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Epigenetic deregulation of genomic imprinting in humans: causal mechanisms and clinical implications

Mammalian genes controlled by genomic imprinting play important roles in development and diverse postnatal processes. A growing number of congenital disorders have been linked to genomic imprinting. Each of these is caused by perturbed gene expression at one principal imprinted domain. Some imprinting disorders, including the Prader–Willi and Angelman syndromes, are caused almost exclusively by genetic mutations. In several others, including the Beckwith–Wiedemann and Silver–Russell growth syndromes, and transient neonatal diabetes mellitus, imprinted expression is perturbed mostly by epigenetic alterations at ‘imprinting control regions’ and at other specific regulatory sequences. In a minority of these patients, DNA methylation is altered at multiple imprinted loci, suggesting that common *trans*-acting factors are affected. Here, we review the epimutations involved in congenital imprinting disorders and the associated clinical features. *Trans*-acting factors known to be causally involved are discussed and other *trans*-acting factors that are potentially implicated are also presented.

KEYWORDS: Beckwith–Wiedemann syndrome • DNA methylation • epigenetics
 • genomic imprinting • pseudohypoparathyroidism • Silver–Russell syndrome
 • *trans*-acting factors • transient neonatal diabetes mellitus • ZFP57

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Almost 30 years ago, experiments in mice formally established that the maternal and paternal genomes are both required for embryonic and fetal development. Mouse embryos engineered to contain only maternal (gynogenetic/parthenogenetic embryos) or only paternal (androgenetic embryos), genomes failed to develop to term [1,2], demonstrating the functional nonequivalence of the parental genomes. In addition, in other placental mammals, including humans, it was found that parthenogenetic (with two maternal genomes) and androgenetic (with two paternal genomes) conceptuses are developmentally aberrant and die *in utero*. The nonequivalence of the parental genomes in eutherians is due to genomic imprinting, an epigenetic phenomenon that gives rise to parent-of-origin-specific, monoallelic expression of genes during development [3]. So far, genomic imprinting has been observed in eutherians and marsupials, but not in monotremes, and approximately 120 imprinted genes have been discovered in humans, mice and other placental mammals [201]. These unusual genes are organized in evolutionarily conserved gene clusters that can comprise up to several megabases of DNA. The monoallelic expression of imprinted genes is mediated by so-called ‘imprinting control regions’ (ICRs). These essential *cis*-acting sequences of several kilobases in size are CpG-rich and marked by cytosine methylation on one of the two parental alleles only. The monoallelic

DNA methylation imprints at ICRs are acquired during male or female gametogenesis. After fertilization, they are maintained somatically throughout development and postnatal life, which confers an epigenetic ‘memory’ to the ICR [3]. During embryonic development, the differentially methylated ICRs produce parental allele-specific expression at adjacent genes through diverse epigenetic mechanisms, which are different between imprinted domains [4]. Because of their allelic DNA methylation, most ICRs are functionally active on one of the two parental chromosomes only, which explains their parental allele-specific effects on gene expression [3,5].

Genetic alterations affecting imprinted gene expression include translocations, deletions, duplications, inversions and uniparental disomy (UPD), and have been shown to be causally involved in different imprinting disorders. Furthermore, epigenetic alterations were found to be causally involved in imprinting-related diseases. These so-called epimutations involve gains or losses of DNA methylation at ICRs and at other differentially methylated regions (DMRs), and affect the regulation of one or multiple imprinted domains. Although in most cases, such epimutations are thought to arise early in development and, in exceptional cases, are due to perturbed imprint acquisition in germ cells; their precise causes remain poorly understood [6]. Nevertheless, the aberrant DNA methylation

profiles at ICRs often result from perturbation of methylation maintenance mechanisms. Several studies in animals and humans have indicated that this intrinsic maintenance process can be readily influenced by environmental cues [5]. For instance, superovulation, embryo culture and other *in vitro* procedures used in the fertility clinic could be responsible for the reported increased occurrence of imprinting disorders in babies conceived by assisted reproduction [7].

DNA methylation changes at ICRs may also be the consequence of point mutations and micro-deletions at imprinted gene loci [8], or may result from genetic mutations at genes elsewhere in the genome that code for essential *trans*-acting factors [9]. The latter possibility, that *trans*-acting factors are causally involved, has arisen from different reports of patients who presented with DNA hypomethylation at multiple ICRs (see 'Multi-locus DNA methylation defects in imprinting disorders' section). During recent years, genetic studies in the mouse have pinpointed *trans*-acting proteins that are involved in the establishment and/or somatic maintenance of parental imprints at imprinted gene clusters. Recently, clinical studies in humans also revealed several genes that encode *trans*-acting factors involved in imprinting. Here, we review recent advances in our understanding of epigenetic perturbation of genomic imprinting in human congenital disorders and discuss the clinical implications. Genomic imprinting is also frequently perturbed in cancer; this related theme is covered in detail elsewhere [10] and is not part of the current review.

Human disorders caused by epigenetic alterations at imprinted loci

Epimutations at imprinted gene loci can be classified as primary or secondary alterations [11]. Primary epimutations concern direct modification of the methylation state of regulatory DNA sequences, affecting imprinted expression without changes in the DNA sequence. Secondary epimutations also involve aberrant DNA methylation and chromatin modifications, but these epigenetic changes are caused by adjacent genetic mutations at the imprinted locus itself, or by genetic mutations elsewhere in the genome controlling the epigenetic regulation of the imprinted loci in *trans*. In most cases, it is unclear whether an apparent primary epimutation is truly primary, since it is not known whether *trans*-acting factors are affected in the patient. Another complication in the interpretation of clinical studies is that DNA methylation levels can often only be

determined in peripheral blood, and not in the actual tissues and organs affected by the disease. In many cases, therefore, it has been difficult to directly link ICR DNA methylation levels to the observed severity of the clinical phenotype in patients [12].

