leading to LOI on the cluster and pathologic gene expression. Aberrant methylation can occur due to microdeletions inside the ICR sequence that affect the CTCF binding sites, as shown in BWS patients. However, not all such deletions implicitly lead to the disease. For example a larger deletion which eliminates the binding sites 3 to 5 does not lead to aberrant methylation on the maternal allele. The reason for this is not known, but there are two possible explanations. One is that the deletion leads to an ICR sequence similar to the full-length version, the other states that unidentified regulatory factors play a role which would be affected by shorter deletions. (KANDURI & al., 2002; BLIEK & al., 2006; CERRATO & al., 2008)

A disease that is strongly related to the imprint status of genes is cancer. In this case LOI is generally related to either expression of physiologically silent alleles of imprinted growth factor genes or oncogenes or silencing of physiologically active alleles of imprinted tumour suppressor genes. IGF-2 appears to play a role here as well. Wilm’s tumour for example is a kidney tumour that affects children who suffer from BWS caused by errors in the *H19/IGF-2* cluster. A biallelic expression of *IGF-2* is stated to be responsible for the typically enlarged kidneys of the patients and the tumour. Other types of

cancer related to *IGF-2* overexpression are lung cancer, breast cancer, ovarian cancer and glioma. However, not only *IGF-2* overexpression, but also *M6P/IGF-*2R underexpression is likely to play a role in cancer. In several tumours this receptor gene was downregulated by abnormal imprinting status, which likely amplifies the effect of *IGF-2* overexpression. (KANEDA & FEINBERG, 2005; IDERABDULLAH & al., 2008)

LOI in the *DLK1/GTL2* gene is related to Wilm's tumour as well. Other genes for which LOI is involved in cancer development are *ARHI* and *PEG1* and more are expected to be identified by upcoming research. (FEINBERG, 2007)

5.2 Metastable epialleles

The term of metastable epialleles describes a phenomenon similar to genomic imprinting, yet in some aspects different enough to be called a condition on its own. Metastable epialleles are a property of mammalian genetics and constitute a remarkable link between nutrition and other environmental influences to gene expression. While the epiallele by itself describes an allele that can exist in a different methylation state than its counterpart and therefore should be categorized within genomic imprinting (see according chapter), a metastable epiallele is more flexible in its methylation state. (RAKYAN & al., 2002) Whether it is silenced by hypermethylation or normally expressed is determined either by chance or by the provision of methyl donating nutrients, e. g. folic acid or choline, or hormones, e. g. genisteine, during embryonal growth and early development. Either way, mitotic inheritance is generally given. Furthermore, some of the best known metastable epialleles are considered 'infected' with transposable DNA elements, mostly IAP, but also L1. (DOLINOY & al., 2007)

Different methylation states of metastable epialleles lead to extensively or partly altered phenotypes in genetically identical organisms. This latter phenotypical occurrence is referred to as mosaicism or variegation and can express itself in e. g. mottled coat colour or kinked tails in mice. However, a difference has to be made from the term position effect variegation (PEV). This

phenomenon is not limited to mammals and was first discovered in D. melanogaster and A. thaliana. It describes the phenotypical effects that occur when normally active genes are translocated near heterochromatic regions and thus silenced. Heterochromatin means a rather tightly packed series of nucleosomes (see introduction) whose DNA sequence is physically silenced by its inaccessibility for the transcription machinery. PEV can also affect a complete organism or embody itself as mosaicism. (GREWAL & ELGIN, 2002)

Another difference has to be made from genomic imprinting (see accoding chapter). While in the latter case it is determined whether the maternally or the paternally inherited allele is silenced, the consequences of the methylation state of a metastable allele is not dependent on the parental origin. Moreover, both alleles might be affected by silencing via CpG island methylation without immediately making development unfeasible. It should be mentioned as well that, as noted, genomic imprinting marks are set during gametogenesis and fertilisation, whereas metastable epialleles appear to be created during early embryonal growth. (WEICHMAN & CHAILLET, 1997; SUTHERLAND & al., 2000)

The question which allele is dominating can by now not be answered uniformly. Since the methylation state can repress an otherwise dominant allele and therefore excludes the assumption of the traditional dominant-recessive classification, the term ‘semi-dominant’ seems appropriate, yet should not be taken too literally. (RAKYAN & al., 2002) As follows, some examples of genes with metastable epialleles are described.

5.2.1 A^{vy} epiallele

Probably the most popular metastable epiallele is the murine *Agouti* gene. Its name refers to the coat colour of the wild type rodent species agouti which is light to dark brown. A variant of this gene is also found in mice. Laboratory mice with yellow fur-colour combined with a high incidence of overweight, insuline resistance and tumour development compared to control animals have been observed. It was later found out that a mutation in the *Agouti* gene was responsible for the altered phenotype and disease susceptibility and

therefore the altered gene was termed *Agouti viable yellow* gene (A^{vy}). Notably, one allele or both can be affected. (MORGAN & al., 1999)

The normal variant in mice encodes an endocrine signal protein that causes melanocytes to produce the pigment protein eumelanin. This pigment is responsible for black to brown skin, hair and fur colour. In contrast, the altered variant in mice encodes a different protein commanding melanocytes to synthesize phaeomelanin, a different pigment protein which causes blonde to yellow colour and pigmentation. Furthermore, this gene variant leads to the noted physiological disadvantages in some way. (DUHL & al., 1994)

The noteworthy aspect at this point is that A^{vy} contains an IAP transposon insertion next to its upstream transcription start site. A cryptic promoter inside the IAP insertion leads to the expression of an altered protein und eventually to different fur colour in mice. (MILTENBERGER & al., 1997; MORGAN & al., 1999) An animal homozygous for the unchanged *A* allele exhibits a regular phenotype, whereas A^{vy} is the dominant allele, thus heterozygous animals show the yellow coloured phenotype (see Fig. 11).

