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Genomic Imprinting

Genomic Imprinting—The Struggle of the Genders at the Molecular Level

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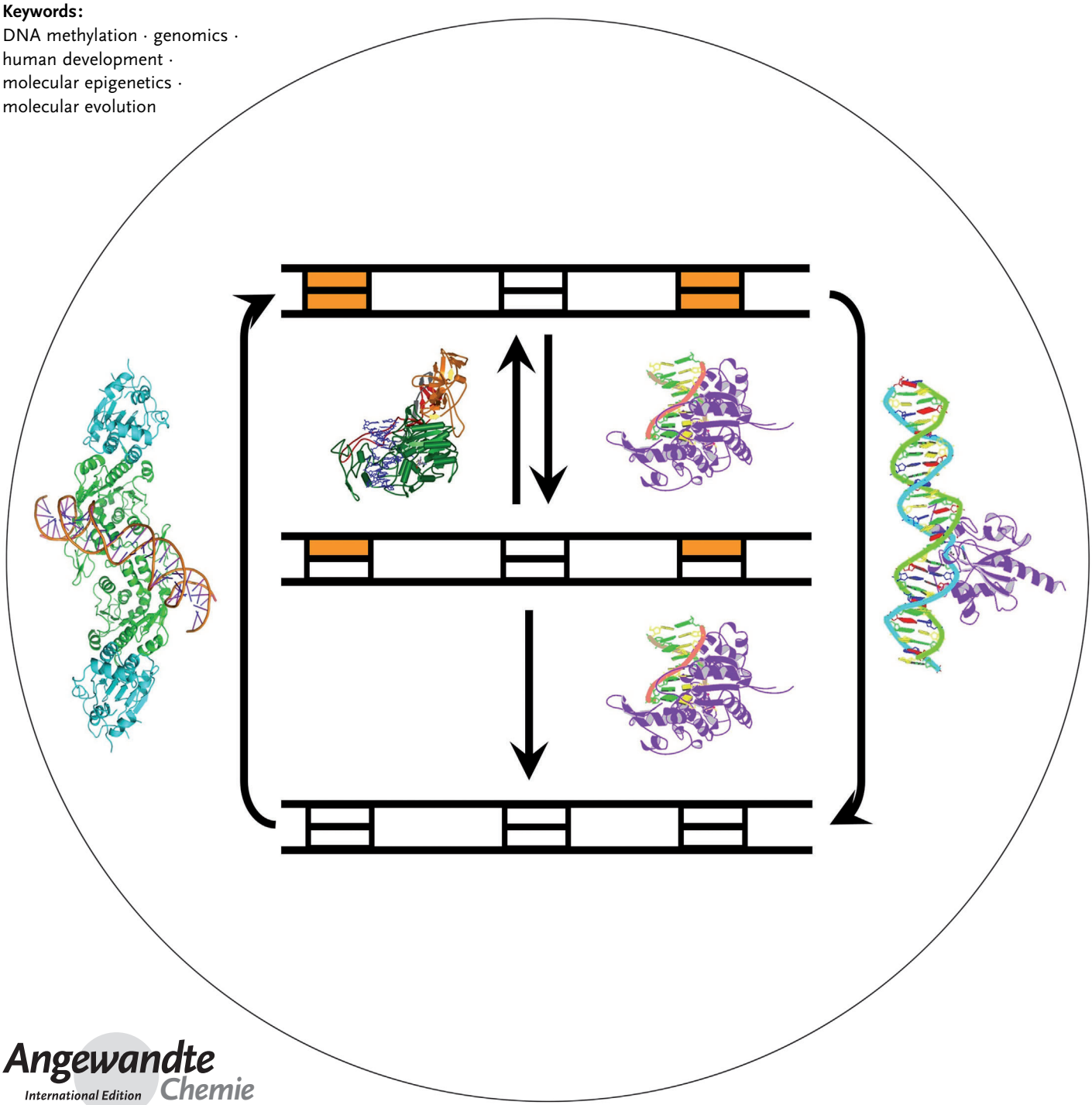

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Genomic imprinting, the parent of origin-dependent expression of genes, has been discovered as a fascinating example of the control of gene expression by epigenetic processes in the human body. It affects about 100 genes, which are often involved in growth and development. In this Review, we discuss the mechanisms leading to the generation of gender-specific imprints in form of DNA methylation marks, their preservation during growth and development of the organism, and the processes that translate parental methylation marks into monoallelic gene expression. We discuss the gender-specific dimorphic nature of imprints from an evolutionary point of view and present the prevalent model that molecular imprinting mediates a conflict of interest between the parents that occurs in viviparous animals. Finally, we summarize the relevance of parental imprinting for human health.

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1. Discovery of Imprinting as a Non-Mendelian Form of Inheritance

Since mammals are diploid, each cell carries two copies of the genetic information, one inherited from the mother via the egg cell, the other from the father via the sperm. In spite of this seemingly "easy" setting, examples of non-Mendelian, parent-of-origin specific inheritance were discovered in the 1970s, suggesting that at some loci one of the two gene copies carries an "imprint" that might repress its activity.[1] In 1984, first molecular evidence appeared that the two copies of the parental genomes are not equivalent, when nuclear transplantation experiments in mice showed that one maternal genome and one paternal genome is needed for a successful development[2–4] (Figure 1). In this study, the paternal pronucleus in fertilized mouse eggs was replaced with a maternal one to generate a bimaternal (gynogenetic or parthenogenetic) zygote. Conversely, the maternal pronucleus was exchanged with a second paternal one to generate a bipaternal (androgenetic) cell. In both settings, these manipulated uniparental zygotes could not complete embryogenesis. Control experiments showed that the failure in development was not caused by the manipulation itself, clearly indicating that the presence of one maternal and one paternal genome is essential to successful embryonic development. This was attributed to the existence of imprinted genes, which are exclusively expressed either from the maternal or the paternal allele and, hence, not expressed at all in one of the uniparental zygotes. First examples of imprinted genes, the insulin-like growth factor 2 (*Igf2*), expressed from the paternal allele, and the insulin-like growth factor type-2 receptor (*Igf2r*) and the H19 noncoding RNA, both expressed from the maternal allele were identified in mice in 1991.[5–8] Later, it was found that the molecular mark, which leads to the allele-specific expression of imprinted genes, is a DNA methylation signal present distinctively on one allele, but not on the other one.[9–11]

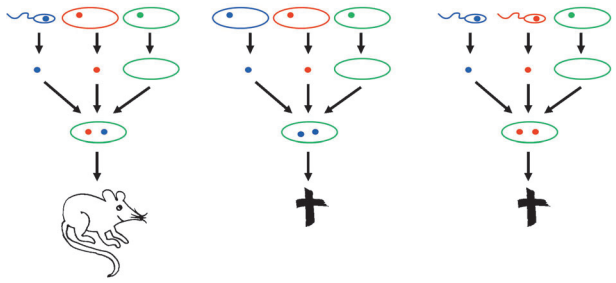

Figure 1. Discovery of imprinting in nuclear transplantation experiments. Transfer of one male and one female nucleus into an enucleated oocyte can lead to successful development, while the transfer of two male or female nuclei never does.

