

1 **Causes and consequences of multi-locus imprinting disturbances in humans.**

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34 **Abstract**

35 Eight syndromes are associated with loss of methylation at specific imprinted loci. There  
36 has been increasing evidence that these methylation defects are not isolated events  
37 occurring at a given disease-associated locus but that some of these patients may have  
38 multi-locus imprinting disruptions (MLID) affecting additional imprinted regions.

39 With the recent advances in technology, methylation profiling has revealed that  
40 imprinted loci represent only a small fraction of the methylation differences observed  
41 between the gametes. To figure out how imprinting anomalies occur at multiple imprinted  
42 domains, we have to understand the interplay between DNA methylation and histone  
43 modifications in the process of selective imprint protection during pre-implantation  
44 reprogramming, which if disrupted leads to these complex imprinting disorders.

45

#### 46 **Key words**

47 Imprinting, germline methylation, ZFP57, NLRPs, multi-locus imprinting disturbances

48

#### 49 **Life cycle of Imprints.**

50 DNA methylation on imprinted differentially methylated regions (DMRs) is transmitted to  
51 the embryo from the gametes, where the asymmetrical marking is established during  
52 gametogenesis. Studies in mice reveal that *DNMT3L* regulates the *de novo* methylation  
53 activity of DNMT3A on DMRs by stimulating its enzymatic activity and facilitating  
54 binding to unmodified H3K4 (H3K4me0) [1-5]. During epigenetic reprogramming in the  
55 embryo imprinted methylation is protected against erasure and is subsequently maintained  
56 by DNMT1-UHRF1 [6-7](Figure 1). Two proteins have been implicated in the  
57 maintenance of the maternal and paternal DNA methylation at DMRs, DPPA3 (also known  
58 as PGC7/Stella) and the KRAB zinc finger protein ZFP57 protein, both of which are  
59 conserved between mice and humans [8, 9](Figure 2). Conversely to the demethylation  
60 wave in the pre-implantation embryo, there is a *de novo* DNA methylation wave at the time  
61 of implantation from which the unmethylated alleles of DMRs require protection, which  
62 has been shown to involve CTCF, OCT4 and the permissive histone modification  
63 H3K4me2/3 [10-12].

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#### 67 **Imprinting disorders and aberrant DNA methylation.**

68 Alterations in any of the above processes can lead to aberrant imprinting, which can result  
69 in either the reactivation of the original silent allele or the silencing of the previously active  
70 allele. Since methylation profiles are faithfully copied during replication, an abnormal  
71 imprinted methylation profile will be maintained through somatic development and be  
72 present in multiple tissues. If methylation defects occur in only a few cells of the pre-  
73 implantation embryo, then somatic mosaicism will result [13].

74 It is currently unknown if the DNA methylation defects associated with imprinting  
75 syndromes are due to primary epimutations that result from the direct disruption of  
76 methylation at imprinted DMRs, which may be influenced by transient environmental  
77 exposures, or to secondary epimutation resulting from an initial genetic mutation in a *cis*-  
78 acting element or *trans*-acting factor involved in establishment or maintenance of imprinted  
79 methylation.

80

#### 81 **Multi-locus imprinting defects by ID:**

##### 82 *Chromosome 6q24- TNDM.*

83 Transient Neonatal Diabetes Mellitus (TNDM, OMIM 601410) is caused by loss of  
84 imprinting of the *PLAGL1* domain (LRG\_1035), with affected patients suffering from  
85 severe intrauterine growth restriction [14] and transient neonatal diabetes mellitus that often  
86 becomes permanent in teenage years. Approximately half of the patients with *PLAGL1*  
87 methylation defects also have additional hypomethylation of other maternally methylated  
88 imprinted regions. Mackay and colleagues coined the term “maternal hypomethylation  
89 syndrome” [15], with the same group identifying recessive mutations of *ZFP57* in TNDM  
90 cases associated with hypomethylation of *PLAGL1* and invariably *GRB10* and *PEG3* [16-  
91 18]. Interestingly individuals with multi-locus imprinting disturbances (MLID) without  
92 *ZFP57* mutations were more severely affected than those with *ZFP57* aberrations, with  
93 additional DMRs (*DIRAS3*, *IGF2R*, *MEST*, *KCNQ1OT1*, *IGF1R*, *ZNF331*, *WRB* and  
94 *SNU13*) frequently being hypomethylated [17, 18]. However it must be noted that these  
95 observations are based on genome-wide methylation screening using high-density or  
96 imprint-targeted arrays in only two ID cohorts, so the frequency and affected loci may  
97 change with additional investigations.