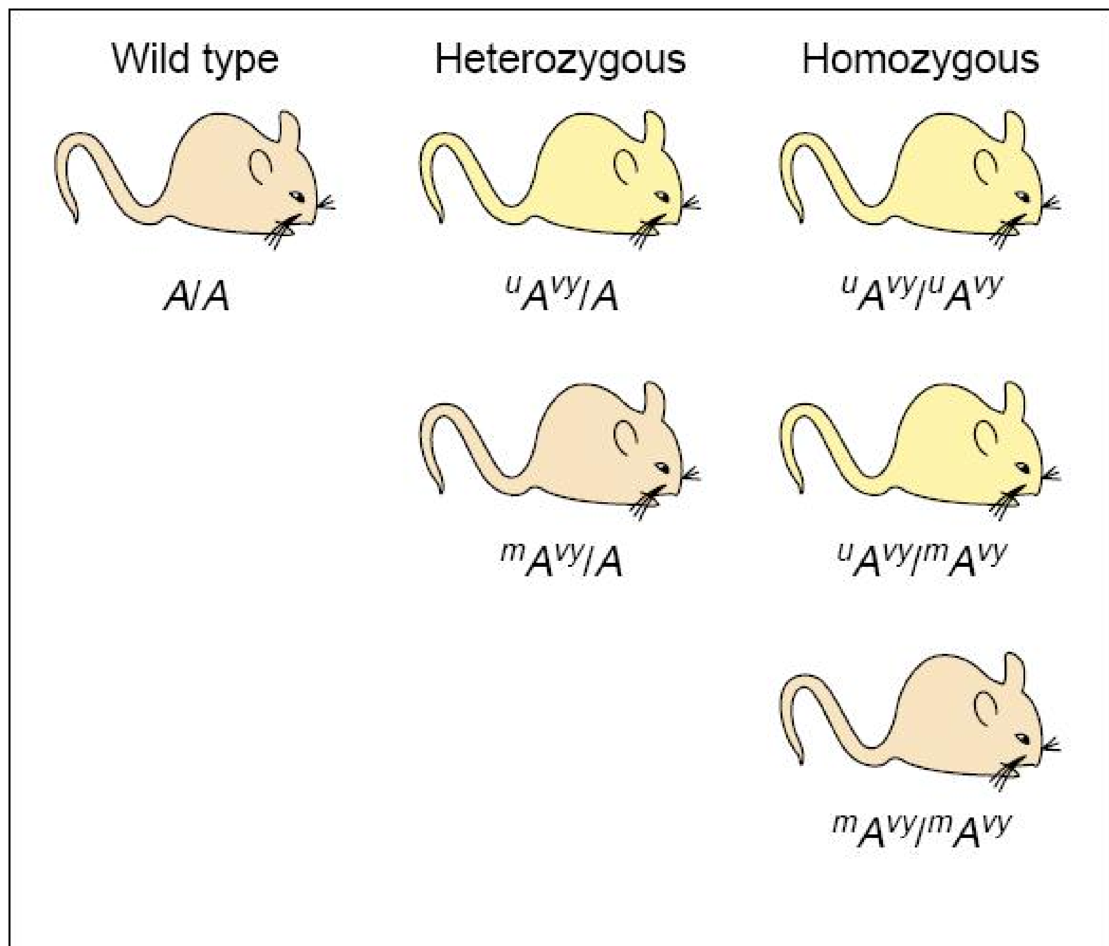

Fig. 11: Methylation states of the A^{vy} *allele*

Schematic overview of phenotypical traits of A^{vy} *mice depending on the methylation state of the affected allele; for explanations, see text (RAKYAN & al., 2002)*

However, the issue is not handled with that yet. As noted, transposable elements belong to the group of DNA sequences that are subject to repression by pronounced CpG island methylation. In vivo feeding studies and genetic examinations on Agouti-mice revealed interesting results on the offspring. Pregnant animals were fed with diets containing different amounts of methyl group donators (see according chapter), which lead to corresponding results visible at the offspring. High dosages of nutritional methyl donators or methylation-inducing hormones led to a phenotype which was superficially not distinguishable from wild type mice. Moreover, the described physiological

disadvantages fell away. These offspring were therefore termed ‘pseudoagouti’ (see Fig. 12). Lower dosages led to mottled fur colour. Concurrently, it was found out that CpG island methylation of the IAP insertion was responsible for the altered condition. Moreover, it was shown that mice homozygous for the A^{vy} allele exhibited the regular yellow phenotype when one allele was methylated. Contrary, if both alleles were methylated, the animals would exhibit the pseudoagouti phenotype (see Fig. 11). Interestingly, methylation of the noxious sequence seems to shut down the gene, leading to the expression of the *A* allele in heterozygous animals. (MORGAN & al., 1999; DAY & al., 2002; NAAZ & al., 2003)

Fig. 12: A^{vy} *and ‘pseudoagouti’ mice*

Results of feeding experiments demonstrate that the left mouse shows the characteristic yellow-coloured phenotype based on the A^{vy} *allele, whereas the ‘pseudoagouti’ offspring lost these traits due to high methylation of this allele; for explanations see text (DOLINOY & al., 2007)*

A definite milestone in epigenetic research was an observation made in connection with metastable epialleles and especially with the A^{vy} variant. The distinct methylation patterns are inherited transgenerationally, i. e. pseudoagouti mothers deliver the same phenotypical traits to their offspring. The reason, like in conjunction with the phenomenon of genomic imprinting (see according chapter), was stated to be ineffective demethylation during the formation of gametes. Therefore, it was hypothesized that changes in the methylation pattern that an organism had obtained in early development could affect not only its own gene expression, but also that of its progeny. (RAKYAN & BECK, 2006)

5.2.2 $Axin^{Fu}$ epiallele

Another known gene with metastable epialleles is the murine *Axin* gene and its epigenetic variation $Axin^{Fu}$. The latter is again originated in the insertion of an IAP sequence, however, in contrast to the A^{vy} allele this insertion is not located near the promoter, but within intron 6 of the original gene. Thus, the cryptic promoter induces the creation of an abbreviated protein with lower or without proper function. *Axin* encodes a protein that inhibits the WnT pathway. This latter biochemical network is indispensable for embryonal growth. Targeted inhibition causes embryonal tissue to develop axes, therefore the name *Axin.* The decrease of function associated with the $Axin^{Fu}$ mutation brings about kinks in the tails of affected mice. Observations concerning the methylation status of the insertion revealed similar results as with the A^{vy} allele. A high degree of CpG island methylation leads to a more wild type-resembling phenotype and the metastability can be meiotically inherited. (VASICEK & al., 2004; WATERLAND & al., 2006)

5.2.3 $Cabp^{IAP}$ epiallele

A bioinformatics approach recently yielded another IAP insertion in a gene that would be specific for the mouse strain C57BL/6J . This gene codes

for the CDK5 activator binding protein (Cabp). The mutated gene was termed *Cabp*^{IAP}. The specialty of this case is that instead of one functional transcript of about 2 kB, a number of shorter transcripts are created. Starting from the original promoter at the 5' end, the non-functional transcripts are terminated shortly before the cryptic promoter of the IAP sequence. Beginning from the 5' end of the new promoter, another RNA sequence is transcribed. Thus the *Cabp*^{IAP} allele is the first with an upstream and downstream impact on gene transcription. (DRUKER & al., 2004)

5.2.4 Other epialleles

Other murine genes with metastable epialleles are known, e. g. *Axial defects* and *Disorganisation*. Mutations in the first cause neural tube defects and mutations of the latter bring forward skeletal abnormalities. However, it is not known if the mutations are based on the insertion of transposable elements. Genes like *BLG*, *RSVIgmyc*, or genes from the lac operon that have been transgenetically introduced into mice strains have metastable properties too. The reason is suggested to be a protective mechanism against DNA sequences that have been recognized as foreign, as is the case with repetitive sequences and transposable elements. However, variegation effects have not been closely examined for some of the above mentioned genes, nor has the question of mutation by insertion of transposable elements been answered. (RAKYAN & al., 2002)

6 COMPLEX DISEASES ASSOCIATED WITH CYTOSINE METHYLATION – CANCER

6.1 General aspects

Cancer is a complex disease and has its origins in a variety of causes and at least as many influencing factors. In industrialized countries cancer is ranked among the most prominent causes of death next to coronary heart diseases and diabetes. Some of the main factors influencing cancer are nutrition, environment, genetic background, age, sex and disease history. However, within these deeply intertwined factors, epigenetic phenomena have been hypothesized to play an essential role in development and progression of this disease.