In eukaryotic cells, the DNA is packed in chromatin, where about 147 base pairs of DNA are wrapped around a histone octamer consisting of two copies of each of the four histone proteins called H3, H4, H2A, and H2B. We now know

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that genomic imprinting is an epigenetic process that involves both DNA methylation and histone modifications in order to achieve monoallelic gene expression from one parental locus without altering the DNA sequence. These epigenetic marks are established in the germline and are generally maintained throughout development in all somatic cells of an organism, although examples of tissue-specific loss or modulation of imprinting have been described.[12] About 100 of the roughly 20000 human or mouse genes are imprinted,[123] and given the fact that several genome-wide DNA methylation studies have been conducted during the last years, which would have detected allele-specific methylation, it is unlikely that this number will greatly increase in the future. Imprinted genes play important roles in regulating growth of the embryo and development of lineages, as well as in behavior and metabolism.[13] They are often located in clusters that typically contain both paternally and maternally expressed genes but also other not imprinted genes. The clusters are controlled by an imprinting control region (ICR) that contains a differentially methylated region (DMR), where parent-specific DNA methylation is present. Typically, one ICR regulates all genes in one cluster, sometimes at long genomic distances.[12,14] A well-known example of the regulation of the expression of imprinted genes in mice is the *Igf2r* gene (Figure 2), where a large noncoding RNA called Airn (antisense Igf2r RNA noncoding) is expressed from the paternal allele. Airn is transcribed in an antisense direction to *Igf2r*, which prevents expression of the *Igf2r* on the paternal chromosome.[15] In the maternal allele, methylation of the DMR prevents the expression of the noncoding RNA, such that the *Igf2r* gene is transcribed. Imprinting may use more complicated modes of regulation, as illustrated by the *Igf2/H19* locus (Figure 2). Here, the methylation of the DMR regulates the binding of the CTCF (zinc-finger protein CCCTC-binding factor) insulator protein. The DMR is unmethylated in the maternal genome, leading to the binding of CTFC, which restricts the access of a downstream enhancer to the *Igf2* gene, finally causing loss of its expression. The enhancer drives the expression of the noncoding H19 RNA instead. In the paternal genome the DMR is methylated; therefore, CTCF cannot bind and the *Igf2* expression is driven by the enhancer.[16–18] For more detailed descriptions of the genetics and molecular biology of imprinting several excellent reviews can be consulted.[1,12,14,19]

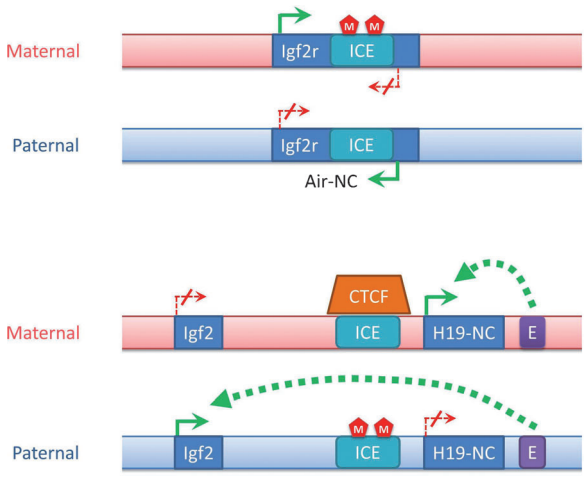

Figure 2. Establishment of allele-specific gene expression at the imprinted *Igf2* and *Igf2r* loci. At the *Igf2r* locus, the imprinting control element (ICE) including the differentially methylated region (DMR) is situated within the Igf2r gene (indicated by a blue rectangle). In the *Igf2* locus, the ICE is located between the *Igf2* and *H19* genes. At the Igf2r locus, the ICE is unmethylated in the paternal allele. This enables expression of the Airn noncoding RNA from an antisense promoter that leads to silencing of the *Igf2r* gene. Methylation of the ICE in the maternal allele prevents the activity of the antisense promoter and this allows expression of *Igf2r*. At the *Igf2* locus, the ICE is unmethylated in the maternal allele. This allows binding of the CTCF insulator, which restricts the access of an upstream enhancer on the *H19-NC* gene. In the paternal allele, methylation of the ICE prevents activity of the *H19-NC* gene and binding of CTCF and this allows the enhancer to drive the expression of the *Igf2* gene. Methylation is indicated by red pentagons. Active alleles are indicated by green arrows, inactive ones by red arrows.

2. Molecular Mechanism of DNA Methylation and Demethylation

The methylation of the C5 position of cytosine is introduced by a class of enzymes called DNA-(cytosine C5)-methyltransferases (MTase), which all share a common catalytic mechanism. Since the cytosine is an electron-poor heterocyclic aromatic system, its C5 position is not nucleophilic and the spontaneous transfer of the methyl group of *S*-adenosyl-L-methionine (AdoMet) to the cytosine C5 position is impossible. The enzymatic catalysis follows the mechanism

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Albert Jeltsch studied biochemistry at University Hannover, where he finished his PhD under the direction of Prof. A. Pingoud on restriction endonucleases. Working on DNA methyltransferases, he finished his habilitation at the Justus-Liebig University Giessen and afterwards moved to Jacobs University Bremen. Since 2011, he has been Professor of Biochemistry at the University Stuttgart. He has received the Gerhard-Hess award (DFG) and the BioFuture award (BMBF). His research deals with molecular epigenetics, methylation of DNA and proteins, and the design of enzymes and artificial gene regulation systems.

of a Micheal addition (Figure 3). It is initiated by the nucleophilic attack of a cysteine thiol on the C6 position of the cytosine, which leads to the formation of a covalent bond

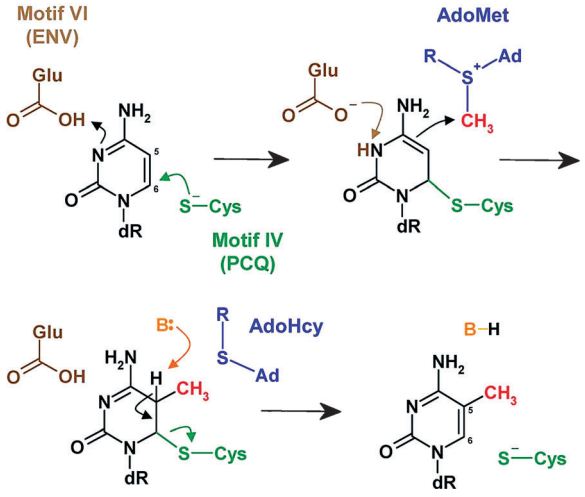

Figure 3. Catalytic mechanism of DNA-(cytosine C5)-methyltransferases. For details refer to the text. The cytosine base is colored black, AdoMet blue, and the methyl group red. The catalytic amino acids are colored brown and green and the not-yet-identified base is in orange.