Primary epimutations at imprinted gene clusters involve the ICR, which shows either reduced or increased DNA methylation ('hypomethylation' and 'hypermethylation', respectively). In several imprinting-related disorders, this seems to be the most frequent causal mechanism [6]. Well-studied examples are provided by two imprinted domains at the telomeric region of the short arm of chromosome 11 (11p15.5). These two growth-related gene clusters are involved in Silver–Russell syndrome (SRS), characterized by intrauterine growth restriction (IUGR), and in Beckwith–Wiedemann syndrome (BWS), characterized by fetal overgrowth and high birth weight and height. A third imprinting disease, which is also often caused by primary epimutations, is transient neonatal diabetes mellitus (TNDM), a neonatal type of diabetes genetically linked to chromosome 6q24. Below, we review these rare congenital disorders caused most frequently by epigenetic mutations, and also briefly present several other imprinting-related diseases in which epimutations are less frequently involved.

■ Silver–Russell syndrome

SRS (OMIM180860) is a rare (incidence one in 100,000 [13]) and mostly sporadic disorder characterized by IUGR, postnatal growth retardation, facial dysmorphism, feeding difficulties and body asymmetry [14]. It is a genetically heterogeneous disorder in which, in rare cases, maternal UPD of chromosome 7 and sometimes other chromosomal alterations are detected [15,16]. Recent epigenetic studies have linked this syndrome to one of the two imprinted gene clusters on human chromosome 11p15.5, the *IGF2/H19* domain [17]. This growth-related locus comprises the paternally expressed *IGF2* and *INS* (imprinted in the yolk sac only) genes and a growth-regulating ncRNA, called H19, expressed from the maternal chromosome. The domain is controlled by an intergenic ICR located several kilobases upstream of *H19*, called the *H19*-ICR. In 2005, for the first time, Gicquel *et al.* reported epimutations at the *H19*-ICR in SRS patients, namely, hypomethylation on the paternal allele, which induced a loss of *IGF2* expression [17]. These results were confirmed by several subsequent studies [18,19], indicating that more than 50% of SRS cases are caused by hypomethylation at the ICR of the

IGF2/H19 locus, which provides a molecular explanation for this severe growth restriction syndrome [20]. Interestingly, complete [18] or partial [17,18] hypomethylation was detected at the *H19*-ICR in SRS patients. This finding suggested that the loss of methylation occurred early in development or could sometimes be caused by a constitutive genetic mutation(s). Whether other imprinted genes also contribute to SRS remains unclear. However, the maternal UPD of chromosome-7 observed in a minority of the patients suggests that a growth-regulating imprinted gene on this chromosome could also be involved. One candidate on this chromosome is the maternally expressed *GRB10* gene [21]. A recent study reported an aberrant gain of DNA methylation at this gene and at several other imprinted genes in SRS patients [22]. Further studies are required to conclude whether or not *GRB10* is genetically involved in SRS.

■ Beckwith–Wiedemann syndrome

Contrary to SRS, BWS (OMIM 130650) is characterized by fetal and postnatal overgrowth and also presents an increased risk of childhood cancers (10% of all patients). A minority of children with BWS develop Wilms' tumor (kidney cancer) and some present with adrenocortical carcinoma. Other clinical features of this rare syndrome (incidence one in 15,000 [23]) include macroglossia, midline abdominal wall defects, earlobe creases or ear pits and neonatal hypoglycemia [21,24,25]. BWS maps to chromosome 11p15. Besides the distal *IGF2/H19* imprinted domain, this chromosomal region also comprises the large (1 Mb) growth-regulating *KCNQ1* imprinting cluster. The key gene in this second, proximally located cluster is *CDKN1C* (previously called *p57KIP2*), a negative regulator of the cell cycle. A frequently observed loss of *CDKN1C* expression in cancer suggests that this is also a tumor suppressor gene [10]. Approximately 5% of sporadic BWS cases and approximately 40% of cases with a positive family history present with point mutations in *CDKN1C* [26,27]. Epimutations are responsible for approximately 60–70% of BWS cases. In approximately 10% of the patients, there is hypermethylation at the *H19*-ICR, which leads to biallelic (and therefore increased) *IGF2* expression responsible for the observed overgrowth phenotype [20]. Strikingly, this is the exact opposite epigenetic change to that observed in SRS, in which this ICR loses its DNA methylation during early development (see 'Silver–Russell syndrome' section above). The most commonly observed epimutation in BWS, however, affects imprinting

along the *KCNQ1* imprinted domain, leading to a strong reduction in *CDKN1C* expression. Particularly, at the domain's ICR (called the KvDMR1) there is loss of DNA methylation early in development. The lack of DNA methylation on both of the alleles of this ICR induces gene silencing along the domain on both the parental chromosomes, including that of *CDKN1C*. The resulting loss of *CDKN1C* expression is thought to be the main determinant in these cases of BWS, since deleterious point mutations in *CDKN1C*, on their own, also induce the overgrowth syndrome, in less than 10% of the patients [27].

A striking observation has been that cohorts of BWS babies present an increased frequency of monozygotic twinning. The affected twins are female and are discordant, with only one of the two girls showing the clinical features of the overgrowth syndrome [28]. Methylation studies have shown that there is often loss of methylation at the ICR of the *KCNQ1* domain, with the epimutation occurring in the affected twin girl. Methylation changes in the peripheral blood, however, are often observed in both twins. In some monozygotic female twins, hypomethylation was also detected at the *H19*-ICR and the ICR of the *DLK1-DIO3* imprinted domain [28]. Significantly, in SRS, discordant monozygotic female twins have been reported that have hypomethylation at the *H19*-ICR, again, affecting one of the two girls only [29]. One interpretation for this intriguing phenomenon is that the methylation losses somehow trigger the twinning process [28,29]. Alternatively, female preimplantation embryos are more prone to loss of DNA methylation than early male embryos, and this effect is exacerbated by the increased number of cell divisions due the monozygotic twinning process.

