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

Marta Sanchez-Delgado[1], Andrea Riccio[2], Thomas Eggerman[3], Eamonn R Maher[4], Pablo Lapunzina[5], Deborah Mackay[6], David Monk[1*].

1. Imprinting and Cancer group, Cancer Epigenetic and Biology Program, Institut d'Investigació Biomedica de Bellvitge, Hospital Duran i Reynals, Barcelona, 08908, Spain.
2. DiSTABiF, Seconda Università degli Studi di Napoli, Caserta; Institute of Genetics and Biophysics – ABT, CNR, Napoli, Italy.
3. Institute of Human Genetics University Hospital Aachen, Aachen, Germany.
4. Department of Medical Genetics, University of Cambridge and NIHR Cambridge Biomedical Research Centre, Cambridge, UK.
5. Instituto de Genética Médica y Molecular (INGEMM)-IdiPAZ, Hospital Universitario La Paz, Madrid, Spain; CIBERER, Centro deInvestigación Biomédica en Red de Enfermedades Raras, ISCIII, Madrid, Spain.
6. Human Genetics and Genomic Medicine, Faculty of Medicine University of Southampton, UK.

[*]Correspondence should be addressed to David Monk (dmonk@idibell.cat).

Abstract

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

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

Key words

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

Life cycle of Imprints.

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

Imprinting disorders and aberrant DNA methylation.

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

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

Multi-locus imprinting defects by ID:

Chromosome 6q24- TNDM.

Transient Neonatal Diabetes Mellitus (TNDM, OMIM 601410) is caused by loss of imprinting of the *PLAGL1* domain (LRG_1035), with affected patients suffering from severe intrauterine growth restriction [14] and transient neonatal diabetes mellitus that often becomes permanent in teenage years. Approximately half of the patients with *PLAGL1* methylation defects also have additional hypomethylation of other maternally methylated imprinted regions. Mackay and colleagues coined the term “maternal hypomethylation syndrome” [15], with the same group identifying recessive mutations of *ZFP57* in TNDM cases associated with hypomethylation of *PLAGL1* and invariably *GRB10* and *PEG3* [16-18]. Interestingly individuals with multi-locus imprinting disturbances (MLID) without *ZFP57* mutations were more severely affected than those with *ZFP57* aberrations, with additional DMRs (*DIRAS3, IGF2R, MEST, KCNQ1OT1, IGF1R, ZNF331, WRB* and *SNU13*) frequently being hypomethylated [17, 18]. However it must be noted that these observations are based on genome-wide methylation screening using high-density or imprint-targeted arrays in only two ID cohorts, so the frequency and affected loci may change with additional investigations.

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

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

Chromosome 11p15- BWS.

Beckwith-Wiedemann syndrome (BWS, OMIM 130650) is a growth disorder characterized by macrosomia, macroglossia, visceromegaly, ear creases, hypoglycemia, hemihypertrophy and abdominal wall defects with an increased risk of pediatric tumours [21]. The molecular alterations in BWS involve two separate imprinted domains on chromosome 11, with sporadic hypomethylation of the *KCNQ1OT1* DMR (also known as *Kv*DMR1, ICR2) (LRG_1052) being the most frequently observed. A gain of methylation at the *H19* intergenic DMR (LRG_1030) is detectable in ~5% of cases, with the remainder of BWS individuals having paternal uniparental disomy of 11p15 or *CDKN1C* mutations (LRG_533) [22, 23].

The first molecular confirmation of MLID involving the chromosome 11p15 locus described two TNDM patients with hypomethylation of both the *PLAGL1* and *KCNQ1OT1* DMRs [24]. Interestingly, one of these TNDM patients had UPD(6)pat, the other a epimutation of the *PLAGL1* DMR. This second patient presented with classic TDNM complicated with umbilical hernia and macroglossia, features commonly seen in patients with BWS. Following this study several groups confirmed MLID in BWS cohorts, with a frequency of up to 30% of those individuals with an underlying *KCNQ1OT1* methylation defect [17, 18, 25-30]. Two recent papers have described methylation anomalies at additional imprinted loci in patients with *H19* hypermethylation [31, 32]. The MLID observed in BWS are notably different from those observed in TNDM, with both gains and losses of methylation observed at maternal and paternal DMRs. The paternally methylated DMRs associated with *ZBDF2*, *NESP* and *ZNF597/NAA60* have been shown to gain methylation in subsets of BWS patients [17, 31]. This acquisition is due to a concomitant loss of methylation in the nearby maternally methylated *GPR1*-AS, *GNAS* and *ZNF597* DMRs, which are known to regulate the methylation of these somatic DMRs in a hierarchical fashion [12, 33, 34].