between the enzyme and the substrate base. Thereby, the negative charge density at the C5 atom of the cytosine increases, such that it can attack the methyl group of the cofactor. The cysteine is located in a characteristic PCQ amino acid motif in the active center of the enzyme that is conserved among DNA-(cytosine C5)-MTases. The nucleophilic attack of the cysteine can be supported by a transient protonation of the cytosine at its endocyclic N3 nitrogen atom by a catalytic glutamate residue located in a conserved ENV amino acid sequence. This residue also contacts the cytosine N4 and thereby positions the target base. The addition of the methyl group to the base is followed by a deprotonation of the C5 atom, catalyzed by a yet unknown proton acceptor, which breaks the covalent bond between the enzyme and the base in an elimination reaction.[20,21] Notably, this mechanism requires an access of the enzyme to the entire Watson/Crick face of the cytosine, which precludes methylation of base-paired target bases. Therefore, DNA methyltransferases flip their target base out of the DNA during catalysis and bury it into a hydrophobic pocket of their active center in a process called base flipping, which is common to all DNA methyltransferases.[20,21] Of note, the mechanism of the methylation of exocyclic amino groups carried out by DNA-(cytosine N4)- and DNA-(adenine N6)-methyltransferases also includes target base flipping, but otherwise is distinct.[21]

Three different enzymatic reactions have been connected so far with DNA demethylation—the conversion of methylcytosine to cytosine: 1) removal of the base by deglycosylation, 2) deamination of methylcytosine, and 3) oxidation of methylcytosine to hydroxymethylcytosine and higher oxidation states.[22,23] These reactions are not mutually exclusive and may act in concert, such that a methylcytosine may be deaminated to thymine, which is then excised from the T:G mismatch by thymine-DNA glycosylase (TDG). Oxidative demethylation pathways can also depend on TDG, which has been shown to act on hydroxymethylcytosine as well, but a direct enzymatic decarboxylation of carboxylcytosine could be possible as well.

3. Role of DNA Methylation As an Epigenetic Modification That Is Heritable but Also Reversible

Due to its stability, but also reversibility, DNA methylation constitutes an ideal signal to mark imprinted loci, where the modification state has to change during germ cell development to reflect the gender of the organism. DNA methylation in humans occurs on the C5 position of cytosine residues, predominantly within CpG dinucleotides, and only rarely at non-CpG sites. However, not all CpG sites in the genome are methylated; therefore, a specific pattern consisting of unmethylated and methylated CpG sites exists. In the human genome approximately 60–80% of all CpGs are methylated in a cell-type-specific pattern. The remaining sites are unmethylated (hemimethylation is a rare state).[24–27] The epigenetic nature of DNA methylation, being at the same time heritable and flexible, can be illustrated by the cycles of DNA methylation occurring during cell division and development (Figure 4). As proposed by Riggs[28] and Holliday and Pugh[29] already almost 40 years ago, the molecular basis for the inheritance of the DNA methylation mark lies in the palindromic nature of the CpG site. Typically, a CpG site exists either in a fully methylated state, where the cytosines from both DNA strands of the CpG site are methylated, or in an unmethylated state, where both strands are unmodified.

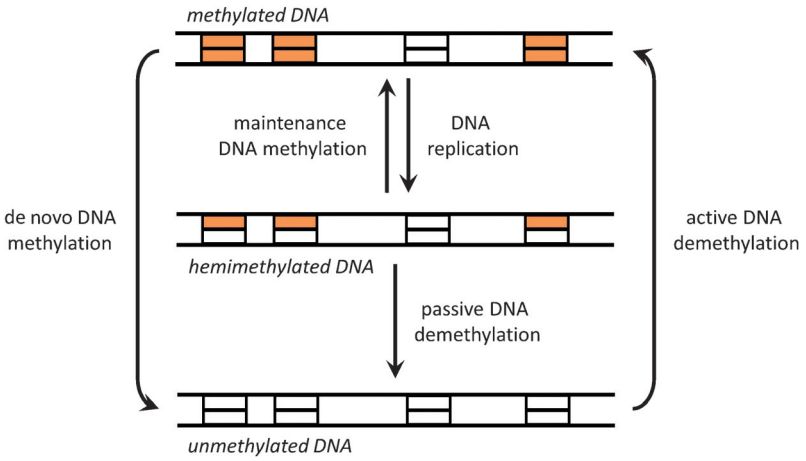

Figure 4. DNA methylation changes in mammalian cells. DNA methylation patterns consist of unmethylated (in white) and methylated CpG sites (in orange). It is maintained after DNA replication by the maintenance DNA methylation and can be lost either through an active replication-independent or passive replication-dependent process. Maintenance DNA methylation is mainly carried out by Dnmt1, while de novo DNA methylation is initially introduced by the Dnmt3 enzymes.

During replication of methylated DNA hemimethylated CpG sites are generated transiently, in which the parental strand carries the methylation marks and the newly synthesized daughter strand is devoid of methylation. Hence, the original pattern of DNA methylation is still available in the parental strand. The daughter strand gets re-methylated by a so-called maintenance DNA methyltransferase, which works as a molecular copy machine to restore the original pattern of methylation. This function is carried out by an enzyme that is specific for hemimethylated DNA, which prevents methylation of previously unmethylated sites and, thereby, preserves the methylation pattern through cell divisions. Methylation of CpG sites in the promoter region of genes usually leads to the repression of gene expression. The regulatory function of DNA methylation is implicated in a variety of important biological processes in humans, including genomic imprinting.

During development, two main cycles of DNA methylation occur in mammals.[30–32] After fertilization, the gamete-specific DNA methylation patterns in the sperm and egg genomes are globally erased by DNA demethylation to achieve totipotency. Despite almost complete genome-wide demethylation, the imprinted genes remain methylated during this phase (Figure 5, step 1), the mechanism of this process is one of the most critical open questions in imprinting research. The erasure of methylation is followed by a wave of de novo DNA methylation. At the time of implantation, de novo DNA methyltransferases introduce methyl groups to the DNA, creating the initial embryo-specific methylation pattern that is propagated though cell division by maintenance DNA methylation. During development smaller DNA methylation changes occur, which generate cell-type-specific methylation patterns. Maintenance of these patterns contributes to the development of cell lineages. A second dramatic resetting of DNA methylation occurs in gametogenesis during differentiation of the primordial germ cells, which are the direct progenitors of oocytes and sperm. In this phase, DNA methylation is also globally removed to restore the developmental potential and erase existing imprints (Figure 5, step 2). This process is very important, because it ensures that the imprints inherited from the parents are not transmitted to the next generation. Afterwards, new DNA methylation patterns including imprints at DMRs are established in an oocyte- and sperm-specific manner. Conceptually, the generation of imprints in this phase can be compared with the generation of tissue-specific DNA methylation patterns. Since sperm and egg are specialized cell types that only occur in one gender, the patterns established at that time automatically are gender-specific as well.