Among epigenetic changes in cancerous diseases, irregular DNA methylation was the first to be discovered. (FEINBERG & VOGELSTEIN, 1983) The pattern of methylated CpG islands frequently differs significantly in tumour cells in comparison to somatic cells (see Fig. 13). However, tumour cells of one single type are characterized by both, hypo- and hypermethylation. This apparent contradiction is resolved by the methylation status of different DNA sequences. Then, the different tumour types also show distinct methylation patterns relative to each other. While the genomes of tumours of the gastrointestinal tract are the most hypermethylated, the genomes of sarcomas and ovarian cancers exhibit the least hypermethylation. These findings are in contrast to a former hypothesis that cancer is solely a genetic disease. However, in the course of transcriptome-wide analyses it was shown that CpG island hypermethylation within gene promoters occur in around 5 percent of genes in individual tumours. This appears to be a far more drastic change in the metabolism of an affected cell than mutations in the DNA sequence. In general, gene sequencing of tumour genomes revealed fewer mutations than would

have been expected by systematic surveys. Moreover, comparative analyses of malignant glioma genomes showed that pathological CpG island methylation patterns play a more important role for tumour development than sequence mutations. Another fact that plays a role in this regard is that most known carcinogens are not genotoxic. These and other recent observations keenly point out that research on the origin of cancer and on ways of treatment cannot neglect the factors that influence the way of gene expression above the pure nucleotide sequence, i. e. epigenetic factors. (JONES & BAYLIN, 2002; HIRST & MARRA, 2008)

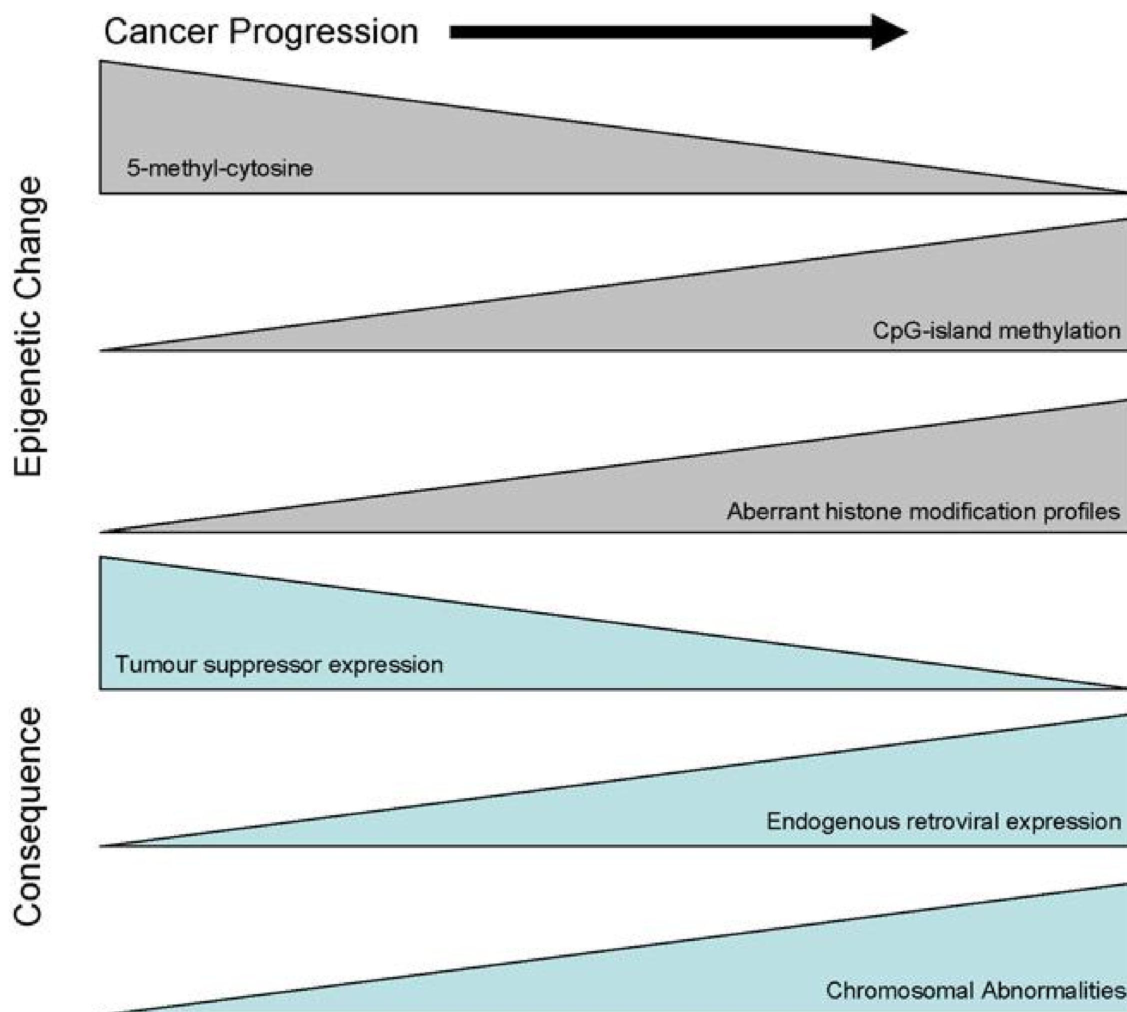

Fig. 13: Epigenetic alterations in cancer

Scheme of epigenetic alterations in cancer cells and consequences for genomic activity and stability (HIRST & MARRA, 2008)

Hypomethylation generally affects the CpG islands in promoters of genes that encode growth factors. Such genes are termed ‘oncogenes’ because of their growth promoting function that favours tumour cell proliferation when out of physiological control. A prominent example is the *IGF-2* gene which might suffer loss of imprinting on the maternal allele (see according chapter) and therefore encourage uncontrolled cell growth. (JIRTLE, 1999) Other genes that function as oncogenes in a hypomethylated state are *HRAS* in gastric cancer, the genes coding for carbonic anhydrase IV in renal cell cancer and for a calcium-binding protein in colon cancer. The *MAGE* gene family is part of a group referred to as cancer/testis (C/T) antigens. These genes are expressed normally in the testicles, but in a hypomethylated state they are also expressed in cancer cells. (WILSON & al., 2007)

Moreover, CpG island hypomethylation affects intragenic and, more importantly, extragenic repetitive regions, like transposable sequences and satellite repeats. No data is available on the interaction between the activation of transposons and tumour cell growth, but it is assumed that moving DNA segments disrupt chromosomal stability. The same may be true for the loss of satellite repeat repression, causing in some way breakage points on a chromosome, especially at the pericentromeric region. (QU & al., 1999) Other parasitic sequences than transposons are directly responsible for certain cancerous diseases. For example, the genome of the HP virus which causes cervical cancer becomes gradually hypomethylated in the course of the disease. (KIM & al., 1994)

On the other hand, hypermethylation in tumour cells generally affect the promoters of tumour suppressor genes. Common to these genes is their regulatory function for a physiological cell existence in common. They code for several kinds of receptors, cell cycle control proteins, apoptosis inducing proteins, DNA repair enzymes, enzymes with antioxidative function, but also DNA mismatch repair enzymes. Examples are *MGMT*, *GSTP-1*, *CDH-1*, *CDKN2A*, *CDKN2B*, *APC*, *VHL*, and *hMLH-1*. (ESTELLER, 2000) Hypermethylation has also been correlated to enhanced gene expression in

tumour cells, albeit not in promoter regions, but in intragenic regions. Exon 5 in the gene *PAX6* serves as an example for colon and bladder cancer. (SALEM & al., 2000)

In addition, hypermethylation affects DNA sequences coding for noncoding micro-RNA (miRNA). While some miRNAs are ascribed oncogenic functions, most seem to be involved in negative regulation of gene expression. A prominent example is the *let*-7 family. This group of miRNAs downregulates the expression of *ras* genes and *HMGA-2* which are known as oncogenes. (GUIL & ESTELLER, 2009)

Resuming, it can be said that both hypo- and hypermethylation can lead to either activation or silencing of cancer-related genes. However, current data advises not to overestimate the extent of CpG island hypermethylation. (FEINBERG & al., 2006)

6.2 The role of stem cells

A striking hypothesis concerning tumour development links uncontrolled growth not to somatic cells, but rather to stem cells. It is stated that stem or progenitor cells located in a specific tissue transform to pre-tumour cells due to epigenetic changes. Such cells have been found in normal tissues of cancer patients. An example for the relevance of DNA methylation in this context is provided by the gene *p16ink4A*. The exact function of this gene has not been clarified, however, its methylation-induced silencing prolongs the life of stem cells and might potentiate possible negative effects for tumour cell formation. Moreover, the protein encoded by the *SFRPs* gene is an antagonist to the Wnt pathway and *SFRPs* silencing causes *Wnt* to be overexpressed in stem cell populations of colorectal carcinoma samples. Together with the rather early nature of epigenetic changes, these observations support the progenitor theory. Cytosine methylation is not the only alteration made responsible for tumour development, lysine methylation of histone 3 (H3K4, H3K27) seems to play a role as well.