98 At the phenotypic level, the cases of MLID associated with TNDM are largely  
99 indistinguishable from other TNDM subgroups [15, 19]. However, subtle heterogeneous  
100 non-diabetes features such as learning difficulties, hypotonia, macroglossia, umbilical hernia  
101 and congenital heart disease may occur more frequently in those with MLID [17, 19, 20].

102 All of the 14 cases reported with *ZFP57* mutations seemed to follow the classical  
103 progression of the disease and any phenotypic differences between *ZFP57* mutated and  
104 idiopathic MLID cases may be due to additional deleterious variants in these highly  
105 consanguineous families.

106

107 *Chromosome 11p15- BWS.*

108 Beckwith-Wiedemann syndrome (BWS, OMIM 130650) is a growth disorder characterized  
109 by macrosomia, macroglossia, visceromegaly, ear creases, hypoglycemia, hemihypertrophy  
110 and abdominal wall defects with an increased risk of pediatric tumours [21]. The molecular  
111 alterations in BWS involve two separate imprinted domains on chromosome 11, with  
112 sporadic hypomethylation of the *KCNQ1OT1* DMR (also known as *KvDMR1*, ICR2)  
113 (LRG\_1052) being the most frequently observed. A gain of methylation at the *H19*  
114 intergenic DMR (LRG\_1030) is detectable in ~5% of cases, with the remainder of BWS  
115 individuals having paternal uniparental disomy of 11p15 or *CDKN1C* mutations  
116 (LRG\_533) [22, 23].

117 The first molecular confirmation of MLID involving the chromosome 11p15 locus  
118 described two TNDM patients with hypomethylation of both the *PLAGL1* and *KCNQ1OT1*  
119 DMRs [24]. Interestingly, one of these TNDM patients had UPD(6)pat, the other a  
120 epimutation of the *PLAGL1* DMR. This second patient presented with classic TNDM  
121 complicated with umbilical hernia and macroglossia, features commonly seen in patients  
122 with BWS. Following this study several groups confirmed MLID in BWS cohorts, with a  
123 frequency of up to 30% of those individuals with an underlying *KCNQ1OT1* methylation  
124 defect [17, 18, 25-30]. Two recent papers have described methylation anomalies at  
125 additional imprinted loci in patients with *H19* hypermethylation [31, 32]. The MLID  
126 observed in BWS are notably different from those observed in TNDM, with both gains and  
127 losses of methylation observed at maternal and paternal DMRs. The paternally methylated  
128 DMRs associated with *ZBDF2*, *NESP* and *ZNF597/NAA60* have been shown to gain  
129 methylation in subsets of BWS patients [17, 31]. This acquisition is due to a concomitant  
130 loss of methylation in the nearby maternally methylated *GPRI-AS*, *GNAS* and *ZNF597*  
131 DMRs, which are known to regulate the methylation of these somatic DMRs in a  
132 hierarchical fashion [12, 33, 34].

133 Although the techniques used to determine MLID vary between laboratories, it  
134 seems that the DMRs associated with *PLAGL1*, *GRB10*, *MEST*, *GNAS*, *IGF2R* and *ZNF331*  
135 are the most frequently disrupted in BWS with MLID (Figure 3) [17, 18, 30]. The

136 maternally methylated region within intron two of *IGF2R* has been observed to be hypo- or  
137 hypermethylated in BWS and TNDM patients [17, 18, 30] with lower rates observed in  
138 control individuals [30], suggesting that this may be in part a stochastic event.

139 Numerous studies have revealed LOM of the *H19* and *KCNQ1OT1* DMRs  
140 coexisting in the same patient. Hypomethylation at *H19* is normally associated with growth  
141 restriction associated with SRS, while *KCNQ1OT1* hypomethylation is associated with  
142 macrosomia [17, 26, 35]. It is unclear why different patients with apparently similar  
143 patterns of LOM in these two loci may have different predominating presentations. It has  
144 been proposed that the dominant phenotype is defined by the locus with the most severe  
145 hypomethylation or the most affectedness in a target organ [13]. This may not always be  
146 apparent by molecular testing, which is often performed on blood-derived DNA.