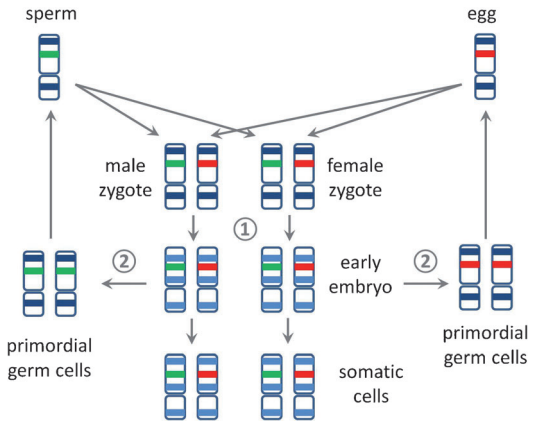

Figure 5. The imprinting cycle in mammals. Sperm and egg cells contain tissue-specific DNA methylation (dark blue) and imprints (green and red). After fertilization the gamete-specific DNA methylation patterns are erased and replaced by a new pattern specific for the embryo (light blue). Imprints are preserved in this process (step ①). In primordial germ cells DNA methylation patterns are re-set again and at this time, imprints are erased as well and newly generated in a gender-specific pattern (step ②).

4. Mechanism of the Generation of Imprints in Germ Cells by Dnmt3a/3L and Dnmt1

Gender-specific DNA methylation imprints are generated by the action of de novo DNA methyltransferases in developing germ cells. The key de novo DNA MTases in mammals are Dnmt3a and Dnmt3b, which are highly expressed in both female and male germlines, as well as in embryonic tissues.[33–36] Both enzymes were discovered in 1998 and shown to have de novo DNA methylation activity.[34,37–39] Genetic studies demonstrated that both Dnmt3a and Dnmt3b are essential for the embryonic development in mice.[34] Specifically, conditional knockout experiments showed that Dnmt3a is required for the proper establishment of imprints during gametogenesis, whereas Dnmt3b is dispensable for this process.[40–42] Later, a third member of the Dnmt3 family, Dnmt3L (Dnmt3-like), was discovered, which is also expressed during gametogenesis and at early embryonic stages.[1,43–45] Although catalytically inactive, Dnmt3L colocalizes with both Dnmt3a and Dnmt3b, directly interacts with the catalytic domains of the enzymes, and stimulates their activity, both in vitro and in vivo.[40,46–49] Hence, it acts as a positive regulator for de novo methylation. While the targeted disruption of the Dnmt3a or Dnmt3b gene in mice is lethal, mice lacking Dnmt3L are viable.[40,44] However, female mice missing Dnmt3L in spite of being fertile fail to produce viable pups due to defective female imprinting, clearly demonstrating the requirement of Dnmt3L for the proper establishment of imprints in the female germline. This phenotype is reminiscent of that of female Dnmt3a-conditional germline knockout animals,[40,41] suggesting that both Dnmt3a and Dnmt3L cooperate in the establishment of DNA methylation imprints during gametogenesis.

Further molecular insight into the mechanism of Dnmt3a stimulation by Dnmt3L came from structural and biochemical studies. The structure of the C-terminal catalytic domain of Dnmt3a in complex with the C-terminal domain of Dnmt3L has been solved and showed that these proteins form a linear heterotetramer, consisting of two Dnmt3L subunits (at the edges of the tetramer) and two Dnmt3a subunits (in the center).[50] The structure revealed that the Dnmt3a C-terminal domain contains two interfaces for protein/protein contacts: a hydrophobic one, generated by the stacking interaction of two phenylalanine residues (called the FF interface), which can mediate Dnmt3a/Dnmt3a and Dnmt3a/Dnmt3L interac-

tions, and a polar one, generated by a hydrogen-bonding network between arginine and aspartate residues (called RD), which can only mediate Dnmt3a/Dnmt3a interactions, since the corresponding region is absent in Dnmt3L.[51] Importantly, residues from the FF interface directly interact with the key catalytic or AdoMet binding residues, which may explain the stimulatory effect Dnmt3L exerts on Dnmt3a AdoMet binding and catalysis. The RD interface of the Dnmt3a subunit generates the DNA binding cleft of the heterotetramer. The arrangement of the two Dnmt3a subunits in the Dnmt3a/Dnmt3L heterotetramer positions the active centers of the two Dnmt3a molecules at a distance of about 10 base pairs (bps) on the DNA,[50,52] suggesting that two CpG sites separated by 10 bps could be methylated by Dnmt3a in one binding event. Additionally, Dnmt3a polymerizes on DNA, forming Dnmt3a–DNA filaments, which allow methylation of multiple CpG sites by a Dnmt3a oligomer.[52,53] Indeed, in vitro methylation experiments demonstrated that there is a correlation of methylation between the sites localized roughly 10 bps apart.[50,52,53] Interestingly, DMRs methylated in the female germline by Dnmt3a/Dnmt3L contain an enrichment of CpG sites in that spacing, which makes these sequences good substrates for the Dnmt3a/Dnmt3L complex.[50]

The presence of two interfaces for homotrophic protein/protein interaction leads to the possibility of Dnmt3a to form linear protein oligomers, which have been observed in different studies.[48,51] Since each RD interface constitutes a DNA binding site, such protein oligomers can bind to more than one DNA molecule, which are oriented roughly in parallel.[51] This ability was shown to play an important role in the binding of Dnmt3a to heterochromatin in cells, possibly because heterochromatic DNA is densely packed and provides several DNA strands for interaction with Dnmt3a oligomers in matching geometry. Since Dnmt3L lacks the RD interface, the binding of Dnmt3L to Dnmt3a oligomers leads to the resolution of the Dnmt3a oligomers and the formation of stable Dnmt3a/Dnmt3L heterotetramers. In vivo, it was shown that Dnmt3L releases Dnmt3a from heterochromatin. Hence, expression of Dnmt3L can increase the availability of Dnmt3a for the methylation of imprinted DMRs and other targets in gene promoters, which generally are euchromatic.[51] This observation implies that Dnmt3L redistributes Dnmt3a in the cell nucleus, in addition to its stimulatory role on Dnmt3a. This model is further supported by the observation that in differentiated cells lacking Dnmt3L, Dnmt3a and Dnmt3b are tightly associated with condensed chromatin containing methylated DNA.[54,55]

The de novo generation of DNA methylation is complicated by the fact that the Dnmt3 enzymes show strong differences in activity depending on the sequences flanking the target CpG site.[56–58] At most CpG sites this leads to a preferential introduction of methylation in one of the two DNA strands, generating a hemimethylated site, while only few sites carry a favorable flanking sequence in both strands, such that the Dnmt3 enzymes can easily generate a fully methylated CpG site. However, hemimethylated CpG sites generated by Dnmt3 enzymes represent ideal substrates for Dnmt1. Therefore, the cooperation of Dnmt3 enzymes with Dnmt1 constitutes a powerful system for introducing double-strand methylation into DNA,[59,60] suggesting that they all are needed for efficient de novo methylation of DNA.