■ Transient neonatal diabetes mellitus

TNDM (OMIM 601410) is a growth deficiency syndrome characterized by IUGR and persistent hyperglycemia during the first 6 weeks of life owing to a lack of insulin. This extremely rare syndrome (incidence <one in 100,000 [30]) has been linked to paternal UPD of chromosome 6 and to large duplications of region 6q24 on the paternal chromosome. In approximately 50% of cases, however, the disease seems to be not genetic in origin and is associated with defective DNA methylation at the imprinted transcription factor gene *ZAC/PLAG1*, localized on chromosome 6q24. Particularly, these TNDM patients with a normal karyotype show hypomethylation at exon 1 of *ZAC* on the maternal chromosome [31,32]. This putative ICR region is thought

to regulate the imprinted expression of *ZAC* and of another, close-by imprinted gene called *HYMAI* [33]. The resulting loss of imprinting (biallelic expression) of *ZAC*, particularly in the pancreas and/or pituitary, is thought to induce TNDM [34,35]. Specifically, the *ZAC* transcription factor is involved in cell-cycle control and insulin secretion in the pancreas. Mouse studies have shown that it regulates the expression of several other imprinted genes, including *Igf2* and *Cdkn1c*, and influences the allelic silencing activity of the ICR of the *Kcnq1* imprinted domain [36]. It is possibly through the effects of *ZAC* on other imprinted loci that TNDM shows clinical overlap with BWS (see also ‘Multilocus DNA methylation defects in imprinting disorders’ section).

■ Pseudohypoparathyroidism

Pseudohypoparathyroidism (PHP; OMIM 103580) represents a heterogeneous group of disorders whose common feature is end-organ resistance to parathyroid hormone leading to hypocalcemia, hyperphosphatemia and obesity [37]. Patients have a short stature, a rounded face and often mild mental retardation, and other characteristic features jointly termed Albright’s hereditary osteodystrophy. These syndromes are due to genetic or epigenetic mutations affecting the *GNAS* locus on chromosome 20q13.11, which encodes the stimulatory G-protein subunit G_{α} , necessary for hormone signal transduction. *GNAS* transcription is predominantly biallelic with selective imprinting and alternative promoter usage in different tissues. PHP1a arises upon maternal inheritance of molecular defects at *GNAS*. Epimutations – particularly loss of allelic DNA methylation at exon 1A – result in PHP1b. PHP1b patients have renal parathyroid hormone resistance, decreased cAMP response to parathyroid hormone infusion and normal erythrocyte Gs activity, resulting in loss of the maternal allele expression in renal tissue, but without other features of Albright’s hereditary osteodystrophy, presumably because activity is maintained in tissues where G_{α} is expressed biallelically. Approximately 15–20% of PHP1b patients display a specific loss of methylation at the *GNAS*DMRs, whereas most PHP1b patients (80–85%) display a broad loss of imprinting at the *GNAS* domain [38,39].

■ Prader–Willi syndrome & Angelman syndrome

The Prader–Willi (PWS; OMIN 176270) [40] and Angelman (AS; OMIN 105830) [41,42]

syndromes are rare neurodevelopmental genetic syndromes (incidence one in 10,000) and were the first reported imprinting diseases. They both map to human chromosome 15q11–13, a large imprinted domain comprising many genes that are expressed from the paternal chromosome in the brain, and one gene (*UBE3A*) expressed from the maternal chromosome. Whereas PWS is most often caused by maternal UPD of chromosome 15, AS is caused most frequently by maternal deletion of chromosome 15q11–q13. The clinical phenotype of PWS is highly complex and caused by loss of expression of multiple paternally expressed genes on the domain. By contrast, AS is caused by loss of expression of the single maternally expressed gene *UBE3A*, which maps to 15q11–13. Since PWS and AS are caused almost exclusively by genetic mutations affecting the imprinted domain itself [6,7], it has not been systematically explored in humans whether *trans*-acting factors could also be affected. Mouse studies, however, have identified several proteins involved in the embryonic maintenance of DNA methylation imprint at the domain’s ICR (see ‘*Trans*-acting factors involved in imprinting-related diseases’ section).

■ Uniparental disomy 14 syndromes

Maternal UPD of chromosome 14 is an extremely rare condition, which causes pre- and post-natal growth retardation, congenital hypotonia, joint laxity, motor delay and mild mental retardation. Paternal UPD of chromosome 14 (OMIM 608149) has a more severe phenotype, with polyhydramnios (excess of amniotic fluid), thoracic and abdominal wall defects, growth retardation and severe developmental delay. Cases of segmental UPD of chromosome 14 indicated that the distal portion of chromosome 14q is the critical region in these syndromes [43]. The 14q32.2 region contains a large cluster of imprinted genes that include the *DLK1*, *RTL1* and *DIO3* genes expressed from the paternal chromosome, and the *GTL2* (*MEG3*) and *MEG8* (*RIAN*), the antisense genes to *RTL1* (*RTL1as*), and miRNA and snoRNA gene clusters expressed from the maternal chromosome [44]. This imprinted domain is under the control of an ICR called the intergenic germline DMR [45], which is methylated on the paternally inherited chromosome. Gene-targeting studies in mice suggest that excessive *Rtl1* expression could explain the phenotype observed in patients with paternal UPD of chromosome 14, while decreased *Dlk1* and *Rtl1* gene expression could account for maternal UPD of chromosome 14 [43].