147 Complex phenotypes may also be observed when loci other than *H19* and  
148 *KCNQ1OT1* are involved. Recently BWS and PHP1B were described in a single patient  
149 with MLID [36]. Alternatively, one phenotype can dominate over another: for example, an  
150 infant with severe LOM at both *PLAGL1* and *KCNQ1OT1* presented neonatally with BWS  
151 and without neonatal diabetes, but later relapsed with adult diabetes (D Mackay, personal  
152 communication). However, not all BWS cases with hypomethylation of *PLAGL1* or *GNAS*  
153 have a history of TNDM or pseudohypoparathyroidism, respectively [17, 26, 35]. In a  
154 patient with a clinical diagnosis a BWS after assisted reproductive technology, Lim et al  
155 [27] found normal methylation at *KCNQ1OT1* DMR but LOM at *H19*, *PLAGL1* and *MEST*  
156 DMRs. In sum, the clinical presentation of a MLID patient probably reflects the severity  
157 and tissue mosaicism of hypomethylation in different tissues; but further molecular and  
158 clinical research is needed to understand and predict the resultant phenotypes in different  
159 individuals.

160 For the majority of BWS patients with MLID no additional clinical features have  
161 been noted [30, 35]. Two studies with deep phenotyping data suggest that developmental  
162 delay and abnormal glycemic control are slightly more prevalent in those patients with  
163 additional loci affected, as are additional congenital abnormalities [17, 29].

164

#### 165 *Chromosome 11p15- SRS.*

166 Silver Russell syndrome (SRS, OMIM 180860) is a clinically heterogeneous disorder  
167 characterized by severe IUGR, postnatal growth failure, craniofacial features such as a  
168 triangular shaped face and broad forehead, body asymmetry and a variety of minor  
169 malformations. In ~40% of patients hypomethylation of the *H19* intergenic paternally

170 methylated region is observed [37]. MLID has been described in ~15% of SRS cases with  
171 *H19* hypomethylation with common deregulated methylation at *DIRAS3*, *PLAGL1*, *GRB10*,  
172 *MEST*, *IG-DMR*, *ZNF331*, *WRB* and *SNU13* DMRs (figure 3) [17, 18 35, 38, 39].  
173 Therefore, similar to BWS cases with MLID both paternally and maternally methylated  
174 DMRs are affected. Remarkably, the hypomethylation observed in SRS is often less severe  
175 when compared to other IDs with MLID, an observation probably associated with the high  
176 levels of mosaicism reported.

177         Recently several SRS patients have been reported with hypomethylation of both  
178 *H19* and *KCNQ1OT1* DMRs. In 2011 Begemann and coworkers reported the molecular  
179 findings in three cases, with one child also having hypomethylation of the *MEST* DMR  
180 [38]. It is striking that, apart from one patient having an umbilical hernia these three  
181 children did not present with any phenotypic features consistent with BWS.

182         In most cases the SRS phenotypes are grossly indistinguishable between isolated  
183 *H19* hypomethylation and individuals with MLID [17, 35]. However in two large studies it  
184 was suggested that SRS with MLID have less severe growth phenotypes and an increased  
185 prevalence of developmental delay and other congenital abnormalities [29].

186         Two individuals with epimutation of the *IG-DMR* and *MEG3* promoter at the  
187 14q32.2 imprinted domain, a region associated with Temple syndrome (TS, OMIM  
188 616222)[40], have been reported with SRS-compatible phenotypes [41]. Both syndromes  
189 have largely overlapping phenotypic features including low birth weight, relative  
190 macrocephaly, body asymmetry and feeding difficulties. A SRS patient with UPD7mat is  
191 also described with hypomethylation within the chromosome 14 imprinted domain [42].  
192 This report highlights that two of the molecular mechanisms giving rise to the same  
193 phenotype have occurred in parallel. This may represent a coincidence, but it may also  
194 suggest the two loci either physical interact as has been reported for other imprinted  
195 domains [43] or that a *trans*-acting factor specific for paternally methylated loci is  
196 involved.