The specific DNA methylation pattern of a human cell is the outcome of targeted and regulated DNA methylation and demethylation. Currently, it is not known exactly how de novo DNA methyltransferases are brought to particular genomic regions and how the specific DNA methylation patterns (including the imprints) are generated in male and female germ cells, which is in part due to the fact that these cells can only be obtained in small amounts and, hence, are difficult to study. Several alternative, but compatible models have been proposed to guide DNA methyltransferases to their target regions (including DMRs) and lead to preferential methylation at some loci but prevent methylation at others.

1. Direct targeting of Dnmts: In general, the Dnmts are recruited to particular genomic regions by other DNA-binding or chromatin-interacting proteins, like sequence-specific transcription factors (Figure 6a). In the context of imprinting, gender-specific methylation marks could be generated if specific sets of DNA-interacting proteins are differentially expressed in developing egg and sperm and they recruit Dnmts to distinct genomic loci. Several transcription factors with important roles in development have been discovered that could be involved in this process. For example, more than 300 KRAB (Krüppel-associated box) domain containing zinc finger proteins have been found in the human genome,[61,62] which show tissue-specific expression and could contribute to the generation of germline DNA methylation patterns. These proteins interact with the tripartite-motif-containing protein 28 (Trim28, also known as Kap1), which by itself interacts with Dnmt3a and Dnmt3b.[63–64] Novel methods of isolating and manipulating developing sperm and egg cells are becoming available, such that the identification of specific transcription factors involved in the generation of imprints is expected in the near future.

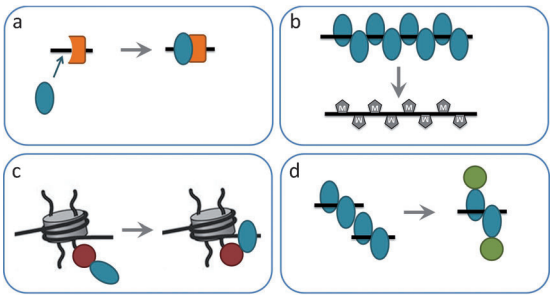

Figure 6. Mechanisms that can influence DNA methylation patterns generated by Dnmt3 enzymes (blue ovals). a) Recruitment by DNA binding proteins (orange). b) Influence of the DNA sequence, here a periodical arrangement of the target sites. c) Direct or indirect binding of the Dnmts to chromatin marks presented on the nucleosome. d) Changes of the subnuclear localization of Dnmt3a, here by addition of Dnmt3L (green), which changes the quaternary structure of Dnmt3a and reduces binding to heterochromatin.

2. Protection of DNA against methylation: Conversely, the specific binding of other proteins could protect regions of the DNA from de novo methylation, a model that is also supported by clear experimental evidence.[65,66] In the context of imprinting it was suggested that DNA methylation at maternally methylated DMRs could be a default process and the lack of methylation at the corresponding sequences in sperm might be due to the specific protection of these sequences from de novo methylation. Some evidence for this model comes from recent studies showing that most maternally methylated DMRs are flanked by regions that are methylated in both oocytes and sperm, and therefore, the DMRs can be seen as unmethylated islands in the male germ cells.[67–70] Additionally, several maternal DMRs in male germ cells are enriched in H3K4 di- and trimethylation at the time of imprint establishment, providing another possible mechanism for their protection from de novo methylation.[71,72]
3. Intrinsic sequence preferences of Dnmts: All Dnmt3 enzymes have intrinsic sequence preferences, which could mediate the specificity for certain target regions, like DMRs (Figure 6b). Examples of this mechanism include the preference of Dnmt3a for methylation of CpG sites embedded in certain flanking sequences and for methylation of CpG sites periodically placed at distances of 8–10 bp. Both of these effects were shown to leave a global footprint on genomic DNA methylation patterns.[50,52,57] Maternally imprinted DMRs contain enrichment of CpGs in favorable distances of 8–10 bp, which could facilitate their methylation by Dnmt3a.[50–52] However, recent analyses have shown that a periodic positioning of CpG sites with a separation of 8–10 bp is more widespread and could be related to the periodic alteration of AT- and GC-rich DNA sequences, which occur at good nucleosome binding sites.[73] However, sequence preferences of Dnmts are unlikely to be a direct determining factor for the preferential methylation of DMRs, since they would affect methylation in both alleles equally. Nevertheless, it is reasonable to assume that evolution will streamline important loci, which need to be methylated in a fast and reliable fashion, to contain CpG sites in a favorable sequence context and distance, both of which facilitate the methylation reaction.
4. Interaction of Dnmts with chromatin: Specific histone modifications are directly involved in the control of the activity and recruitment of Dnmt3s to particular loci, including DMRs (Figure 6c). This model has been supported by the finding that all three Dnmt3 proteins directly bind with their N-terminal ADD domains to histone H3 tails unmethylated at K4.[74–76] Moreover, the PWWP domain of Dnmt3a binds H3 di- and trimethylated at K36.[77,78] Interestingly, binding to H3 tails allosterically stimulates Dnmt3a.[79] This stimulation is disrupted by methylation of H3 at K4, suggesting that H3K4 trimethylation could prevent methylation of chromatin by the Dnmt3 enzymes. This model is consistent, because H3K4me3 is an activating mark correlated with high transcription and anticorrelated with DNA methylation.[80a] Further evidence for this model came from the observation that the histone H3K4 demethylase KDM1B, which is highly expressed in growing oocyte, is required for methylation of several maternal DMRs in oocytes.[80b]
5. Role of Dnmt3L in imprinting: As described above, Dnmt3L plays an important role in the setting of maternal imprints. It stimulates the activity of Dnmt3a, and additionally changes Dnmt3a's quaternary structure and by this alters the subnuclear localization of Dnmt3a. By converting Dnmt3a oligomers into Dnmt3a/Dnmt3L heterotetramers, Dnmt3L leads to a release of Dnmt3a from heterochromatin and thereby makes the enzyme available for the methylation of euchromatic targets, like DMRs (Figure 6d).
6. Role of chromatin remodeling in DNA methylation: As DNA is wrapped on histone octamers in nucleosomes and packed into chromatin in mammalian cells, the accessibility of the DNA to de novo DNA methylation machinery could determine which regions are subject to methylation. This model is supported by the finding that the lymphoid specific helicase (LSH) protein, which belongs to the SNF2 family of chromatin remodelers, is essential for de novo methylation of DNA in mice.[81,82] LSH was shown to stimulate Dnmt3a- as well as Dnmt3b-directed DNA methylation, but elucidation of the mechanistic details of this process awaits further studies.