Concerning aberrant methylation, three example genes should be noted. These are *IGF-2*, *MLL-1* and the genes that encode the polycomb group proteins. Loss of imprinting (see according chapter) of *IGF-2* leads to an enlarged accumulation of intestinal progenitor cells and increased activity of progenitor cell-related markers in *Apc*-mutated mice before tumour development. The same observation has been made in humans. The other two genes are crucial regulators in stem cells, responsible for keeping the undifferentiated state of these cells. In tumour cells, these genes are reactivated and overexpressed, further strengthening the role of stem cells in cancer development.

Finally, nuclei of tumour cells, e. g. melanoma or medulloblastoma that have been inserted into oocytes were able to develop to blastocyst stage and even viable mice. (TSAI, 2004; FEINBERG, 2007)

These findings strongly indicate that epigenetic alterations in stem and progenitor cells are the origin in the development of cancerous diseases or at least play an important role.

6.3 Epigenetic biomarkers for cancer diagnosis

Associative markers for cancer detection include the lower methylation state of peripheral blood cells in connection with bladder cancer. The associated risk is classified as high and samples are rather easy to obtain. Epigenetic changes also seem to be involved in the H. pylori mediated mechanisms that cause gastric cancer in infected individuals. (TAHARA, 2004)

A rather novel approach for cancer diagnosis comprises the examination of the methylation status of certain genes. E. g. hypermethylation of *MGMT* or *p16ink4A* can be observed up to three years prior to the diagnosis of squamous cell lung carcinoma. *GSTP-1* hypermethylation is a suitable marker for the diagnosis of prostate cancer, because it occurs in up to 90 percent of patients. Other genes or their methylation status, respectively, like *THBS-1*, *SFRP-1* and some *cadherin* genes provide information about survival chances.

The administration of appropriate medication (see below) should lead to demethylation of cytosine residues. This effect can, among others, be observed by the activity of O(6)-methylguanine-DNA methyltransferase (MGMT). This enzyme restores guanine after alkylation. Its increased sensitivity towards alkylating agents is a sign of increased expression due to demethylation at the gene's promoter. Other markers for the success of a therapy include the activity of the base mismatch repair enzyme hMLH-1 and GSTP-1, as well as the methylation status of *BRCA-1*, a gene related to familial breast cancer. (MULERO-NAVARRO & ESTELLER, 2008; ZHU & al., 2008)

6.4 Epigenetic drugs for cancer treatment

Candidate drugs (see Fig. 14) for the treatment of cancer on the level of cytosine methylation can be divided into two classes. The first comprises cytidine analogues, the other consists of various biomolecules most of which are in use for several clinical applications. Experiments credited inhibitory effects on cytosine methylation to the latter group. All drugs are hypothesized to interfere with DNMT enzymes and thus inhibit hypermethylation and silencing of anti-proliferative genes (see above).

Two cytidine analogues are 5-aza-cytidine (5-aza-CR) and 5-aza-2'-deoxy-cytidine (5-aza-CdR or decitabine, respectively). Neither chemical is stable in aqueous solution or well bioavailable and especially 5-aza-CR is toxicologically not impeccable. These drugs have already been approved and in addition, three more cytidine analogues are currently being tested against haematological malignancies. These are dihydro-5-azacytidine (DHAC), 5-fluoro-2'-deoxy-cytidine (FCdR) and zebularine. These analogues are more stable in aqueous solution and show less toxic properties. All mentioned drugs have in common that they are incorporated into the DNA instead of regular cytosine and form a covalent and stable complex with the methyl transferring enzyme. After the methyl transfer to the C5 position of the incorporated analogue, the remaining proton cannot slide to the C6 position (see according

chapter). The thiol group of the enzyme remains bound to the C6 position and therefore the enzyme cannot be released.

Potential non-analogue drugs are procaine and procainamide, hydralazine, (-)-epigallocatechin-3-gallate (EGCG) and RG108. Procaine and procainamide, an anesthetic and anti-arrhythmic drug, respectively, are hypothesized to bind sequences with high CpG island occurrence and thus prohibit methyl transferring enzymes from binding to the cytosine position to be methylated. Hydralazine, intended as an anti-hypertensive drug, and EGCG, a herbal phenolic compound, have also shown inhibitory effects on DNA methylation. RG108 is a promising candidate because of its specificity towards the catalytic centre of DNMT1, especially in regard to hypermethylated tumour suppressor genes. Furthermore it shows little toxicity. However, the major drawback is the low solubility in aqueous solution and therefore a drastically limited applicability. (JAIN & al., 2008; MAI & ALTUCCI, 2008)

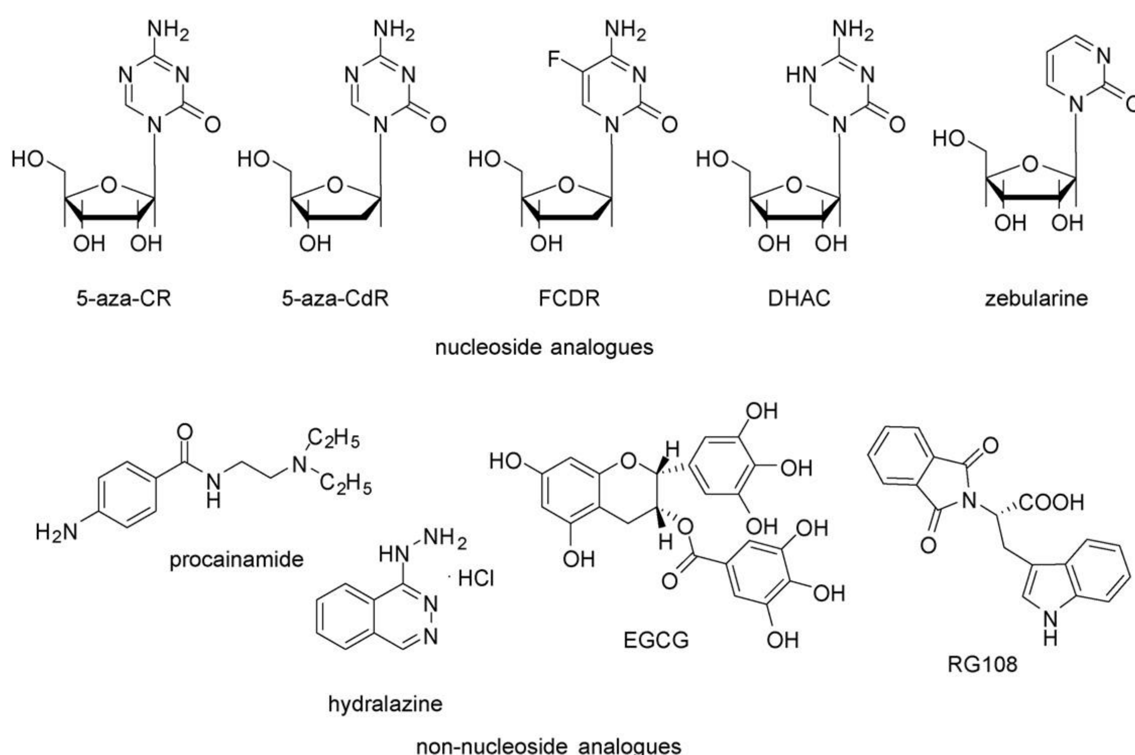

Fig. 14: DNMT inhibitors

Overview on the chemical structure of various drugs that inhibit cytosine methylation (MAI & ALTUCCI, 2008)