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

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

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

Complex phenotypes may also be observed when loci other than *H19* and *KCNQ1OT1* are involved. Recently BWS and PHP1B were described in a single patient with MLID [36]. Alternatively, one phenotype can dominate over another: for example, an infant with severe LOM at both *PLAGL1* and *KCNQ1OT1* presented neonatally with BWS and without neonatal diabetes, but later relapsed with adult diabetes (D Mackay, personal communication). However, not all BWS cases with hypomethylation of *PLAGL1* or *GNAS* have a history of TNDM or pseudohypoparathyroidism, respectively [17, 26, 35]. In a patient with a clinical diagnosis a BWS after assisted reproductive technology, Lim et al [27] found normal methylation at *KCNQ1OT1* DMR but LOM at *H19*, *PLAGL*1 and *MEST* DMRs. In sum, the clinical presentation of a MLID patient probably reflects the severity and tissue mosaicism of hypomethylation in different tissues; but further molecular and clinical research is needed to understand and predict the resultant phenotypes in different individuals.

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

Chromosome 11p15- SRS.

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

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

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

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

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

Chromosome 20q13- PHP.

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

chromosome 20 (LRG_1051) suggesting that imprinting alterations are the basis of the disorder since no *cis*-acting causes have been reported [44, 45]. MLID in sporadic patients is rare, but when methylation changes are observed they are often mild and affect isolated additional DMRs (Figure 3) [17, 46-48]. These additional methylation defects have not been reported to influence growth trajectories, BMI or biochemical measurement. One fascinating observation gained from these studies is that methylation defects at the *GNAS* locus are frequently observed in BWS with MLID with normal hormonal levels, whereas epimutated PHP cases rarely have MLID.

MLID in other imprinting disorders.

Very little is known about the frequency of MLID in Angelman syndrome (AS, OMIM 105830), Prader-Willi (PWS, OMIM 176279), Temple or Kagami-Ogata (KOS, OMIM60814) syndromes because either epigenetic anomalies in these patients are rare (<5% for AS and PWS) or the disorder itself is so rare that cohort-based studies are difficult. To date, no cases of MLID have been reported KOS with epimutations at the chromosome 14-imprinted domain and only a single case for TS with additional hypomethylation of the *KCNQ1OT1* and *WRB* DMRs [49]. Four patients with features of PWS but molecular diagnosis of AS have been reported in literature, a situation termed "Prader-man" [50-52]. These cases presented with partial loss of methylation of the *SNRPN* DMR. The only two reported AS case with MLID have been reported. The first presented with additional hypomethylation of *KCNQ1OT1, PEG3* and *GNAS* and was reported to have a complex phenotype overlapping with BWS and PWS [53] and the second having hypomethylation at *DIRAS3, RB1, IGF1R, ZNF331* and *GNAS* along with *ZDBF2* hypermethylation [53]. The methylation defects involving the *SNRPN* DMR are extremely rare, and only two cases being described, one in a child with MLID and a non-specific clinical phenotype presentation [54] and the second with TNDM but with no additional clinical data reported [18]. Therefore *SNRPN* methylation defects outside the context of AS and PWS are extremely rare suggesting that this specific DMR may employ a unique mechanism to protect methylation. Potential candidates are the Rb-binding proteins ARID4BA/B, which bind specifically to the mouse *Snrpn* DMR, which when ablated alter epigenetic modifications including a reduction in trimethylation of histone H4K20 and H3K9 and DNA methylation on the maternal allele [55].

MLID and Assisted Reproductive Technologies.

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

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

Searching for mutations in *trans*-acting factors.

To identify the underlying genetic insults responsible for MLID numerous studies have performed candidate gene mutation screening. These studies have focused on *ZFP57*, *DNMT3L*, *DNMT1, MBD3, DPPA3*, *NLRP2, NLRP7, KHDC3L* and *TRIM28* [13, 26, 31, 38, 64] with very few pathological variants identified, with the exception of ~50% of TNDM MLID having recessive mutation of *ZPF57* [16,17].

ZFP57 is required to protect imprinted methylation.