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#### 198 *Chromosome 20q13- PHP.*

199 Pseudohypoparathyroidism (PHP) is a rare disorder typified by hypocalcaemia,  
200 hyperphosphataemia and elevated parathyroid hormone levels. The main imprinted form of  
201 the disease is PHP1B (OMIM 603233), characterized by PTH and sometimes TSH  
202 resistance. The majority of cases are sporadic, with PHP1B subjects displaying  
203 paternalization of the maternally methylated DMRs within the *GNAS* locus on human

204 chromosome 20 (LRG\_1051) suggesting that imprinting alterations are the basis of the  
205 disorder since no *cis*-acting causes have been reported [44, 45]. MLID in sporadic patients  
206 is rare, but when methylation changes are observed they are often mild and affect isolated  
207 additional DMRs (Figure 3) [17, 46-48]. These additional methylation defects have not  
208 been reported to influence growth trajectories, BMI or biochemical measurement. One  
209 fascinating observation gained from these studies is that methylation defects at the *GNAS*  
210 locus are frequently observed in BWS with MLID with normal hormonal levels, whereas  
211 epimutated PHP cases rarely have MLID.

212

213 *MLID in other imprinting disorders.*

214 Very little is known about the frequency of MLID in Angelman syndrome (AS, OMIM  
215 105830), Prader-Willi (PWS, OMIM 176279), Temple or Kagami-Ogata (KOS,  
216 OMIM60814) syndromes because either epigenetic anomalies in these patients are rare  
217 (<5% for AS and PWS) or the disorder itself is so rare that cohort-based studies are  
218 difficult. To date, no cases of MLID have been reported KOS with epimutations at the  
219 chromosome 14-imprinted domain and only a single case for TS with additional  
220 hypomethylation of the *KCNQ1OT1* and *WRB* DMRs [49]. Four patients with features of  
221 PWS but molecular diagnosis of AS have been reported in literature, a situation termed  
222 “Prader-man” [50-52]. These cases presented with partial loss of methylation of the *SNRPN*  
223 DMR. The only two reported AS case with MLID have been reported. The first presented  
224 with additional hypomethylation of *KCNQ1OT1*, *PEG3* and *GNAS* and was reported to  
225 have a complex phenotype overlapping with BWS and PWS [53] and the second having  
226 hypomethylation at *DIRAS3*, *RBI*, *IGF1R*, *ZNF331* and *GNAS* along with *ZDBF2*  
227 hypermethylation [53]. The methylation defects involving the *SNRPN* DMR are extremely  
228 rare, and only two cases being described, one in a child with MLID and a non-specific  
229 clinical phenotype presentation [54] and the second with TNDM but with no additional  
230 clinical data reported [18]. Therefore *SNRPN* methylation defects outside the context of AS  
231 and PWS are extremely rare suggesting that this specific DMR may employ a unique  
232 mechanism to protect methylation. Potential candidates are the Rb-binding proteins  
233 ARID4BA/B, which bind specifically to the mouse *Snrpn* DMR, which when ablated alter  
234 epigenetic modifications including a reduction in trimethylation of histone H4K20 and  
235 H3K9 and DNA methylation on the maternal allele [55].

236

237 **MLID and Assisted Reproductive Technologies.**

238 There is unequivocal evidence that within AS and BWS populations, isolated LOM of the  
239 *SNRPN* and *KCNQ1OT1* respectively is more prevalent in patients conceived following the  
240 use of assisted reproductive technologies (ART) [27, 56-60]; however it must be noted that  
241 the absolute risk of having a child with an ID following ART is extremely low [61]. Several  
242 cohorts have identified associations between BWS MLID and ART [25-27, 30], but such  
243 associations are not consistent between publications [62]. Furthermore there are conflicting  
244 reports of ART influencing the BWS phenotype, with no significant associations found in  
245 most reports. However, statistical differences were observed for earlobe anomalies,  
246 advanced bone age and congenital heart disease, in one deep-phenotyping study [62].

247 It remains to be determined whether loss of methylation at imprinted DMRs is  
248 associated with the underlying fertility problems or whether this occurs as a consequence of  
249 the treatment or embryo culture. It has recently been reported that embryos with delayed  
250 first cytokinesis and those who took longer to get to the four-cell stage were associated with  
251 both increased aneuploidy and decreased levels of DNMT3B and NLRP5 [63]. Importantly  
252 these observations were independent of the fertility status, suggesting that aberrant  
253 epigenetic and imprinting profiles maybe linked to slower pre-implantation embryo  
254 cleavage rates during the reprogramming window.