6.5 Cause or consequence

In this regard, the question whether epigenetic changes are cause or consequence of cancer development should be addressed. In the context of LOI, exemplified by that of *IGF-2*, the question seems plain to answer. The erroneous modification exists from early development onwards and an extremely increased risk of embryonal and childhood tumours in connection with BWS are the consequences. LOI of this gene also occurs in adults at a frequency of 5-10 percent and correlates with a fivefold increased risk of developing colorectal benignant and malignant neoplasms. Together with an associated family history of colon carcinomas that indicates heritability of this disease, these findings seem to confirm the causality of cytosine methylation for the development of cancerous diseases.

The answer to this question seems, however, not that simple. One problem is that neither mutations, nor aberrant methylation patterns have yet been identified for genes directly responsible for cytosine methylation. Diseases like the Rett- or the ICF-syndrome for which mutations occur in the MeCP2 protein (see according chapter) and DNMT3B (see according chapter), respectively, are not associated with an increased risk of cancer. Moreover, the use of 5-aza-CdR did not provide continuously stable results, indicating that epigenetic errors could be an effect, but not necessarily a cause, as the development of a tumour progresses.

Other evidence again argues for a causal relation. Next to LOI of *IGF-2*, it has been shown in breast cancer patients that the gene *p16* becomes gradually hypermethylated along with age in both, normal and cancerous tissues. Additionally, the development of T-cell lymphomas appears to be a consequence of chromosomal instability due to hypomethylation of repetitive satellite sequences.

Mice experiments further strengthen the causal role of methylation. When *DNMT1*-knockout mice are crossed with *Apc*-mutated multiple-intestinal-neoplasia (MIN) mice, the result is a higher frequency of intestinal and liver tumours and interestingly a delayed progression of adenomas. This latter

observation indicates that hypomethylation is more crucial in the early development of cancerous tissues and hypermethylation plays a more important role as tumour development progresses.

The hypothesized coherence between DNA methylation and gene mutations should be mentioned as well. As described, epigenetic changes have a negative impact on the expression and activity of DNA sequence repair enzymes. Without sufficient activity of *MGMT*, mutations of guanine to adenine occur, explicitly favouring the oncogenic or tumour suppressor activity, respectively, of members of the *Ras* and the *p53* gene family. Furthermore, radiation-induced cytosine to thymine mutations and the resulting CpG island loss favours the emergence of oncogenic mutations.

Altogether, there are reasonable arguments for a causal relation between aberrant DNA methylation and carcinogenesis. In any case, an interface between genetics and epigenetics can be assumed, whereas epigenetic aberrations seem to function as a ‘collecting pond’ for environmental influences that passes these changes on to gene expression and probably even to gene sequences, thus amplifying the origin of exuberantly growing cells. However, many more aspects remain to be illuminated to further clarify this issue. (BAYLIN & BESTOR, 2002; FEINBERG, 2007)

7 EXPERIMENTAL METHODS OF CYTOSINE METHYLATION RESEARCH – AN OVERVIEW

At the beginning of the 1980s, the first methods for analyzing the degree of cytosine (C) methylation in DNA samples have been developed. The oldest available procedure used high-performance-liquid-chromatography (HPLC), followed by the chemical conversion of nucleotides with several reagents. The temporal difference of more than 30 years between the first occurrence of these methods and the discovery of 5-methyl-cytosine (5mC) (HOTCHKISS, 1948) is surprisingly high. However, the methodology of cytosine methylation measurement has experienced impressive improvement during the last few years and is still expanding.

The methodology for determining cytosine methylation comprises a number of different techniques, but these can be split into two main groups, the global methylation analysis and the gene-specific analysis. The latter can further be divided into genome-wide and target-gene analysis. (SHEN & WATERLAND, 2007)

There are some crossovers between the two groups, for example the use of bisulphite or immunologic methods can be adopted for global and specific analysis. Both of them started as being applied in rather general experiments, but soon became part of more sequence-specific approaches. To allow for this instance, the structure of this chapter is a compromise that does not necessarily reflect experimental standard procedures. The procedural scheme below (see Fig. 15) provides a good overview about the selection of distinct procedures for the respective experimental design.

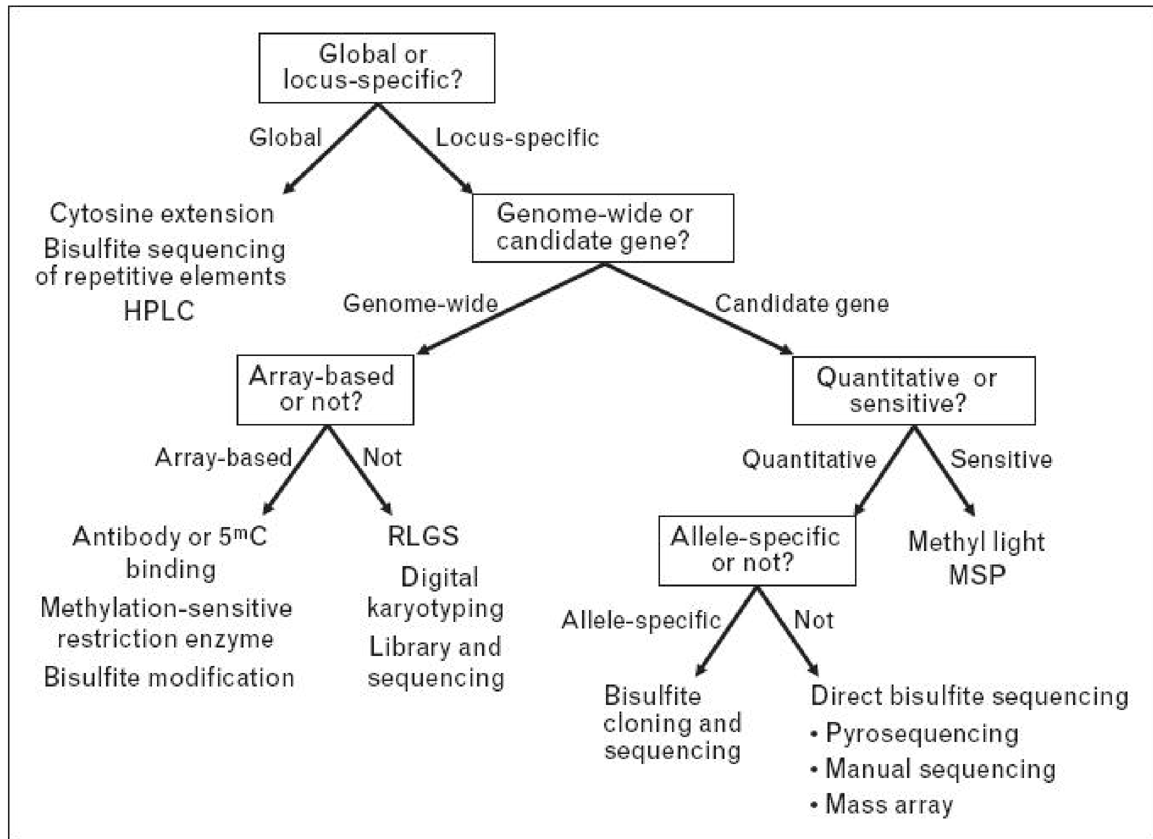

Fig. 15: Experimental decision tree

Overview of experimental methods for the assessment of DNA methylation according to the experiment's objective; the most important approaches are described in the text (SHEN & WATERLAND, 2007)