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

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

67], suggesting that Zfp57 recruits the corepressor complex that includes the H3K9 methyltransferase Setdb1 and the heterochromatin protein HP1γ to specific target sequences [66]. Consistent with this, Zfp57 has been shown to be necessary for the maintenance of allelic DNA methylation and H3K9me3 at imprinted DMRs [9, 66] and to be involved in silencing of a limited number of non-imprinted loci [67]. Zuo and colleagues found that re-introducing *Zfp57* into knock out mES cells failed to re-establish DNA methylation at imprinted loci, indicating irreversible loss at these DMRs [68].

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

Extreme cases of MLID- hydatidiform moles.

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

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

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

Methylation defects associated with maternal effect *NLRP7* mutations.

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

Recently, a family was described in which two fetuses and one child with SRS-like features showed mosaic widespread methylation defects, including maternally and paternally imprinted loci (including *GNAS, KCNQ1OT1, L3MBTL, MEG3, NAP1L5, NNAT, PLAGL1, RB1* and *ZNF597*) in multiple tissues. A mutation screening identified a p.A719V change in *NLRP7* in the mother [64]. However, it remains unclear if this substitution is responsible for the extreme epigenetic aberrations reported. The DNA base change is a low frequency variant in both 1000 Genome and in the dbSNP databases and the mother had inherited the change from her mother, indicating that further stochastic processes would be required in addition to maternal transmission of c.2156C>T. This observation raises interesting yet challenging questions with regards to the role of *NLRP7* non-synonymous variants in the pathogenesis of RHM. It has recently been observed that women suffering from other forms of reproductive loss have missense variants in heterozygous state, suggesting that phenotype variability may frequently be present [79, 80]. It is therefore essential to determine if normal imprinted methylation profiles are maintained in these non-RHM pregnancy outcomes.

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

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

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

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

Conclusions

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

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

Trends box.

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

Outstanding Questions Box

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

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

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

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

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

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

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

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

Figure 1.

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

Figure 2.

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

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

Figure 3.

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

Acknowledgements

We are grateful to Alejandro Martin-Trujillo, Isabel Iglesias-Platas and members of the various laboratories for discussions. We apologize to those whose publications we were unable to cite owing to space limitations. The Monk laboratory is funded by the Spanish Ministerio de Educacion y Ciencia (BFU2014-53093 to DM). All co-authors are members of the "European Network for Human Congenital Imprinting Disorders" COST action (BM1208).

References

1. Bourc'his D. *et al*. (2001) Dnmt3l and the establishment of maternal genomic imprints. *Science*. 294, 2536-2539.
2. Kaneda M. *et al*. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*. 429, 900-903.
3. Suetake I. *et al*. (2004) DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J Biol Chem*. 279, 27816-2723.
4. Ooi S.K. *et al*. (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature*. 448, 714-717.
5. Ciccone D.N. *et al.* (2009) KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature*. 461, 415-418.

501 6. Howell C.Y. *et al*. (2001) Genomic imprinting disrupted by a maternal effect mutation
502 in the Dnmt1 gene. *Cell*. 104, 829-838.
503 7. Sharif J. *et al*. (2007) The SRA protein Np95 mediates epigenetic inheritance by
504 recruiting Dnmt1 to methylated DNA. *Nature*. 450, 908-912.
505 8. Nakamura T. *et al*. (2007) PGC7/Stella protects against DNA demethylation in early
506 embryogenesis. *Nat Cell Biol*. 9, 64-71.
507 9. Li X. *et al*. (2008) A maternal-zygotic effect gene, Zfp57, maintains both maternal and
508 paternal imprints. *Dev Cell*. 15, 547-557
509 10. Engel N. *et al*. (2006) CTCF binding sites promote transcription initiation and prevent
510 DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. *Hum Mol*
511 *Genet*. 15, 2945-2954.
512 11. Demars J. *et al*. (2010). Analysis of the IGF2/H19 imprinting control region uncovers
513 new genetic defects, including mutations of OCT-binding sequences, in patients with
514 11p15 fetal growth disorders. *Hum Mol Genet*. 19, 803-814.
515 12. Court F. *et al*. (2014) Genome-wide parent-of-origin DNA methylation analysis reveals
516 the intricacies of human imprinting and suggests a germline methylation-independent
517 mechanism of establishment. *Genome Res*. 24, 554-569.
518 13. Azzi S. *et al*. (2014) Complex tissue-specific epigenotypes in Russell-Silver Syndrome
519 associated with 11p15 ICR1 hypomethylation. *Hum Mutat*. 35, 1211-1220.
520 14. Diatloff-Zito C. *et al*. (2007) Genetic and epigenetic defects at the 6q24 imprinted locus
521 in a cohort of 13 patients with transient neonatal diabetes: new hypothesis raised by the
522 finding of a unique case with hemizygotic deletion in the critical region. *J Med Genet*.
523 44, 31-37.
524 15. Mackay D.J. *et al*. (2006) A maternal hypomethylation syndrome presenting as transient
525 neonatal diabetes mellitus. *Hum Genet*. 120, 262-269.
526 16. Mackay D.J. *et al*. (2008) Hypomethylation of multiple imprinted *loci* in individuals
527 with transient neonatal diabetes is associated with mutations in ZFP57. *Nat Genet*. 40,
528 949-951.
529 17. Court F. *et al*. (2013). Genome-wide allelic methylation analysis reveals disease-specific
530 susceptibility to multiple methylation defects in imprinting syndromes. *Hum Mutat*. 34,
531 595-602.
532 18. Docherty L.E. *et al*. (2014) Genome-wide DNA methylation analysis of patients with
533 imprinting disorders identifies differentially methylated regions associated with novel
534 candidate imprinted genes. *J Med Genet*. 51, 229-238.