Several recent reports described deletions and epimutations affecting the imprinted *DLKI-DIO3* region at chromosome 14q32.2 in individuals with a phenotype similar to that of maternal UPD of chromosome 14 (but who did not present evidence for the occurrence of UPD) [46–48]. In these studies, loss of methylation was detected at the ICR (the intergenic germline-DMR) and also at the DMR comprising the *MEG3* promoter, indicating the occurrence of an epimutation (hypomethylation) affecting both of these paternally methylated DMRs [46–48].

Multilocus DNA methylation defects in imprinting disorders

In 2005, for the first time, Arima and his colleagues showed that the imprinted genes *ZAC*, *KCNQ1OT1* (*LIT1*) and *CDKN1C* are likely part of a common imprinted gene network that plays a role in BWS [49]. They found that *ZAC* bound to and activated the expression of another imprinted gene, *KCNQ1OT1*. The *KCNQ1OT1* promoter corresponds to the ICR of the imprinted *KCNQ1* domain, and expression of this ncRNA negatively regulates the *CDKN1C* gene in *cis*. The authors discovered that some patients with TNDM (two out of 17) had defects in DNA methylation at the *KCNQ1OT1* promoter/ICR region, further supporting the proposed link between these two imprinted genes. Following this initial report, a breakthrough study on TNDM reported that besides loss of DNA methylation at *ZAC*, hypomethylation also occurred at several other imprinted gene loci, including at *GRB10* (7p12), *PEG1* (7q32), *KvDMR1* (11p15; *KCNQ1* domain) and *PEG3* (19q13) [50]. The imprinted genes *SNRPN* (15q11–q13) and *H19* (11p15) remained unaltered however. In these patients, epigenetic mosaicism was inferred by the extent of the methylation defects and the gene loci and tissues that were affected. Interestingly, TNDM patients with multilocus methylation changes presented a higher weight at birth and a broader clinical spectrum in comparison to ‘classical’ TNDM patients. This could be explained by a deregulation of gene expression occurring at additional imprinted genes. Since there was loss of maternal allele-specific DNA methylation at all loci involved, it was hypothesized that the multilocus cases of TNDM might represent a disease syndrome of maternal hypomethylation. It is postulated that, in these patients, there is not only hypomethylation at *ZAC1/PLAGL1*, but also different degrees of hypomethylation at other ICRs and DMRs as well. The combined changes would make it difficult to readily link

DNA methylation levels at individual loci to a defined clinical etiology [50].

Recent studies on imprinting-related syndromes provide further evidence for multilocus methylation deregulation. One recent study carefully assayed allele-specific DNA methylation levels at 27 DMRs in a total of 65 patients with imprinting-related diseases [51]. The authors thus identified multilocus hypomethylation in numerous BWS, TNDM and PHP1b patients, and also in one individual with SRS. Other studies on SRS [52–54] revealed that 7% of the patients with *H19*-ICR hypomethylation also displayed hypomethylation at additional imprinted loci, and these changes frequently included the developmentally important *DLKI-DIO3* imprinted locus [52]. Other recent work indicates that BWS patients show multilocus loss of DNA methylation in approximately 25% of the cases [55], and the most affected loci, besides *KvDMR1* (the ICR of the *KCNQ1* domain), are *GNAS*, *IGF2R* and *PEG1* [56,57]. Similarly, a recent study reported that in some PHP1b patients, the loss of methylation at the *GNAS* locus had occurred concomitantly with lesser methylation changes at other imprinted loci [58]. Interestingly, in the latter study, both gains and losses of DNA methylation were reported. A recent publication, however, concluded that multilocus hypomethylation is an uncommon event in PHP patients [59]. Combined, novel data reveal the existence of a broad range of epimutations in several different imprinting-related disorders, which show partial overlap in their clinical phenotypes [51]. Based on these novel findings, by many different groups, hypothesis of ‘epidominance’ was put forward, in which the clinical phenotype is caused mostly by the imprinted locus at which the DNA methylation is most severely affected [58]. The new studies also emphasize that molecular testing for the above-described syndromes should not be restricted to the canonical causal locus [12].

Research has started to address whether genetic mutations in genes coding for *trans*-acting factors could explain the concomitant occurrence of methylation changes at multiple loci in some of the patients. So far, however, only few *trans*-acting factors have been causally linked to imprinting-related diseases. One of these is the zinc finger (ZF) protein, *ZFP57*, which is discussed below.

Trans-acting factors involved in imprinting-related diseases

As described previously, the involvement of *trans*-acting factors was first suspected when

BWS and TNDM patients were found to present loss of DNA methylation at multiple loci other than the canonical loci affected in these diseases [9,56]. Below, we discuss the still limited number of *trans*-acting factors known to be perturbed in imprinting-related diseases. We also present *trans*-acting factors that emerged from recent animal studies whose involvement in human diseases remains to be explored. *Trans*-acting factors involved in imprinting maintenance are listed in TABLE 1. Their potential 'mode of action' in imprinting-related diseases is depicted in FIGURE 1.

■ ZFP57 & KAP1

ZFP57 is a nuclear protein, which protects ICRs and other DMRs against loss of methylation in the embryo. Its key role initially emerged from studies on TNDM pedigrees, in which loss of DNA methylation at multiple DMRs was found to be linked to homozygous mutations in the *ZFP57* gene [9,56]. Subsequently, additional loss-of-function mutations were identified at

the human *ZFP57* gene. A recently discovered 1-bp deletion resulting in premature translation termination and a truncated protein, were associated with hypomethylation at multiple maternal-methylated ICRs in TNDM patients [9,51]. Gene-targeting experiments in mice indicated that this KRAB-domain zinc-finger protein (ZFP) is required for the early embryonic maintenance of DNA methylation imprints [60], and binds to a methylated hexanucleotide motif (TGCCGC) that is present in most ICRs [61]. Indeed, similarly to the first and second ZFs, the third and fourth ZFs of human ZFP57 recognize the TGC^{met}CGC motif, and two mutations (R248H and H277N) associated with TNDM were shown to affect binding to this methylated DNA sequence [62]. This critical transcription factor is expressed in pluripotent cells and maintains the somatic methylation of imprinted domains through the recruitment of KAP1 (also called TRIM28 or TIF1 β), which recruits repressive chromatin modifiers, including ESET (also called SETDB1), a lysine

Table 1. *Trans*-acting factors of imprinted gene expression that have emerged from studies on human imprinting disorders and from mouse studies.