255

### 256 **Searching for mutations in *trans*-acting factors.**

257 To identify the underlying genetic insults responsible for MLID numerous studies have  
258 performed candidate gene mutation screening. These studies have focused on *ZFP57*,  
259 *DNMT3L*, *DNMT1*, *MBD3*, *DPPA3*, *NLRP2*, *NLRP7*, *KHDC3L* and *TRIM28* [13, 26, 31,  
260 38, 64] with very few pathological variants identified, with the exception of ~50% of  
261 TNDM MLID having recessive mutation of *ZFP57* [16,17].

262

### 263 ***ZFP57* is required to protect imprinted methylation.**

264 The *ZFP57* gene encodes for a krüppel-associated box domain (KRAB) zinc finger protein  
265 and is located on human chromosome 6q22.1 and mouse chromosome 17qB1. Unlike most  
266 ZNF genes, *ZFP57* is not a part of a large ZNF-cluster [65]. In mouse, maternal effect  
267 mutations that result in the loss of *Zfp57* in the developing zygote (*Zfp57*<sup>-/-</sup> F1 from *Zfp57*  
268 <sup>-/+</sup> mothers) are partially lethal, while eliminating both maternal and zygotic function  
269 (*Zfp57*<sup>-/-</sup> F1 from *Zfp57*<sup>-/-</sup> mother) causes complete embryonic lethality [9].

270 In wild type mouse embryonic stem (mES) cells, *Zfp57* and *Trim28* bind to all  
271 known imprinted DMRs by recognizing the recurrent methylated [TG]GCCGC motif [66,



272 67], suggesting that *Zfp57* recruits the corepressor complex that includes the H3K9  
273 methyltransferase *Setdb1* and the heterochromatin protein HP1 $\gamma$  to specific target  
274 sequences [66]. Consistent with this, *Zfp57* has been shown to be necessary for the  
275 maintenance of allelic DNA methylation and H3K9me3 at imprinted DMRs [9, 66] and to  
276 be involved in silencing of a limited number of non-imprinted loci [67]. Zuo and colleagues  
277 found that re-introducing *Zfp57* into knock out mES cells failed to re-establish DNA  
278 methylation at imprinted loci, indicating irreversible loss at these DMRs [68].

279 Females with homozygous or compound heterozygous mutations of *ZFP57* have  
280 been reported in 13 families [16, 17, 20, 69]. All of these families were identified with  
281 TNDM as a result of hypomethylation at the *PLAGL1* DMR and additional LOM of other  
282 maternally methylated imprinted genes [16-18]. Apart from at the *PLAGL1* locus, the  
283 methylation defects appear mosaic, indicating that *ZFP57* is involved in the maintenance of  
284 methylation at imprinted regions during pre-implantation reprogramming, similar to its  
285 function in mouse. It remains possible that other members of the *ZFP57* complex maybe  
286 involved, such as *AFF3* (also known as *AF4/FMR2*), a protein recently shown to bind to  
287 the methylated allele of imprinted DMRs in a *Zfp57*-dependent fashion [70]. Furthermore,  
288 methylation profiling has identified a folate-sensitive interval upstream of *ZFP57* implying  
289 that environmental exposures may influence expression levels [71]. Consistent with the  
290 hypothesis that the *ZFP57* promoter may be epigenetically liable to periconceptual  
291 environment is the observation that methylation in the same region is subjected to seasonal  
292 fluctuations in Gambian children [72].

293

#### 294 **Extreme cases of MLID- hydatidiform moles.**

295 Hydatidiform mole (HM) is an aberrant human pregnancy characterized by abnormal  
296 trophoblast proliferation. Complete HMs do not contain any embryonic tissues other than  
297 placental villi, whereas partial HMs may contain other tissues. Sporadic complete HMs are  
298 mostly diploid and androgenetic in origin. Occasionally HM can be recurrent (RHM) and  
299 familial in nature (OMIM 231090) [73] with mutations in two interacting proteins, *NLRP7*  
300 (*NACHT*, leucine rich repeat and *PYD* containing 7) and *KHDC3L* (previously known as  
301 *C6ORF221*) being responsible for ~ 80% of biparental RHMs [74, 75].