535 19. Mackay D.J. *et al*. (2015) Multilocus methylation defects in imprinting disorders.
536 *Biomol Concepts*. 6, 47-57.
537 20. Boonen S.E. *et al*. (2008) Clinical characterisation of the multiple maternal
538 hypomethylation syndrome in siblings. *Eur J Hum Genet*.16, 453-461.
539 21. Brioude F. *et al*. (2013) Beckwith-Wiedemann syndrome: growth pattern and tumor risk
540 according to molecular mechanism, and guidelines for tumor surveillance. *Horm Res*
541 *Paediatr*. 80, 457-465.
542 22. Choufani S. *et al*. (2013) Molecular findings in Beckwith-Wiedemann syndrome. *Am J*
543 *Med Genet C Semin Med Genet*. 163C, 131-140.
544 23. Brioude F. *et al*. (2015) Mutations of the Imprinted CDKN1C Gene as a Cause of the
545 Overgrowth Beckwith-Wiedemann Syndrome: Clinical Spectrum and Functional
546 Characterization. *Hum Mutat*. 36, 894-902.
547 24. Arima T. *et al*. (2005) ZAC, LIT1 (KCNQ1OT1) and p57KIP2 (CDKN1C) are in an
548 imprinted gene network that may play a role in Beckwith-Wiedemann syndrome.
549 *Nucleic Acids Res*. 33, 2650-2660.
550 25. Rossignol S. *et al*. (2006) The epigenetic imprinting defect of patients with Beckwith-
551 Wiedemann syndrome born after assisted reproductive technology is not restricted to the
552 11p15 region. *J Med Genet*. 43, 902-907
553 26. Bliek J. *et al*. (2009) Hypomethylation at multiple maternally methylated imprinted
554 regions including PLAGL1 and GNAS loci in Beckwith-Wiedemann syndrome. *Eur J*
555 *Hum Genet*. 17, 611-619.
556 27. Lim D. *et al*. (2009) Clinical and molecular genetic features of Beckwith-Wiedemann
557 syndrome associated with assisted reproductive technologies. *Hum Reprod*. 24, 741-747.
558 28. Hiura H. *et al*. (2012) Characterization of DNA methylation errors in patients with
559 imprinting disorders conceived by assisted reproduction technologies. *Hum Reprod*. 27,
560 2541-2548.
561 29. Poole R.L. *et al*. (2013) Targeted methylation testing of a patient cohort broadens the
562 epigenetic and clinical description of imprinting disorders. *Am J Med Genet A*. 161A,
563 2174-2182.
564 30. Tee L. *et al*. (2013) Epimutation profiling in Beckwith-Wiedemann syndrome:
565 relationship with assisted reproductive technology. *Clin Epigenetics*. 5, 23.
566 31. Maeda T. *et al*. (2014) Comprehensive and quantitative multilocus methylation analysis
567 reveals the susceptibility of specific imprinted differentially methylated regions to
568 aberrant methylation in Beckwith-Wiedemann syndrome with epimutations. *Genet Med*.

16, 903-912.

32. Alders M. *et al*. (2014) Methylation analysis in tongue tissue of SBW patients identifies the (EPI)genetic cause in 3 patients with normal methylation levels in blood. *Eur J Med Genet*. 57, 293-297.

33. Kobayashi H. *et al*. (2013) Epigenetic and transcriptional features of the novel human imprinted lncRNA GPR1AS suggest it is a functional ortholog to mouse Zdbf2linc. *Epigenetic*s. 8, 635-645.