Species	<i>Trans</i> -acting factor	Cofactor(s)	Function	Phenotype due to mutation/protein deficiency	Human disease	Ref.
Human	ZFP57	KAP1	Binds DNA at many methylated ICRs	Hypomethylation of multiple ICRs	TNDM BWS	[9,51]
Mouse	KAP1	SETDB1	Scaffold protein for heterochromatin factors	Hypomethylation of multiple ICRs	–	[64]
Mouse	ZFP42	–	Pluripotency transcription factor, similarity to YY1	Aberrant acquisition of DNA methylation at <i>Gnas</i> and <i>Peg3</i>	–	[5]
Human	NLRP7	C6orf221	Unknown	Hypomethylation at multiple ICRs	FBHM	[74]
	NLRP2	–	Unknown	Loss of methylation at KvDMR1 ICR (<i>KCNQ1</i> domain)	BWS	[67]
Human	C6orf221	NLRP7	Unknown	Hypomethylation at maternal ICRs	FBHM	[76,77]
Human	OCT4	SOX2	Pluripotency transcription factor. Protection against DNA methylation	Mutation of OCT4/SOX2 sites at specific ICRs leads to hypermethylation	BWS	[79,80]
Mouse	CTCF	Cohesin, OCT4	Higher-order chromatin organization	Gain of methylation at <i>H19</i> -ICR	–	[96]
Mouse	DPPA3	RanBP5	Protection against DNA demethylation in mouse	Hypomethylation at several maternal and paternal ICRs in mice	–	[99]
Mouse	ARID4A & ARID4B	SIN3A	Maintenance of DNA methylation and histone methylation	Loss of methylation at <i>Snrpn</i> ICR (<i>PWS</i> domain)	–	[104]
Mouse	MBD3, MTA2	–	Components of the NuRD complex	Hypomethylation at at <i>H19</i> -ICR and <i>Peg3</i>	–	[105,106]

BWS: Beckwith–Wiedemann syndrome; FBHM: Familial biparental hydatidiform mole; ICR: Imprinting control region; PWS: Prader–Willi syndrome; TNDM: Transient neonatal diabetes mellitus.