302 *NLRP7* does not have an orthologue in mouse, but is thought to have originated  
303 from an evolutionary duplication of its nearest family member, *NLRP2* [76]. Intriguingly,  
304 *NLRP2* was shown to be responsible for a single kindred of BWS based on the discovery of

305 a frameshift mutation in a homozygous state in an asymptomatic mother with two children  
306 affected with BWS. Upon methylation analysis, these BWS individuals presented with  
307 methylation defects at multiple loci, including *KCNQ1OT1* and *MEST* DMRs [77].  
308 However, since this report, no other cases of IDs were shown to have mutations in *NLRP2*,  
309 which makes this finding a rare causal event occurring in a small minority of cases.

310

### 311 **Methylation defects associated with maternal effect *NLRP7* mutations.**

312 A recent genome-wide methylation screening in *NLRP7*-mutated molar tissues suggests  
313 that all maternally methylated DMRs lack methylation while the sperm-derived *H19* and  
314 *IG-DMR* are unaffected [78]. This widespread disruption to maternally methylated DMRs  
315 also extends to the newly identified placenta-specific DMRs that orchestrate imprinting  
316 solely in the placenta [12, 78], suggesting that aberrant expression of both ubiquitously and  
317 placenta-specific imprinted transcripts play a role in the pathophysiology of RHM.

318       Recently, a family was described in which two fetuses and one child with SRS-like  
319 features showed mosaic widespread methylation defects, including maternally and  
320 paternally imprinted loci (including *GNAS*, *KCNQ1OT1*, *L3MBTL*, *MEG3*, *NAP1L5*,  
321 *NNAT*, *PLAGL1*, *RBI* and *ZNF597*) in multiple tissues. A mutation screening identified a  
322 p.A719V change in *NLRP7* in the mother [64]. However, it remains unclear if this  
323 substitution is responsible for the extreme epigenetic aberrations reported. The DNA base  
324 change is a low frequency variant in both 1000 Genome and in the dbSNP databases and  
325 the mother had inherited the change from her mother, indicating that further stochastic  
326 processes would be required in addition to maternal transmission of c.2156C>T. This  
327 observation raises interesting yet challenging questions with regards to the role of *NLRP7*  
328 non-synonymous variants in the pathogenesis of RHM. It has recently been observed that  
329 women suffering from other forms of reproductive loss have missense variants in  
330 heterozygous state, suggesting that phenotype variability may frequently be present [79,  
331 80]. It is therefore essential to determine if normal imprinted methylation profiles are  
332 maintained in these non-RHM pregnancy outcomes.

333       Exactly how NLRP-KHDC3L complexes are involved in regulating imprinted  
334 methylation is still a mystery, especially since detailed immunostaining for these factors in  
335 early human embryos and oocytes revealed that this protein is exclusively localized to the  
336 cytoskeleton, within the subcortical maternal complex, and not in the nucleus where it  
337 could associate with chromatin and influence methylation [81, 82]. This profile is similar to  
338 the location of DNMT3A and DNMT3B in human oocytes [83], indicating that NLRP-

339 KHDC3L-complexes may ensure the correct cellular localization and nuclear translocation  
340 during oocyte development. Once in the nucleus, this low abundance complex may  
341 associate to specific DNA sequences by direct interaction with chromatin regulator YY1  
342 [84] or ZBTB16, a methylation-sensitive Krüppel-like zinc finger protein [85].  
343

#### 344 **A new player- maternal effect mutations in *NLRP5*.**

345 The reports of MLID in various IDs have lead many researchers to perform exome-  
346 sequencing screens for the underlying coding changes. Despite much effort only a few  
347 causative *trans*-acting mutations have been found. Recently maternal-effect mutations in  
348 *NLRP5* in five mothers of individuals affected by MLID have been reported [54]. The  
349 clinical presentation of the offspring was heterogeneous with two probands having SRS,  
350 three with BWS and two with non-specific phenotypes. All women suffered multiple  
351 reproductive losses. Unlike RHM with *NLRP7* mutations, these MLID individuals had only  
352 a small number of DMRs affected (*H19*, *PEG3*, *GNAS*, *PLAGL1*, *KCNQ1OT1*, *GRB10*,  
353 *MEST* and *SNRPN* in various combinations) with hypomethylation of both maternally and  
354 paternally methylated DMRs consistent with a role in imprint maintenance. It is interesting  
355 to note that variants identified in 2 of the 5 cases involved non-synonymous SNPs listed in  
356 the dbSNP database. In fact *NLRP5*, 2 and 7 have a large load of non-synonymous SNPs  
357 (182, 153 and 160 respectively) within their ~3 kb coding sequence suggesting that careful  
358 consideration should be given when these variants are observed on both alleles creating a  
359 compound heterozygous state.