34. Liu J. *et al*. (2005) Distinct patterns of abnormal GNAS imprinting in familial and sporadic pseudohypoparathyroidism type IB. *Hum Mol Genet*. 14, 95-102.

35. Azzi S. *et al*. (2009) Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. *Hum Mol Genet*. 18, 4724-4733.

36. Bakker B. *et al*. (2015) A Girl With Beckwith-Wiedemann Syndrome and Pseudohypoparathyroidism Type 1B Due to Multiple Imprinting Defects. *J Clin Endocrinol Metab*. 100, 3963-3966.

37. Gicquel C. *et al*. (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat Genet*. 37, 1003-1007.

38. Begemann M. *et al*. (2011) Silver-Russell patients showing a broad range of ICR1 and ICR2 hypomethylation in different tissues. *Clin Genet*. 80, 83-88.

39. Fuke T. *et al*. (2013) Molecular and clinical studies in 138 Japanese patients with Silver-Russell syndrome. *PLoS One*. 8, e60105.

40. Ioannides Y. *et al*. (2014) Temple syndrome: improving the recognition of an underdiagnosed chromosome 14 imprinting disorder: an analysis of 51 published cases. *J Med Genet*. 51, 495-501.

41. Kagami M. *et al*. (2008) Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes. *Nat Genet*. 40, 237-242.

42. Begemann M. *et al*. (2012) Segmental maternal uniparental disomy 7q associated with DLK1/GTL2 (14q32) hypomethylation. *Am J Med Genet A*. 158A, 423-438.

43. Zhao Z. *et al*. (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet*. 38, 1341-1347.

44. Liu J. *et al*. (2000) A GNAS1 imprinting defect in pseudohypoparathyroidism type IB. *J*

Clin Invest. 106, 1167-1174.

45. Bastepe M. *et al*. (2001) Positional dissociation between the genetic mutation responsible for pseudohypoparathyroidism type Ib and the associated methylation defect at exon A/B: evidence for a long-range regulatory element within the imprinted GNAS1 locus. *Hum Mol Genet*. 10, 1231-1241.

46. Perez-Nanclares G. *et al*. (2012) Detection of hypomethylation syndrome among patients with epigenetic alterations at the GNAS locus. *J Clin Endocrinol Metab*. 97, E1060-7.

47. Maupetit-Méhouas S. *et al*. (2013) Simultaneous hyper- and hypomethylation at imprinted loci in a subset of patients with GNAS epimutations underlies a complex and different mechanism of multilocus methylation defect in pseudohypoparathyroidism type 1b. *Hum Mutat*. 34, 1172-1180.

48. Rochtus A. *et al*. (2016) Genome-wide DNA methylation analysis of pseudohypoparathyroidism patients with GNAS imprinting defects. *Clin Epigenetics.* 8:10.

49. Bens S. *et al*. (2016) Phenotypic spectrum and genomic extent of DNA methylation defects in multi-locus imprinting disturbances. *Epigenomics*. In Press.

50. Gillessen-Kaesbach G. *et al*. (1999) A previously unrecognised phenotype characterised by obesity, muscular hypotonia, and ability to speak in patients with Angelman syndrome caused by an imprinting defect. *Eur J Hum Genet*. 7, 638-644.

51. De Molfetta G.A. *et al*. (2002) A further case of a Prader-Willi syndrome phenotype in a patient with Angelman syndrome molecular defect. *Arq Neuropsiquiatr*. 60, 1011-1014.

52. Camprubí C. *et al*. (2007) Imprinting center analysis in Prader-Willi and Angelman syndrome patients with typical and atypical phenotypes. *Eur J Med Genet.* 50, 11-20.

53. Baple E.L. *et al*. (2011) An atypical case of hypomethylation at multiple imprinted loci. *Eur J Hum Genet*. 19, 360-362.

54. Docherty L.E. *et al*. (2015) Mutations in NLRP5 are associated with reproductive wastage and multilocus imprinting disorders in humans. *Nat Commun*. 6, 8086.

55. Wu M.Y. *et al*. (2006) Deficiency of Rbbp1/Arid4a and Rbbp1l1/Arid4b alters epigenetic modifications and suppresses an imprinting defect in the PWS/AS domain. *Genes Dev*. 20, 2859-2870.