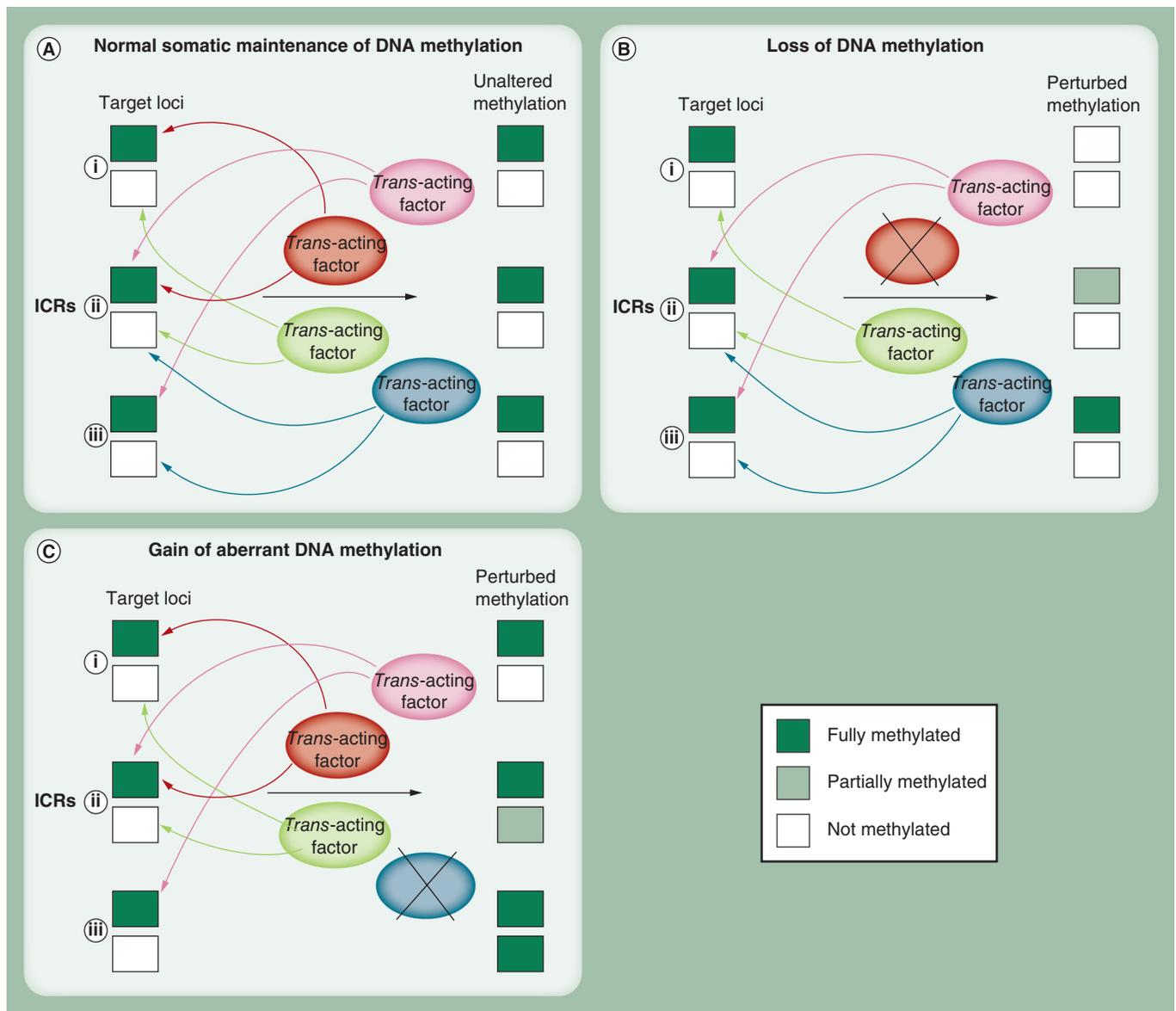


Figure 1. Involvement of *trans*-acting maintenance factors in imprinting-related diseases. (A) Normal maintenance of differential DNA methylation at ICRs. Multiple proteins ensure that the DNA-methylated alleles (dark green boxes) of ICRs remain methylated during development. Many of these *trans*-factors act on subsets of ICRs. For simplicity, three ICRs are depicted, with two methylation maintenance factors (red and pink circles) that have overlapping targets (left part of the figure). Conversely, the unmethylated alleles of ICRs need to remain unmethylated, which involves other *trans*-acting factors that protect against acquisition of *de novo* methylation. For simplicity, only two such protective *trans*-factors are depicted (green circles), each with a different combination of targets. The right part of the figure indicates that the allelic DNA methylation is maintained faithfully at the three ICRs. (B) Aberrant loss of DNA methylation at ICRs in imprinting diseases due to mutations in, or altered expression of, *trans*-acting factors. In the example given, the functional loss of one of the *trans* factors (indicated with a cross) leads to a complete loss of methylation at one, and partial loss of methylation at another of its target ICRs (right part of the figure). (C) Gain of aberrant DNA methylation in disease due to mutations in, or altered expression of, a *trans*-acting factor. In the example given, the functional loss of a protective *trans*-acting factor (depicted with a cross) leads to hypermethylation at one ICR, and a smaller gain of methylation at another ICR (right part of the figure). ICR: Imprinting control region.

For color figure, please see www.futuremedicine.com/doi/full/10.2217/epi.13.66

methyltransferase that deposits H3K9me3 onto chromatin [61]. The latter repressive mark is recognized by the UHRF1 (also known as NP95 or ICBP90), which facilitates recruitment of the maintenance DNMT1 following DNA replication [63]. Although the precise relationship between these different protein factors remains

unclear, combined, these proteins ensure that DNA methylation imprints are efficiently maintained, particularly during the critical preimplantation stages of development. Concomitantly, in KAP1-deficient early mouse embryos and in cancer cells, loss of DNA methylation occurs at multiple ICRs [64,65].

■ ZFP42

Whether related ZF proteins could also be involved in the perturbation of DNA methylation imprints in disease, is unknown. In the mouse, however, loss of ZFP42 expression was found to induce aberrant acquisition of DNA methylation at the *Gnas* and *Peg3* imprinted domains [66].

■ NLRP2 & NLRP7

NLRP2 and *NLRP7* (also called *NALP2* and *NALP7*) encode proteins that comprise a nucleotide-binding oligomerization domain, a leucine-rich repeat and a pyrin domain (NLRP), and belong to a family of cytoplasmic proteins of unknown function comprising 14 members. *NLRP2* is highly homologous to *NLRP7*, and both proteins are expressed in oocytes and pre-implantation embryos. In rare cases of BWS that presented loss of DNA methylation at the KvDMR1 ICR (the *KCNQ1* domain), it was found that this methylation defect was linked to a frame-shift mutation in exon 6 of *NLRP2* [67], revealing, for the first time, a *trans* mechanism in BWS. A complete hydatidiform mole represents a highly aberrant developmental phenotype resulting from a general perturbation of genomic imprinting. In most cases of complete hydatidiform mole, the conceptus is entirely androgenetic, with two paternal genomes [68]. In a similar way as observed in mice [1,2], the lack of a maternal genome mostly results in proliferation of the extra-embryonic tissues, while embryonic development fails [69]. A rare familial, biparental form of this extremely severe clinical phenotype, named familial biparental hydatidiform mole (FBHM), has been identified, in which affected women suffer from recurrent abnormal pregnancy with no embryonic development and cystic degeneration of placental villi [70]. In different cases of FBHM, there was a failure to establish maternal imprints in the oocyte [71–73]. The imprint acquisition failure at multiple maternal ICRs suggested that a *trans*-acting factor was genetically affected. In 2006, recessive mutations in *NLRP7* were identified as one cause of FBHM and further studies demonstrated that *NLRP7* is mutated in a majority of FBHM families [74]. It remains to be determined precisely how *NLRP7* contributes to maternal imprint establishment during oogenesis.

■ C6orf221

Some FBHM families do not present *NLRP7* mutations, showing that there is genetic heterogeneity in familial hydatidiform moles [72,75].

Mutations in *C6orf221* – a member of a rapidly evolving gene family specific to eutherians – were identified as a second genetic cause of FBHM [76]. *C6orf221* (also called *ECAT1*) is part of a group of four highly similar genes within a 100-kb cluster on human chromosome 6 [77]. Members of this gene family display a mostly oocyte and/or early embryonic expression pattern. This highly specific expression pattern, and the fact that genetic mutations in *C6orf221* and *NLRP7* induce identical phenotypes (i.e., absence of maternal imprints), make it likely that *NLRP7* and *C6orf221* participate in an oocyte-specific protein complex involved in imprint establishment. Indeed, the *C6orf221* and *NLRP7* proteins were found to interact [76]. Further studies are required to elucidate the precise molecular mechanisms, and to which extent these factors could also be important in the embryonic maintenance of imprints.