7.1 Global approaches

7.1.1 High performance liquid chromatography

HPLC-based methods for determining the 5mC content in DNA samples have been at the lead to emerge. The early versions of those applied reverse phase HPLC. The first step requires treatment of sample DNA with hydrolyzing enzymes, like DNAse I, nuclease P1 or phosphodiesterase. Afterwards, the processed sample has to be treated with alkaline phosphatase. The released deoxyribonucleotides (dNTs) are chromatographically separated and identified by UV-light detection of their specific absorbances at 254 and 280 nm. This

method has further been improved with mass spectrometric detection. (KUO & al., 1980; DEL GAUDIO & al., 1997)

The amount of DNA sample needed is less than 1 µg, making the method suitable for routine measurements, but less practical when only a small amount of cells is available. (OAKELEY, 1999)

7.1.2 Thin layer chromatography

Thin layer chromatography (TLC) makes use of the restriction enzyme MspI that cuts DNA at specific CCGG sites. The phosphate at the 5' ends of the chopped DNA can be labelled by attachment of [γ^{32}P]ATP with a polynucleotide kinase. Then the sample is hydrolyzed (see above) and applied onto a diethylaminoethyl (DEAE) cellulose plate. (KUCHINO & al., 1987) Common solvent compositions are isobutyrate : ammonia : distilled water (66 : 1 : 33) and isopropanol : HCl : distilled water (70 : 15 : 15) for the first and second dimensional separation, respectively. Parallel to the sample run, a control run with labelled C and 5mC should be carried out. The relative occurrence of the C to the 5mC spots can be determined by phosphor imaging or related methods. Additionally, a second control can be run without MspI digestion to subtract possible non-specific signals.

As with HPLC application this method is limited to qualitative measurements and requires appropriate amounts of sample. (OAKELEY, 1999)

7.1.3 SssI methyl transferase assay

The SssI methyl transferase assay is used for quantifying small changes in global DNA methylation. For this purpose, Tritium-labelled methyl groups of SAM are transmitted to unmethylated Cs at CpG sequences. After immobilisation of the DNA and washing-off of excessive SAM, the amount of incorporated CH_3-groups can be measured by scintillation counting. (SCHMITT & al., 1997)

One of the two major drawbacks of this method is the chemical instability of both SAM and SssI, typically causing large errors between times or

conductors of such an experiment. The other drawback is the challenge of completely dissolving DNA. If this is not performed correctly, the scintillation count might overlook portions of the DNA involved in the reaction. These disadvantages can be allayed by the use of internal standards and the digestion with a restriction enzyme that does not affect CpG islands, respectively. (OAKELEY, 1999)

7.1.4 Chloracetaldehyd assay

The chloracetaldehyde reaction starts with treating the sample DNA with sulphuric acid and removing the liberated purines by silver precipitation or chromatography. C is then converted to uracil by using sodium bisulphite (see below). The following reaction of 5mC with chloracetaldehyde results in an ethenocytosine derivate which is highly fluorescent.

This method contains two major flaws as well. First, it is rather time consuming, second, chloracetaldehyde is a toxic reagent. (OAKELEY & al., 1999)

7.1.5 Restriction endonuclease approaches

The use of bacterial restriction enzymes in determining DNA methylation is quite common, especially in contexts where simple and cheap methods are required. Two such enzymes, HpaII and MspI, can be used in a southern blot. These two enzymes are endonucleases and so-called isoschizomeres, meaning they cleave DNA at specific recognition sites within a given sequence and they have a different sensitivity towards the methylation status of the respective site. For both mentioned enzymes the site is CCGG. It makes no difference for MspI if the site is methylated or not, but HpaII will cleave the site only if it is unmethylated. Depending on the methylation status of the cleavage site, a southern blot reveals different bands when the activity of both enzymes is compared. Two bands of the same size mean an unmethylated sequence and a larger HpaII band means methylation.

The disadvantages of this method are its restriction to qualitative analyses, the limitation of sample numbers, the relatively large amounts of sample needed and the fact that not all methylation in a given genome occurs on the cleavage site of the respective endonuclease. (OAKELEY, 1999)

7.1.6 Immunological approaches

There are several ways of immunological application in measurements of DNA methylation. One method to determine global methylation levels uses an antibody against 5mC against which in turn another antibody is used. The second antibody is linked to fluorescein-isothiocyanate (FITC). The signal that is proportional to the amount of 5mC can be measured fluorometrically. Additional ethidium bromide staining can visualize the total amount of DNA on the plate. For immobilizing the sample DNA, a DEAE cellulose plate is recommended, however, in any case it is necessary to measure the autofluorescence of the plate. Before DNA application, the plate is recommended to be washed with a solution of 1% Triton X-100 in wash buffer. The same solution should be used to wash off excessive antibodies after incubation. (OAKELEY & al., 1997)

The major flaw of this method is the requirement of 5mC not to pair to another base. This is best accomplished by depurination (see above). (OAKELEY, 1999)

7.1.7 The bisulphite approach

One of the most prominent features of several techniques for determining the methylation status of a given DNA sequence is the chemical sodium bisulphite. In single-stranded, but not double-stranded DNA, this reagent converts C to uracil (U) with high reactivity, whereas 5mC is hardly affected. The C6 position of a pyrimidine ring becomes sulphonated and subsequently the bond of the amino group at the C4 position in C and 5mC is weakened. At a pH optimum of 5.8 both bases will deaminate, however, the conversion of C to U is finished before considerable conversion of 5mC to T has occurred. During a probably following polymerase chain reaction (PCR) of

selected sequences, U is exchanged with T. Afterwards the sequences are basically ready for sequence analysis. (HAYATSU & al., 1970; FROMMER & al., 1992; OLEK & al., 1996)

Caution has to be taken in connection with acid-catalyzed loss of purines, because this might cause considerable damage to examinable sequences. The pH conditions used in such experiments usually do not cause remarkable losses, however the incubation time with bisulphite should not exceed a few hours. DNA denaturation is best conducted by 'trapping' it in frozen agarose-gel.