360

#### 361 **Conclusions**

362 From assessing the methylation profiles of the various IDs, it is now established that with  
363 the exception of TNDM, MLID is not restricted to maternally methylated DMRs but can  
364 also affect paternally methylated loci. Given the co-existence of LOM at both parentally  
365 methylated DMRs and the mosaic status of the defects in the majority of cases, this  
366 confirms that these methylation aberrations occur after fertilization as a consequence of not  
367 maintaining imprinted methylation during pre-implantation epigenetic reprogramming. The  
368 processes that erase the majority of the non-imprinted germline methylation are complex,  
369 with only a few *bona fide trans*-acting imprinting protection factors known. MLID in  
370 human provides us with a unique opportunity to identify the regulatory mechanisms  
371 involved in maintaining allelic differences in methylation and the factors involved in the  
372 imprinting life-cycle.

373 In the coming years it will be important to determine the degree of methylation  
374 mosaicism in various cell types, whether at a single disease associated locus or in the  
375 context of MLID, as very few epimutated imprinting disorder cases present with absolute  
376 hypo- or hypermethylated DMRs. As with the detection of somatic UPD, contamination of  
377 normal cells is known to decrease the observed frequency of mosaic epimutations, with  
378 levels of methylation in blood not always reflecting that in other tissues which can  
379 worryingly lead to false negative disease diagnosis.

380

### 381 **Trends box.**

- 382 • Imprinted DMRs represent a small minority of the methylation difference between  
383 gametes, but somatic protection of these elements is essential to avoid developing  
384 imprinting disorders (IDs).
- 385 • A subset of patients with IDs have methylation defects at single disease associated  
386 imprinted DMRs, but other individuals may have multi-locus imprinting  
387 disturbances (MLID) affecting additional imprinted regions.
- 388 • The frequency and loci involved in MLID varies between IDs, with Beckwith-  
389 Wiedemann syndrome presenting with the highest and most severe MLID cases,  
390 whilst this phenomenon has not been reported in Angelman or Temple syndrome  
391 patients.
- 392 • To date, mutations in three *trans*-acting factors (ZFP57, NLRP2 and NLRP5) have  
393 been associated with MLID.

394

### 395 **Outstanding Questions Box**

- 396 • Are multiple loci involved in mosaic MLID deregulated in the same or different  
397 cells? With the advent of technologies to quantify genome-wide methylation it will  
398 be important to determine the extent of methylation defects in multiple tissues at  
399 single cell resolution.
- 400 • How should MLID be defined? Which loci should be tested using which  
401 techniques?
- 402 • Since there is a large degree of clinical heterogeneity in IDs, could MLID and  
403 mosaicism prevent some IDs from being correctly diagnosed?

- 404 • Are cases of MLID without known underlying genetic mutations, such as those with  
405 negative exome sequencing results, caused by environmental insults or is genome  
406 sequencing warranted in these cases?
- 407 • Does protection of imprints during pre-implantation embryonic reprogramming  
408 involve specific factors that function at different developmental time points? The  
409 identification of such factors, and their spatial expression profile (i.e. after  
410 embryonic genome activation) will help elucidate possible recessive and maternal-  
411 effect genes involved in this process.
- 412 • Do the additional loci involved in MLID influence the phenotypes of IDs patients in  
413 the long term? For example, will BWS individuals with MLID involving *GNAS* or  
414 *PLAGL1* develop parathyroid problems or early onset adult diabetes?
- 415 • Are the epigenetic changes involved in isolated and MLID cases reprogrammed in  
416 the germline so that there is no subsequent risk to the offspring of these individuals?
- 417 • There is reported increased prevalence of IDs following assisted reproductive  
418 technologies. Is this true for MLID also?