■ Involvement of pluripotency factors

Dynamic, genome-wide changes occur in DNA methylation during the preimplantation stages of development. Concomitant to these global processes, developmental pluripotency is acquired in the inner cell mass of the blastocyst. Despite the major transitions in the embryonic cells that are becoming pluripotent, remarkably, the differential epigenetic status of ICRs persists during preimplantation development [4]. This has raised the question as to whether pluripotency factors could be involved in epigenetic maintenance processes at imprinted loci. Recent genetic studies indicate that this is indeed the case. Point mutations and small deletions at OCT4- (also called POU5F1) and SOX2-binding sites in BWS patients at the *H19*-ICR were found to correlate with acquisition of DNA methylation at this ICR-controlling *IGF2* expression [78–80]. In the reported cases, the defects were transmitted through the female germline only, with no phenotypic consequences following paternal transmission of the genetic mutations [79,80]. This interesting finding strongly suggests that binding of pluripotency factors to this ICR, on the unmethylated maternal allele, is essential in keeping this parental allele unmethylated through preimplantation development.

A potential role of OCT4 and SOX2 binding was also discovered at the ICR linked to the neurodevelopmental AS. In mechanistic studies in the mouse, mutation of either OCT4- or SOX2-binding sites resulted in a significant maternal allele-specific gain of methylation at this control region in oocytes [81].

Recently, it was experimentally confirmed that SOX2–OCT4 motifs (octamer motifs and their flanking sequences) in the ICR determine the cell type, DNA region and allele specificity of DNA methylation [82,83]. Another pluripotency factor, which contributes to the maintenance of the differential DNA methylation, at least in the mouse, is ZFP42 (also called REX1) [66]. This protein is highly expressed in the early embryo and is similar to the broadly expressed YY1 transcription factor.

Even if the mechanism of action of pluripotency factors themselves remains unclear, it is interesting to note that OCT4 and SOX2 binding has also been reported at the X-chromosome inactivation center, and both transcription factors are required for appropriate X-chromosome inactivation [84]. OCT4 interacts directly with chromatin structural CTCF–cohesin complexes and its depletion induces inactivation on both X chromosomes upon differentiation of embryonic stem cells [84]. Mechanistically, OCT4 antagonizes cohesin binding. It thus acts as a negative regulator of chromosome-loop formation mediated by cohesin association to CTCF-binding sites [85]. It should be interesting to explore whether, besides their apparent protective effects against *de novo* DNA methylation, similar structural mechanisms are mediated by OCT4 binding to specific ICRs.

■ Involvement of CTCF & cohesins

CTCF is to be a major DNA-binding protein, which is able to establish insulators [86]. Specifically, CTCF binding can block enhancer function when it occurs between enhancers and promoters [87,88]. One of the best illustrations of such an enhancer-insulating function is provided by the *H19/IGF2* locus. The differentially methylated ICR of this imprinted locus contains a cluster of CTCF-binding sites, and imprinted *H19/IGF2* expression is regulated by selective binding of CTCF to the unmethylated allele. CTCF binding on the maternal chromosome mediates an allele-specific higher-order chromatin configuration at the domain and thus constitutes an enhancer-blocking transcriptional insulator of the *IGF2* gene [89,90]. By contrast, methylation of the paternal allele prevents CTCF binding and thus prevents the establishment of the insulator function, allowing *IGF2* expression on this parental chromosome [89–92]. Several genome-wide studies have shown frequent colocalization of CTCF and cohesin, and CTCF binding is strictly required to recruit cohesin to the chromatin [93]. Conversely, cohesin seems to play a

critical role in maintaining CTCF-mediated chromatin conformation [94]. Mutation of all four CTCF-binding sites in the *H19*-ICR leads to aberrant gain of DNA methylation on the maternal allele during postimplantation development [95,96]. In BWS, small maternally inherited ICR deletions (1.4–2 kb) that remove one or more of the CTCF sites were reported to be associated with an aberrant gain of DNA methylation at the remaining target sites, leading to increased expression of *IGF2* [97,98].

■ DPPA3

Mouse studies have shown that maternal proteins contribute to the protection against DNA demethylation following fertilization. DPPA3 (also called PGC7 or STELLA) is highly expressed during oogenesis and persists in the preimplantation embryo. Gene targeting experiments in the mouse have shown that after fertilization, up to the two-cell stage, *Dppa3* plays a crucial role in protecting the maternal genome against DNA demethylation. This action includes the maternally methylated ICRs at the *Peg1 (Mest)*, *Peg3* and *Peg10* loci. Additionally, on the paternal genome, DPPA3 protects the paternally methylated gene loci *Dlk1-Dio3*, *H19-Igf2* and *Rasgrf1* against active DNA demethylation in the early embryo [99,100]. Interestingly, DNA methylation of *H19* and *Rasgrf1* ICRs was not maintained in pronuclear five-stage embryos derived from DPPA3-null oocytes, whereas that of the *Dlk1-Dio3* domain ICR was. These observations suggest that the mechanisms of epigenetic maintenance at the *H19-Igf2* and *Rasgrf1* domains could differ from that at the *Dlk1-Dio3* domain. Possibly, some of the observed effects could also be linked to the reported role of DPPA3 in chromatin condensation during oogenesis [101], which might trigger additional histone modifications that protect against DNA demethylation. Interestingly, chromatin immunoprecipitation studies showed that the repressive H3K9me2 is present at the *H19* and *Rasgrf1* ICRs, but not at the ICR of the *Dlk1-Dio3* domain [102]. DPPA3 appears to protect the maternal genome against the TET3 protein-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine through its competing association with H3K9me2-marked chromatin [102,103]. Combined, the studies are consistent with a model in which inhibition of TET3 activity, through the binding of DPPA3 to H3K9me2, provides protection against DNA demethylation during the very early stages of development.