The bisulphite method alone contains a number of noteworthy flaws. Bisulphite conversion may be incomplete or excessive, leading to artifacts in both cases. In addition, the complexity of a sequence is reduced because of the conversion of C to U, making the primer design more difficult. Furthermore, the C to T ratio will most likely not reflect the true 5mC to C ratio in a given sequence. Because of these caveats, combinations of the use of bisulphite with other methods have been developed. (OAKELEY, 1999; BECK & RAKYAN, 2008)

7.1.7.1 Combined bisulphite restriction analysis

One of the mentioned bisulphite modifications is the combined bisulphite restriction analysis (COBRA). For this method, bisulphite conversion and PCR amplification are first conducted (see above). The next step is the application of two restriction endonucleases. The first one must recognize CpG containing sequences as its restriction site. The second is used as a control and must recognize a restriction sequence containing C, but not CpG. This procedure serves for the determination of possible incomplete conversion or methylation at CpNpG sites. If the control is negative, the location of the cleavage sites can proceed. (XIONG & LAIRD, 1997)

This method provides more accuracy than sequencing after bisulphite reaction alone, but it is still limited to specific restriction sites and can probably not capture the complete methylome of a given sequence. (OAKELEY, 1999; SHEN & WATERLAND, 2007)

7.2 Sequence-specific approaches

7.2.1 The permanganate / hydrazine approach

The first reported chemical reactions to modify methylation-related bases involved hydrazine and permanganate. Hydrazine breaks DNA at C and T residues, whereas permanganate oxidizes 5mC and T residues. Subsequent pyridinolysis breaks the DNA at those oxidized residues. The break parts can then undergo a so-called ligation-mediated PCR for later sequencing. Therefore, a gene-specific primer and a linker that binds to the hydrazine or permanganate site of fracture are added to the sample. The DNA sequence between the primer and the linker is replicated by PCR. After sequencing, e. g. on a gel, a methylation pattern can be determined. (MAXAM & GILBERT, 1980; FRITZSCHE & al., 1987; PFEIFER & al., 1989)

It should be noted that the permanganate reaction is more variable and provides less clear information than the hydrazine reaction. (OAKELEY, 1999)

7.2.2 Methylation-sensitive single nucleotide primer extension

Another modification of bisulphite-based methods is the methylation-sensitive single nucleotide primer extension (MS-SNuPE) method. Bisulphite reaction and PCR are carried out as usual and the amplification product is purified by gel-electrophoresis. Afterwards, a specific primer is added which should attach to the amplified sequence in 5' direction immediately next to the opposite strand's C residue in question. For the following primer extension reaction, either [^{32}P]-labelled cytidine triphosphate or the equally labelled thymidine triphosphate is added. The outcome can be measured by denaturating the strand on a gel and phosphor imaging of the elongated primer. Depending on the original methylation status of the examined C residue, either the labelled C or the labelled T will show a signal. (GONZALGO & JONES, 1997)

7.2.3 Bisulphite pyrosequencing

Bisulphite pyrosequencing is an advanced technique of bisulphite conversion and PCR. Hereby, a biotinylated primer is aligned to the amplified conversion product. Alternatively, a specific primer together with a biotinylated primer can be used. The result should in any case be single-stranded DNA for the following reaction. The basis of this method is the luciferase reaction creating measurable light after processing luciferin to oxyluciferin with ATP. The production of ATP is catalyzed by the enzyme ATP sulfurylase which uses the liberated pyrophosphate (PP_i) after nucleotide adherence to the DNA strand.

The reaction chemicals next to the processed DNA sample are luciferin, luciferase, DNA-polymerase, apyrase, ATP sulfurylase and adenosine-5'-monophosphate (APS) as the substrate for ATP production. In the next step, nucleotide triphosphates are added one by one. If one of these can be added to the primer or synthesized DNA strand because of a fitting opposing base, PP_i is released and the sulfurylase-luciferase reaction is employed. The chemiluminescent output is measured and excessive nucleotide triphosphates are degraded by apyrase. This method provides a methylation pattern of the examined DNA sequence in comparison to the same sequence that has not been bisulphite-processed. (COLELLA & al., 2003)

7.2.4 Microarray-based bisulphite approaches

The first genome-wide microarray-based method involved the use of bisulphite (see above). Application of the amplified product to microarrays with original DNA probes reveals the original methylation state of a CpG island. Probe design, however, proves difficult because of the reduction of the C content of the examined sequence and it is not clear if these methods can be used on the whole genome of an organism.

Nevertheless, they have been improved in the course of their development. In one extension not only a specific sequence, but the complete sample is amplified after bisulphite reaction, raising the number of identifiable CpG islands. Another extension is the application of bisulphite-treated DNA to

microarrays with probes complementary to the converted sequence. (BECK & RAKYAN, 2008)

7.2.5 Microarray-based non-bisulphite approaches

Array-based methods without bisulphite conversion use a combination of restriction enzymes, either methylation-specific or non-specific. Because of the non-existent conversion of C to T, these methods offer a wider genomic range to be analysed. The constraint therefore is that only the restriction sequences can be analyzed, but probably not a complete genome.

An advancement of the non-bisulphite array-based method uses the affinity of some proteins to restriction sequences with methylated DNA, especially 5mC in methylated CpG islands. One of these methods is the CpG island recovery assay (MIRA) which purifies methylated sequences on a column with the respective binding proteins, e. g. MeCP2. This method is suited for identification of different methylation marks in cancer. (RAUCH & PFEIFER, 2005)

7.2.5.1 Methylated DNA immunoprecipitation / Methylcytosine immunoprecipitation

Two methods, methylated DNA immunoprecipitation (MeDIP) and methylcytosine immunoprecipitation (mCIP), make use of antibodies directed against 5mC. The precipitate is applied to a microarray. Immunoprecipitation does not show resolution below a few hundred basepairs, however, methylated CpG islands at close quarters in a given sequence can sum up to about 1000 base pairs. This and the independence from bisulphite conversion and methylation-specific restriction enzymes probably make this application the most sensitive of all array-based methods. (WEBER & al., 2005; KESHET & al., 2006)

7.2.6 Restriction landmark genome scanning

Restriction landmark genome scanning (RLGS) was the first location-specific method to be used in methylation analysis. Restriction enzymes that recognize methylated sites split up DNA samples which are then separated by two-dimensional gel-electrophoresis.

Although the sensitivity is limited because measureable effects can only be observed at 30% methylation or higher, the detection of numerous different methylation profiles at certain loci is possible. Based on this method, different methylation patterns in various normal tissues in comparison to cancerous tissues have been reported. (COSTELLO & al., 2002; SHEN & WATERLAND, 2008)

7.2.7 Methylation-specific digital karyotyping

A method similar to RLGS is the methylation-specific digital karyotyping (MSDK). Methylation-specific restriction enzymes are used as well, followed by another step of fractional digestion by a restriction enzyme. Eventually, the pieces of DNA are sequenced and mapped. Sensitivity is about as high as in RLGS. (HU & al., 2005)

7.2.8 Methylation sensitive PCR

Methylation analysis has seen the employment of a specialised version of PCR. The methylation sensitive PCR (MSP) is based on primers that bind on either methylated or unmethylated sites. This way, differences in the methylation status of paternal and maternal alleles of a gene can be determined, however, only on a qualitative basis. Real time PCR-like methods have been developed, but quantitative analysis is limited to either strongly methylated or almost unmethylated sequences. (HERMAN & al., 1996; ZESCHNIGK & al., 2004)

7.2.9 Mass spectrometric approaches

The method for general mass spectrometry (MS) is relatively simple. A DNA sample is hydrolyzed and derivatized with a reagent, mainly N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide + 1% tert-butyldimethylchlorosilane (MTBSTFA + 1%TBDMCS). The volatile derivatisation products of nucleotides can be separated and analyzed by GC-MS, whereas C and 5mC show distinct retention times and ion fragment patterns. (SAN ROMERIO & al., 2005; GLAVIN & al., 2006)

An approach that has proved useful for the determination of single nucleotide polymorphisms (SNPs) makes use of MALDI mass spectrometry and with some extensions has been presented for the analysis of general and location-specific DNA methylation patterns. The actual MALDI application comes into play in connection with the GOOD assay for SNP determination. (SAUER & al., 2000) This assay is based on primer extension (see above) for allele distinction and MALDI-MS is typically used for analysis. Together with the bisulphite method, this assay is able to determine the methylation status of various CpG positions within a given sequence, e. g. a promoter or an intron, in a relatively precise way. (TOST & al., 2003)

8 CONCLUSION

The present work provides a survey of the most important issues in connection with DNA methylation. Clearly, this topic and epigenetics in general have for the last few decades up to date been relevant research fields in molecular biology and medicine. New experimental methods have been developed and crucial results have been yielded. And yet many more results are to be expected, because the momentary knowledge on DNA methylation might cover only a diminutive part of the processes going on in a cell nucleus. In any case it can be regarded certain that the exclusive knowledge of nucleotide sequences and divergent mutations will not be sufficient to establish an adequate model for the developmental background of an organism. The same is true for the treatment of human disease. During the past few years, alterations of genetic events beyond the DNA sequence have gained considerable attention and a wave of clinical results for a new level of treatment may be expected.