5. Mechanism of the Inheritance of DNA Methylation and Imprints by Dnmt1

After its establishment in germ cells, the methylation pattern at the DMRs of the imprinted genes is inherited throughout the lifetime of the organism (with the eventual loss of imprints in some tissues). The long-term inheritance of this molecular mark is particularly challenging in the context of genome-wide demethylation and reprogramming that occurs after fertilization. Which factors specifically protect the imprinted loci from demethylation? Is the DNA maintenance methylation machinery preferentially targeted to these regions? These questions cannot be answered at present, since the molecular mechanism underlying the protection of the imprints during early embryogenesis remains unknown. As described above, the maintenance of DNA methylation patterns over cell divisions requires an enzyme that has a preference for the methylation of hemimethylated CpG sites, a property that was described for the first time in 1981 for a DNA MTase that was later identified as Dnmt1.[83] Recent data indicate that Dnmt1 has a roughly 10–20-fold preference for hemimethylated DNA over unmethylated substrates.[84–88] Conceptually, understanding of the copy process of DNA methylation patterns can be divided into two unrelated tasks: first, the mechanism of Dnmt1 that allows the enzyme to achieve its preference for hemimethylated DNA must be comprehended; second, additional factors enhancing the inherent preference of Dnmt1 must be identified, since a 10–20-fold preference for hemimethylated DNA is not sufficient to explain the inheritance of DNA methylation patterns through numerous cell divisions.

Dnmt1 is a processive enzyme, able to methylate long stretches of DNA without dissociation from the DNA, a property that fits perfectly to its function at the DNA replication fork.[87,89–90] Interestingly, processive methylation is possible only in one strand of the DNA, which indicates that Dnmt1 does not change its binding orientiation while moving along its substrate.[89] Due to its high processivity, Dnmt1 is a very effective enzyme, ideally suited to follow DNA replication and methylate the newly synthesized DNA strand before the chromatin is reassembled. Dnmt1 contains multiple functional domains located in its N-terminal part which is joined by a flexible linker to the C-terminal part containing the catalytic center. The N-terminal part of Dnmt1 encloses a CXXC domain, which contains eight conserved cysteine residues and two zinc ions and binds unmethylated DNA.[84,91,92] The structure of a truncated Dnmt1 protein encompassing the CXXC, BAH, and catalytic domains in complex with an unmethylated DNA showed specific binding of the unmethylated CpG site to the CXXC domain, but not to the catalytic domain.[92] Based on this observation, an autoinhibition model was proposed for the regulation of Dnmt1 activity and specificity, in which the binding of unmethylated CpG sites to the CXXC domain would prevent their methylation and constitute the mechanism for Dnmt1 specificity; this was supported by kinetic data with the truncated Dnmt1.[92] However, in the context of full-length Dnmt1, exchanges in the CXXC domain, which led to loss of its DNA binding capacity, did not change the preference of the enzyme.[84] This finding argues against an important role of the CXXC domain in the specificity of Dnmt1. Instead, it has been revealed that the specificity for hemimethylated DNA lies most probably in the catalytic domain of Dnmt1.[84,85,88]

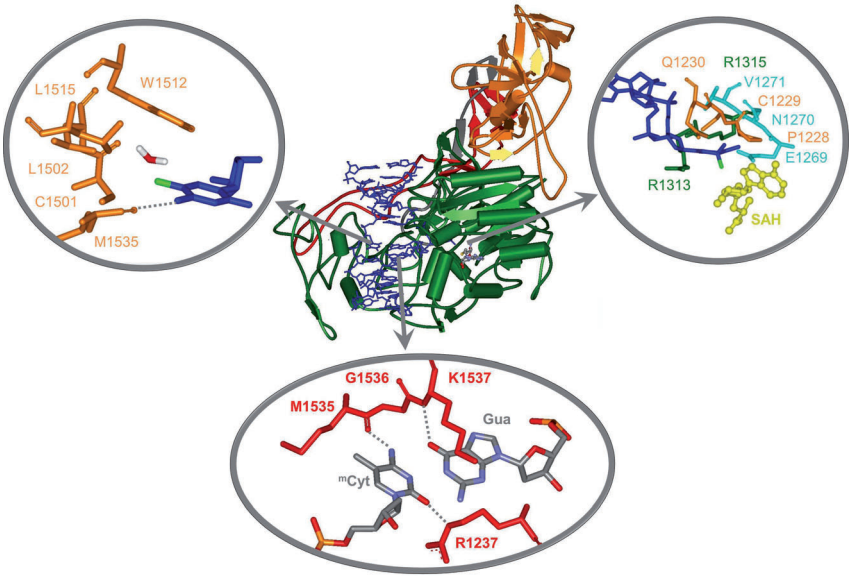

Figure 7. Structural basis of the specificity of Dnmt1 for methylation of hemimethylated CpG sites. The central part shows the structure of a fragment of Dnmt1 comprising the BAH1 and BAH2 domains (orange and red) and its catalytic domain (green) bound to *S*-adenosyl-L-homocysteine (SAH, colored by atom) and hemimethylated DNA (blue). The Dnmt1 enzyme is shown in a ribbon representation. The enlargement on the left side shows the interaction of hydrophobic amino acid residues with the methyl group of the hemimethylated CpG site (light green), which is the basis of the preference for methylation of hemimethylated DNA. The enlargement at the bottom shows the recognition of the methylcytosine (mCyt)/guanine (Gua) base pair by several hydrogen bonds. The enlargement on the right side shows the active site pocket with the characteristic PCQ, ENV, and RXR motifs (colored in orange, cyan, and green, respectively). The target cytosine is rotated out of the DNA helix and it has been methylated in this structure (the transferred methyl group is colored light green). SAH is shown in yellow.

The structure of a shorter C-terminal Dnmt1 fragment lacking the CXXC domain in complex with hemimethylated DNA[88] showed that the DNA is bound to the catalytic domain of the enzyme and revealed several base-specific contacts of Dnmt1 to its target sequence at the methylated cytosine–guanine base pair (Figure 7), which are all essential for catalysis.[85] The methyl group of the 5mC approaches a hydrophobic pocket formed by residues from the catalytic domain, which can explain the preference of Dnmt1 for hemimethylated sites (Figure 7). In addition, flipping of the target base was observed in the Dnmt1–DNA complex structure,[88] which is consistent with other structures of DNA MTases with substrate DNA. Here, the target cytosine is flipped out of the DNA helix and bound to a pocket in the protein, which presents the conserved active site residues described above.[20,21]

A structure of full-length Dnmt1 in the absence of DNA showed that its RFTS (replication foci targeting sequence) domain occupies the DNA binding site of the catalytic domain of Dnmt1,[93] suggesting that the RFTS domain allosterically regulates the activity of the enzyme. This observation was supported with kinetic data, indicating that the RFTS domain inhibits DNA binding and methylation by Dnmt1.[93,94] Hence, Dnmt1 exists in at least two different conformations: a catalytically inactive one, in which the RFTS domain blocks the active site, and a catalytically active one, in which DNA is bound in the active site. Moreover, interaction of Dnmt1 with secondary (non-substrate) DNA molecules at additional DNA binding sites (like the CXXC domain) has been shown to influence the activity and specificity of the enzyme.[95] In the cell, the arrangement of different domains like the CXXC and the RFTS domains might be regulated by the interaction with other proteins or posttranslational modifications. Thereby, Dnmt1 could be activated during the S-phase but its activity could be kept low during the other phases of the cell cycle. Consistent with its function, Dnmt1 is expressed at high levels in proliferating cells and its amount peaks at the entry into S-phase, when the replication occurs.[96,97] Additionally, its stability is regulated by various posttranslational modifications, further ensuring the availability of the enzyme only during S-phase.[98,99]