■ ARID4A & ARID4B

Arid4A and *Arid4B* are closely related members of the *ARID* gene family. They encode RBP1 and RBP1L1, respectively, which are both members of chromatin-remodeling complexes and could recruit the histone deacetylase-dependent SIN3A transcriptional corepressor. Targeted deletion of both of these genes in mice resulted in loss of DNA methylation and reduced H3K9me3 and H4K20me3 on the maternal allele at the *Snrpn* ICR controlling the PWS imprinted domain [104]. The study suggests that these proteins are involved in the maintenance of imprinting; however, their function in humans remains to be explored.

■ Other chromatin-associated proteins

Several other chromatin regulatory proteins are involved in the embryonic maintenance of allelic DNA methylation at individual ICRs in the mouse. These include two components of the NuRD complex: MBD3 and MTA2. Knock-down studies were performed in embryonic cells on both of these proteins and this was found to lead to a partial loss of the paternal DNA methylation at the *H19*-ICR, but not at several other ICRs [105,106]. These data suggest that recruitment of the NuRD chromatin remodeling complex contributes to the DNA methylation at the *H19*-ICR, and therefore constitutes a candidate for involvement in SRS.

Conclusion & future perspective

Several novel insights have emerged into the genetic and epigenetic origins of imprinting disorders. Possibly the most relevant novel finding is that in some patients, altered DNA methylation affects not only the canonical ICR involved in the disease, but multiple other ICRs/DMRs are affected to different extents. A multilocus aberrant DNA methylation phenotype has now been reported in different diseases and strongly suggests that common *trans*-acting factors are genetically affected in some of the patients. One major challenge for the future will be to identify these factors. Besides the exceptional involvement of ZFP57, no other *trans*-acting maintenance factor has been reported to be mutated in the multilocus methylation phenotype. A recent study on the DNMT3-L protein (involved in imprint establishment) shows that this candidate protein is not involved in aberrant multilocus methylation [51], but few other systematic studies have been performed on other candidates so far.

The recent reports on *H19*-ICR microdeletions and point mutations in BWS raise the question of what percentage of patients presenting small

genetic mutations, including single nucleotide changes, could be responsible for the observed epimutations (hypomethylation and hypermethylation) at specific ICRs? To address this issue, it should be relevant in future studies to not only assess DNA methylation levels at ICRs, but to also carefully study their DNA sequences.

The novel insights also present additional challenges for the future. For instance, are changes confined to specific ICRs, and which ones, or are other DMRs in the genome affected? New technologies are already being developed to systematically assess DNA methylation at multiple ICRs and DMRs [31] or to detect UPDs with a high sensitivity for the estimation of their size and low-rate mosaicism [51,107]. Ideally, such studies should encompass all of the regulatory gene regions of the genome, an approach which becomes feasible with the emergence of affordable high-throughput sequencing approaches [108]. Additionally, it should be interesting to explore repressive histone modifications, particularly those that are linked to the somatic maintenance of DNA methylation imprints [109]. A major handicap remains the limiting availability of suitable tissue material, particularly to address whether aberrant methylation patterns are tissue-specific or are detected broadly in different lineages.

The occurrence of ICR point mutations and microdeletions highlights the importance of 'hunting' for genetic defects in *cis* in case the methylation change is confined to the canonical ICR associated with the imprinting disorder. In case the methylation change affects multiple imprinted (and nonimprinted) gene loci, however, it should seem relevant to explore whether *trans*-acting factor genes are mutated or altered in their expression. In such genetic studies, it might not be sufficient to include only the known *trans*-acting factors of imprinting disorders. Novel candidates emerging from mouse studies could be included in a targeted screen as well. Particularly when a familial component is suspected, it should be worthwhile to use broad, high-throughput sequencing approaches for mutation detection (e.g., exome sequencing). Hopefully, in the years to come, it will become clearer how commonly *trans*-acting factors are perturbed in imprinting-related diseases in comparison with other causal mechanisms. Despite the many technological challenges ahead, these are exciting novel avenues of research. The coming years will undoubtedly pinpoint new players involved in imprinting-related diseases, and may shed further light on the extent to which these epigenetic diseases could be genetic in origin.

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Executive summary**Imprinting disorders**

- Many congenital disorders are caused by perturbed imprinted gene expression. Some of these complex diseases are caused almost exclusively by genetic mutations at specific imprinted domains.
- Other imprinting disorders seem to be caused mostly by epigenetic alterations ('epimutations').

Epimutations that produce imprinting disorders

- DNA methylation defects at specific imprinted gene domains are causally involved in several imprinting disorders.
- In most cases, there is decreased or increased DNA methylation at imprinting control regions; the *cis*-regulatory elements that control imprinted gene expression.
- Although the reported DNA methylation changes at imprinting control regions are often not accompanied by gross genetic changes, there could nevertheless be a genetic origin of the epimutation(s) in many of the patients.

Multilocus DNA methylation defects in some patients

- Several recent studies show that in some patients with specific imprinting disorders, methylation defects occur in concert at multiple imprinted loci.
- This novel finding strongly suggests that common *trans*-acting factors are (genetically) affected in these patients.

Trans-acting factors involved in imprinting disorders

- Several recent studies have identified *trans*-acting factors that are mutated in some patients with human imprinting disorders.
- Other candidate *trans*-acting factors have emerged from recent animal studies.

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