Among the prospects in regard of human disease, epigenetic therapy in cancer appears to provide a most promising outlook. Several drugs have been developed, although only the fewest of them have already been permitted for clinical application. However, doctors and patients have to mind some limitations. Cancer is stated to be a highly multicausal health problem with a variety of possible origins, peculiarities and consequences. Therefore, directed interruption of methylation metabolism will most probably be only one innovative way of treatment, but not the sole cure. Even though a decent portion of hope is put into the medical treatment of such diseases, the enormous potential of a preventive lifestyle is never to be underestimated.

Moreover, epigenetic research and techniques may provide a solid base for stem cell research. The discussion of a potential role of stem cells in cancer development has been discussed (see according chapter) and more data can be expected. The isolation of adult stem cells and the retransformation

of differentiated cells to a pluripotent embryonic stem cell status will also be an interesting issue. Regulation of DNA methylation seems to play a role here as well.

Developmental records of humans and other organisms can be achieved by keeping track of epigenetic profiles and the changes that these are subject to. Therefore, insights into the epigenetic relevance of physiological and pathological phenomena like ageing, disease or the different development of twins may be looked forward to.

And last but not least, in the face of this year's 150th birthday of Darwin's famous publication "On the Origin of Species" some revelations of the evolutionary context of epigenetics and especially DNA methylation may be encouraged as well.

9 LITERATURE

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10 ABSTRACT

One of the most investigated epigenetic mechanisms is the methylation of DNA. This survey is concentrated on cytosine methylation which concerns a variety of organisms. 5-methyl-cytosine (5mC) mainly occurs in clusters of cytosine and guanine, so-called CpG islands. In plants CpNpG islands are also found, whereas N stands for any nucleotide. CpG islands can occur in a mosaic pattern or be spread across the genome. The methylation of cytosine is basically associated with gene silencing.

5mC does not exist as a ready nucleobase for DNA synthesis. Rather than that, a variety of enzymes catalyzes the transfer of a methyl group from their co-enzyme S-adenosyl-L-methionine (SAM) to a target cytosine position within the genome. These enzymes are basically divided into three families which are DNMT1, DNMT2 and DNMT3. Plants contain an additional family, termed chromomethylases (CMTs). The removal of the methylated state of cytosine is accomplished by nucleotide excision.

Several nutrients can provide the requisite methyl group. One of them is methionine itself, as a part of SAM. Others are folic acid, cobalamine and choline.

Epigenetic phenomena based on cytosine methylation are genomic imprinting and the formation of metastable epialleles. Marks of genomic imprinting are set during gametogenesis and early embryonal development. One allele of certain genes is deactivated while the other is normally expressed, depending on its parental origin. Metastable epialleles are formed during embryogenesis and fetal growth. Their formation is influenced by the nutrient composition provided by the mother organism. In general, this applies to genes containing an insertion. A high methylation of this insertion can cause considerable phenotypical and physiological alterations.

Among civilisation diseases, cancer is currently the most popular object of examination in connection with epigenetic mechanisms. Several biomarkers

and related genes have been identified and the development and clinical testing of efficient drugs is currently in progress. It is also hypothesized that degenerate stem cells play a role in cancer development. In any case there is, however, some concern about the succession of changing epigenetic phenomena and the development of a tumour. There are some arguments that epigenetic alterations may be merely consequences after a somatic cell has proceeded to its cancerous state.

An overview of the current methodology concerning DNA methylation research is given in the last chapter.

Eine der am meisten erforschten epigenetischen Mechanismen ist die Methylierung von DNA. Diese Arbeit beschränkt sich auf die Cytosin-Methylierung, von der eine Vielfalt an Organismen betroffen ist. Das hauptsächliche Vorkommen von 5-Methyl-Cytosin (5mC) besteht in Anhäufungen von Cytosin und Guanin, sogenannten CpG-Inseln. In Pflanzen findet man auch CpNpG-Inseln vor, wobei N für ein beliebiges Nukleotid steht. CpG-Inseln können mosaikartig verteilt oder über das gesamte Genom verstreut sein. Die Methylierung von Cytosin wird gemeinhin mit der Funktionsstille eines Gens in Verbindung gebracht.

5mC kommt nicht als Baustein für die DNA vor. Stattdessen katalysiert eine Vielzahl an Enzymen den Transfer einer Methylgruppe vom gemeinsamen Coenzym S-Adenosyl-L-Methionin (SAM) auf das jeweilige Ziel-Cytosin innerhalb des Genoms. Diese Enzyme werden grundsätzlich in drei Klassen eingeteilt, welche DNMT1, DNMT2, sowie DNMT3 genannt werden. Pflanzen enthalten eine zusätzliche Klasse, die sogenannten Chromomethylasen (CMTs). Die Entfernung des Methylierungsstatus erfolgt mittels Ausschnitt des ganzen Nukleotids.

Unterschiedliche Nährstoffe liefern die notwendige Methylgruppe. Einer davon ist Methionin direkt, als Bestandteil von SAM. Andere sind Folsäure, Cobalamin und Cholin.

Epigenetische Vorgänge, die auf Cytosin-Methylierung beruhen, sind genomisches ‚imprinting' und die Formierung von metastabilen Epiallelen. ‚Imprint'-Markierungen werden im Zuge der Genese von Keimzellen und im früh-embryonalen Stadium reguliert. Dabei ist ein Allel eines bestimmten Gens deaktiviert, während das andere normal exprimiert wird. Welches Allel exprimiert wird, ist abhängig von dessen elterlicher Herkunft. Metastabile Epiallele bilden sich während der Embryonal- und Fötalentwicklung. Ihre Bildung ist von der Nährstoffzusammensetzung durch den Mutterorganismus abhängig. Allgemein sind Gene mit einer Insertion betroffen. Ein hoher Methylierungsgrad dieser Insertion kann beträchtliche phänotypische und physiologische Änderungen bewirken.

Unter den Zivilisationskrankheiten wird der Krebs momentan vorrangig im Zusammenhang mit epigenetischen Mechanismen untersucht. Mehrere Biomarker und damit in Beziehung stehende Gene sind identifiziert worden und die Entwicklung und klinische Erprobung von Medikamenten schreitet voran. Allerdings bestehen in jedem Fall Bedenken bezüglich der Abfolge von sich verändernden epigenetischen Erscheinungen und der Entstehung eines Tumors. Es gibt einige Ansätze, die dafür sprechen, dass epigenetische Veränderungen nur die Folge sind, nachdem sich eine somatische Zelle zu einer Krebszelle entwickelt hat.

Das letzte Kapitel der vorliegenden Arbeit gibt einen Überblick über die aktuelle Methodik bezüglich der DNA-Methylierungsforschung.

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