An important mechanism of enhancing the specificity of Dnmt1 in cells is to target the enzyme to newly synthesized DNA. This is ensured by at least two independent processes: Dnmt1 directly interacts with PCNA, a component of the DNA replication machinery that forms a ring around the DNA helix.[100] Disruption of this interaction delayed DNA re-methylation after replication.[101] In a parallel pathway, Dnmt1 interacts with the Uhrf1 (ubiquitin-like with PHD and ring finger domains 1) protein that binds specifically to hemimethylated DNA through its SRA domain and recruits Dnmt1 to hemimethylated DNA after replication.[102,103] Showing an almost complete loss of DNA methylation, the molecular phenotype of the targeted disruption of the *Uhrf1* gene in mouse embryonic cells[102,103] resembles that of the Dnmt1 knockout.[104] This observation indicates that Uhrf1 plays an essential role in the maintenance of DNA methylation in mammals. Furthermore, the binding of Uhrf1 to histone H3 tails di- and trimethylated at K9 during the S-phase recruits Dnmt1, which connects DNA methylation with H3K9me3, another important repressive mark, and provides an example of the cooperative action of epigenetic modifications.[105] In addition, the de novo enzymes Dnmt3a and Dnmt3b play a role in the maintenance of DNA methylation at heterochromatic DNA, particularly in densely methylated or repetitive sequences.[106] However, at imprinted loci—which are the focus of this Review—DNA methylation is mainly maintained by Dnmt1, as indicated by the finding that Dnmt1 knockout cells lose their methylation imprints.[107]

Despite these significant advances in the understanding of DNA maintenance methylation, it remains elusive how imprints are specifically preserved during the phase of genome-wide loss of DNA methylation in early embryonic development. This phase constitutes a particular challenge, because the methylation signal must be maintained in one allele against the massive genome-wide demethylation wave. Clearly, sequence-specific targeting of DNA MTases, introduced above as the basic mechanism for the generation of tissue-specific methylation patterns by de novo DNA MTases, would not suffice for this task, because it could not discriminate between the two alleles. A tempting extension of this model could involve a combined readout of the sequence and the methylation state. In this concept, a DNA binding protein would specifically bind to imprinted DMRs, but only if they are methylated, and thereby recruit DNA MTases specifically to the methylated allele of DMRs to enforce the stability of the methylation mark. Recent studies provided first experimental evidence for this model. They identified Trim28 and zinc finger protein (ZFP) 57 as key players involved in the maintenance of methylation imprints in mouse embryos[63,108,109] and showed that mutations in ZFP57 in humans are correlated with a loss of methylation at several imprinted loci.[110] Importantly, ZFP57 recognizes a methylated CpG-containing hexanucleotide motif and specifically targets Trim28 to the methylated allele of imprinted genes.[63] Interestingly, this motif was found at an average of two copies in all mouse ICRs and in the majority of the human ones.[63] In addition, it was shown that Trim28 forms a complex with all three Dnmts as well as with other components of the epigenetic machinery, including the SETDB1 histone H3K9 methyltransferase, heterochromatin protein 1 (HP1), the NURC chromatin remodeling complex, and also Uhrf1. This complex could therefore specifically target the DNA methylation machinery to methylated imprinted alleles and thereby reinforce the stability of the monoallelic methylation mark. Remarkably, more than 300 KRAB-ZFP have been identified in the human genome, many of which have been suggested to bind DNA in a methylation-sensitive manner.[61] Hence, the proposed "methylation enhancement" could be a general mechanism used to specifically target methylation to differentially methylated sequences present at different DMRs.

6. Sexual Dimorphism of DMRs and Gene-Silencing Mechanisms

It has been pointed out first by Bestor and colleagues[111,112] that both the structure of DMRs and the gene-silencing mechanisms of imprinted alleles differ strongly between the maternal and paternal genome. In general, maternally methylated DMRs are CpG-rich, often located in gene promoters, and function by a direct regulation of the corresponding gene. In contrast, paternal DMRs are often CpG-poor, located in the intergenic regions, and not directly involved in gene regulation. Instead, the paternally repressed genes are often regulated by a noncoding RNA, which itself is regulated by methylation of the maternal allele. This process is illustrated in the paternal silencing of *Igfr2* (shown in Figure 2), which is achieved by the action of a noncoding RNA. In the maternal allele the noncoding RNA is repressed by methylation and the *Igfr2* gene is expressed. In contrast, in the paternal allele, the noncoding RNA is expressed and silences the *Igfr2* gene. Therefore, surprisingly, the repression of the paternal *Igfr2* allele is mediated by an allele-specific methylation in the maternal allele and not by a specific methylation mark deposited in the paternal one.

The intriguing structural and mechanistic dimorphism between maternally and paternally methylated DMRs can be explained from an evolutionary point of view, when one considers the development of primordial germ cells. The presence of 5-methylcytosine in the DNA of higher eukaryotes is mutagenic, because it promotes cytosine deamination, and T/G mismatches (arising from deamination of 5-methylcytosine) have a lower repair efficiency than U/G mismatches (arising from deamination of unmethylated cytosine).[21] This difference is due to the fact that uracil, a nonnatural base in DNA, is efficiently recognized and excised by the uracil DNA glycosylase, whereas thymidine is a natural component of DNA. The DNA sequence of the human genome clearly documents the strong impact of the methylation-mediated mutagenesis on the genome evolution, because the CpG dinucleotides are threefold underrepresented with respect to their expected frequency, while, for example, GpC dinucleotides occur almost at their expected frequency. No CpG depletion is observed in some regions of the genome, called CpG islands, which overlap with the annotated transcriptional start sites of about 70% of all human genes,[113] including most housekeeping genes. Importantly, these gene promoters are

usually not methylated in the germline, meaning that they do not experience the mutagenic pressure of 5-methylcytosine. A similar mechanism may have led to the gender-specific dimorphism in imprinting, because the methylation imprints are set at different developmental points in both genders—at early embryonic stages in male germ cells, but only relatively late (after birth during the growth of oocytes, shortly before ovulation) in the female germline (Figure 8).[111,112] This

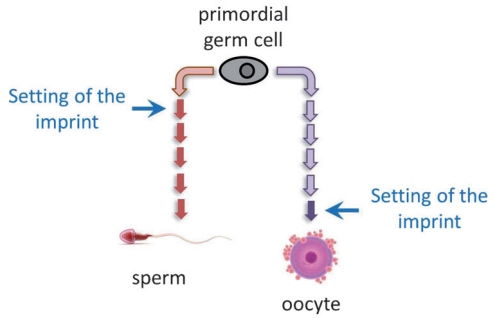

Figure 8. Differences in the timing of the establishment of imprints in male and female germ cell development leads to the differential genomic characteristics of the maternally and paternally methylated DMRs. In male germ cells the imprint is set early in embryonic development, such that the methylated DNA undergoes many duplications before the mature sperm develops; each duplication is accompanied by the risk of cytosine deamination mutations that are promoted by DNA methylation. In contrast, in the developing oocyte the imprint is set only late in development, such that there is less chance for methylation-induced mutations to occur.

implies that the methylated DMRs in the oocyte were under lower mutational pressure caused by the DNA methylation, since the maternal imprints have to be propagated through only few divisions before fertilization. This is drastically different in the case of the sperm, where imprints are established early in development and the DNA methylation is propagated through numerous cell divisions before a mature sperm is formed. Hence, the paternally methylated DNA experiences a strong mutational pressure, which led to the loss of many CpG sites in paternally methylated DMRs. This process finally undermined the regulatory power of the paternally methylated DMRs, because with the reduced CpG density the DNA methylation signal was also diluted. Therefore, the direct silencing of paternally repressed genes by DNA methylation became inefficient, such that the indirect mechanism described above was invented by evolution. Hence, the sexual dimorphism of DMRs and gene-silencing mechanisms beautifully illustrate the connections between development, epigenetics, and evolution.