419

420 **Glossary** (Terminology and abbreviations, for the benefit of students, 450 words)

421 **Imprinting disorders (IDs):** There are eight classical imprinting disorders including  
422 Angelman syndrome (AS), Prader-Willi syndrome (PWS), Beckwith-Wiedemann  
423 syndrome (BWS), Silver-Russell syndrome (SRS), Pseudohypoparathyroidism (PHP),  
424 Transient Neonatal diabetes (TNDM), Kagami-Ogata syndrome (KOS) and Temple  
425 syndrome (TS). All result from abnormal imprinted gene dosage caused by cytogenetic  
426 changes (deletions and duplications), uniparental disomy, coding mutations and epigenetic  
427 defects. The frequency of the cause varies between disorders, but for the purpose of this  
428 review we have focused on those with methylation defects only.

429 **Loss-of-methylation (LOM):** Hypomethylation at imprinted differentially methylated  
430 regions (DMRs) occurs on only one allele. The majority of imprinted DMRs are maternally  
431 methylated inheriting methylation from oocytes, with only two known examples of paternal  
432 germline DMRs at the *H19-IGF2* loci on chromosome 11 and the *IG*-DMR on chromosome  
433 14. Full annotation of imprinted DMRs in humans is available at [http://www.imprinting-](http://www.imprinting-disorders.eu)  
434 [disorders.eu](http://www.imprinting-disorders.eu).

435 **Multi-locus imprinting disturbance (MLID):** These are methylation changes, often  
436 hypomethylation, at additional imprinted loci in addition to those classically causing the  
437 ID.

438 **Mosaic methylation disturbances:** The vast majority of methylation changes observed in  
439 IDs are not absolute as would be expected for a germline methylation defect (only observed  
440 in recurrent hydatidiform moles with *NLRP7* mutations). Rather they may deviate from the  
441 expected ~50% methylation by as little as 10%. This is thought to reflect mosaicism with  
442 some cells maintaining the correct allelic methylation while others are abnormal.  
443 Furthermore DNA-derived from different tissues from the same patient may present with  
444 different LOM patterns.

445 **Embryonic epigenetic reprogramming:** Within a few hours of fertilization a wave of  
446 global demethylation ensures that methylation in the blastocysts are at their lowest levels  
447 erasing the majority of this germline epigenetic information compatible with blastomere  
448 totipotency. However the specific sequences associated with imprinted DMRs survive this  
449 reprogramming, through binding specific factors including ZFP57 and DPPA3/STELLA.

450

451 Figure 1.

452 **The life cycle of epigenetic changes at imprinted loci in mouse.** Regions of differential  
453 methylation are established in the germline and protected from pre-implantation  
454 reprogramming by the maintenance factors ZFP57 and DPPA3. The allelic methylation is  
455 then preserved by the semi-conservative action of DNMT1-UHRF1. In primordial germ  
456 cells of the developing embryos the DNA methylation at imprinted DMRs is erased so that  
457 the new profiles can be established according to the sex of the embryo. This complex  
458 procedure involves histone demethylation of H3K4 and the subsequent recognition and  
459 DNA remethylation by the DNMT3L-DNMT3A complex. \* Note that *DNMT3L* is not  
460 expressed in human oocytes suggesting different recruiting methods between species.

461

462 Figure 2.

463 **Schematic showing the complexes involved in protecting imprinted methylation of**  
464 **pre-implantation reprogramming.** (A) DPPA3 selectively binds to YYCAGSCTSS sites  
465 (where Y is cytosine or thymine and S is cytosine or guanine) associated with underlying  
466 H3K9me2 and DNA methylation predominantly observed in the maternal pronucleus  
467 selective protecting methylation from TET3-mediated hydroxylation. (B) Imprinted DMRs  
468 containing the TGCC<sup>meth</sup>GC hexanucleotide motif are protected from demethylation by the

469 ZFP57-TRIM28 complex during pre-implantation reprogramming. DNA methylation and  
470 K3K9 methylation are maintained at these loci by the recruitment of DNMT1 and  
471 SETBD1, respectively.

472

473 Figure 3.

474 **Ideogram showing the positions of known imprinted domains and the frequency they**  
475 **are hypomethylated in IDs with MLID.** The size of the circle is proportional to the  
476 frequency of hypomethylation at each imprinted loci for 10 SRS, 17 BWS, 6 TNDM and 12  
477 PHP patients with MLID. The white circle depicts the primary DMR associated with each  
478 disorder. Data taken from studies that assessed a minimum of 10 imprinted DMRs (mainly  
479 those employing the Illumina Infinium HumanMethylation450 BeadChip array), therefore  
480 some inaccuracies may exist due to coverage, molecular or bioinformatics techniques  
481 employed.

482

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