7. Imprinting and the Evolutionary Conflict between Male and Female in Mammalian Development

The most popular model for the biological role of imprinting in mammals presented by Haig and Moore in 1991[114] also rests on evolutionary arguments. In viviparous animals with a large input of resources from the mother to support the growing embryo (as in mammals), a fundamental evolutionary conflict of interest exists between the mother on one side and the embryo and father on the other side. Since a high birth weight clearly is correlated with good health and survival perspectives of the embryo, both the embryo and the father in order to optimize their survival and reproduction perspective, respectively, would be interested to direct as many resources as possible from the mother to the developing embryo. The mother, in contrast, needs to control the growth of the embryo and provision of resources in order not to compromise her own survival and the chances of future pregnancies. She will, therefore, tend to restrict the growth of the embryo to a reasonable extent (Figure 9). This tug of war

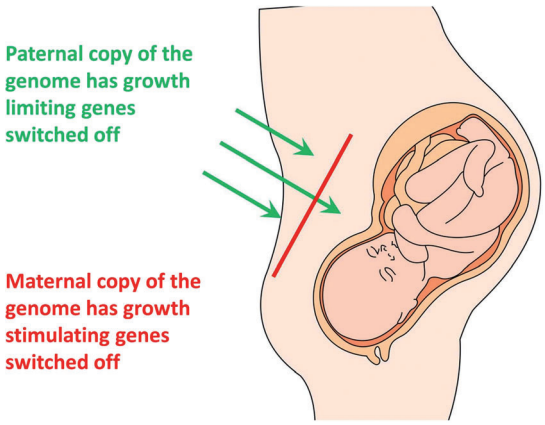

Figure 9. Evolutionary conflict between parents in animals giving live birth is mediated by genomic imprinting. The paternally expressed genes tend to increase the growth of the embryo, while maternally expressed ones tend to limit it.

between genders cannot be resolved by conventional Mendelian inheritance, because evolutionary advantages and disadvantages of any change depend on the gender of the offspring. While, for example a mutation that promotes the growth of the embryo is an advantage for male offspring, it would be a disadvantage for female descendants. Consequently, a mechanism of gender-specific gene regulation would be needed, which is exactly what genomic imprinting provides. In this concept, the female genome in the embryo should restrict the embryonic growth, while the male one would tend to stimulate it. This model is based on the general observation that paternally expressed genes are often growth promoting, while maternally expressed genes are growth inhibiting.[13] Accordingly, many imprinted genes are expressed in the placenta and play a role in the transfer of nutrients across the placenta to the embryo. For example, the maternally expressed Igf2 is a growth factor, while the paternally expressed Igf2 receptor (Igf2r) acts as an inhibitor of growth, because it sequesters Igf2. Consequently, mice lacking Igf2 are reduced in size,[6] whereas and embryos lacking Igf2r show overgrowth.[115,116] In agreement with this interpretation, the parthenogenetic zygotes (derived from two female pronuclei) showed reduced size of the extra-

embryonic tissue. In contrast, the androgenetic zygotes (derived from two male pronuclei) developed oversized extra-embryonic tissue, but had underdeveloped embryonic components.[4] Since the extra-embryonal lines later give rise to the placenta and thereby ensure nutrient flow to the embryo, these results are in agreement with the prediction that maternally expressed genes should restrict the embryo growth and the paternally expressed genes should promote it. Similar arguments hold for the regulation of the mother/child interaction during lactation, which explains why imprinted genes also have roles in the control of behavior. Similarly, human disorders associated with disturbances of imprinting often manifest with corresponding growth phenotypes as described in Section 8. Additional evidence in favor of this model to rationalize the biological role of imprinting is that imprinting appeared in the mammalian line with the marsupials, while the egg-laying mammals, as well as birds and reptiles do not have it.[117] In further support of this model, higher flowering plants, in which seedlings are heavily supplied with nutrients by the mother as well, independently also developed imprinting, which uses very similar epigenetic mechanisms, including DNA methylation, which represents an example of a convergent evolution. In summary, this model suggests that the molecular process of imprinting developed as an evolutionary response to strategic conflicts between male and female animals, which appeared with the development of live birth in the mammalian lineage.

8. Medical Implications: Imprinting Defects and Loss of Imprinting in Cancer Cells

Imprinted genes are susceptibility targets for numerous human pathologies, because their functional haploid state enables a single genetic or epigenetic change to deregulate their expression and cause health effects. Because of their essential role in development, imprinting anomalies are often manifested as developmental and neurological disorders when they occur during early development. Examples of imprinting disorders include the pediatric Beckwith–Wiedeman, Silver–Russel, and Angelman syndromes.[13,118–120] The first two are caused by alteration of the human *Igf2* locus (see Figure 2). In individuals with Beckwith–Wiedeman syndrome, the maternal genome also carries methylation at the CTCF binding site, which is characteristic of the paternal setting and yields a twofold increase (= biallelic) of *Igf2* expression. Affected children have an increased birth weight. Conversely, in children with the Silver–Russel syndrome the methylation at the *Igf2* locus is lost in both alleles, such that both alleles are in the "maternal" state. *Igf2* expression is lost and affected individuals have a low birth weight.

Since imprinted genes regulate growth, imprinting abnormalities often contribute to cancer as well. In the case of growth-promoting genes, the organism is adapted to a single gene dosage and the loss of the epigenetic silencing of the second copy will lead to overgrowth. In the case of an imprinted growth-controlling gene (which often have important roles as tumor suppressor genes), since one allele is already epigenetically silenced, a single critical mutation in the active allele suffices to result in a complete loss of the gene product. Somatic loss of imprinting has been observed in several cancers, including bladder, breast, cervical, colorectal, esophageal, hepatocellular, lung, ovarian, prostate, and testicular cancers, and leukemia, among others.[121,122] As indicated by the high incidence of imprinting abnormalities in cancer, the loss of heterozygosity at imprinted genes constitutes a high risk. The observation that imprinting nevertheless was established in evolution and remained stable indicates the important role it plays in mediating the interests of both genders at molecular level during pregnancy and early childhood.

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