56. Maher E.R. *et al*. (2003) Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs? *Hum Reprod*. 18, 2508-2511.

57. Halliday J. *et al*. (2004) Beckwith-Wiedemann syndrome and IVF: a case-control study.

637 *Am J Hum Genet*. 75, 526-528.
638 58. Lidegaard O. *et al*. (2005) Imprinting diseases and IVF: Danish National IVF cohort
639 study. *Hum Reprod*. 20, 950-954.
640 59. Cox G.F. *et al*. (2002) Intracytoplasmic sperm injection may increase the risk of
641 imprinting defects. *Am J Hum Genet*. 71, 162-164.
642 60. DeBaun M.R. *et al*. (2003) Association of in vitro fertilization with Beckwith-
643 Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet.*
644 72, 156-160.
645 61. Sutcliffe A.G. *et al*. (2006) Assisted reproductive therapies and imprinting disorders-a
646 preliminary British survey. *Hum Reprod*. 21, 1009-1011.
647 62. Tenorio J. *et al*. (2016) Clinical and molecular analysis of Beckwith-Wiedemann
648 syndrome: Results of 187 patients and comparison between natural conception and
649 assisted reproductive techniques. *Clin Epigenentic*. In Press.
650 63. Vera-Rodriguez M. *et al*. (2015) Prediction model for aneuploidy in early human
651 embryo development revealed by single-cell analysis. *Nat Commun*. 6, 7601.
652 64. Caliebe A. *et al*. (2014) A familial disorder of altered DNA-methylation. *J Med Genet*.
653 51, 407-412.
654 65. Seetharam A. *et al*. (2013) A study on the distribution of 37 well conserved families of
655 C2H2 zinc finger genes in eukaryotes. *BMC Genomics*. 14, 420.
656 66. Quenneville S. *et al*. (2011) In embryonic stem cells, ZFP57/KAP1 recognize a
657 methylated hexanucleotide to affect chromatin and DNA methylation of imprinting
658 control regions. *Mol Cell*. 44, 361-372.
659 67. Strogantsev R. *et al*. (2015) Allele-specific binding of ZFP57 in the epigenetic
660 regulation of imprinted and non-imprinted monoallelic expression. *Genome Biol.* 16,
661 112.
662 68. Zuo X. *et al*. (2012) Zinc finger protein ZFP57 requires its co-factor to recruit DNA
663 methyltransferases and maintains DNA methylation imprint in embryonic stem cells via
664 its transcriptional repression domain. *J Biol Chem*. 287, 2107-2118.
665 69. Boyraz M. *et al*. (2013) Transient neonatal diabetes mellitus in a Turkish patient with
666 three novel homozygous variants in the ZFP57 gene. *J Clin Res Pediatr Endocrinol*. 5,
667 125-128.
668 70. Luo Z. *et al*. (2016) Regulation of the imprinted Dlk1-Dio3 locus by allele-specific
669 enhancer activity. *Genes Dev*. 30, 92-101.
670 71. Amarasekera M. *et al*. (2014) Genome-wide DNA methylation profiling identifies a

folate-sensitive region of differential methylation upstream of ZFP57-imprinting regulator in humans. *FASEB J*. 28, 4068-4076.

72. Silver M.J. *et al*. (2015) Independent genomewide screens identify the tumor suppressor VTRNA2-1 as a human epiallele responsive to periconceptional environment. *Genome Biol.* 16, 118.

73. Judson H. *et al*. (2002) A global disorder of imprinting in the human female germ line. *Nature*. 416, 539-542.

74. Murdoch S. *et al*. (2006) Mutations in NALP7 cause recurrent hydatidiform moles and reproductive wastage in humans. *Nat Genet*. 38, 300-302.

75. Parry D.A. *et al*. (2011) Mutations causing familial biparental hydatidiform mole implicate c6orf221 as a possible regulator of genomic imprinting in the human oocyte. *Am J Hum Genet*. 89, 451-458.

76. Duéñez-Guzmán E.A. and Haig D. (2014) The evolution of reproduction-related NLRP genes. *J Mol Evol*. 78, 194-201.

77. Meyer E. *et al*. (2005) Germline mutation in NLRP2 (NALP2) in a familial imprinting disorder (Beckwith-Wiedemann Syndrome). *PLoS Genet*. 5, e1000423.

78. Sanchez-Delgado M. *et al*. (2015) Absence of Maternal Methylation in Biparental Hydatidiform Moles from Women with NLRP7 Maternal-Effect Mutations Reveals Widespread Placenta-Specific Imprinting. *PLoS Genet*. 11, e1005644.

79. Messaed C. *et al*. (2011) NLRP7 in the spectrum of reproductive wastage: rare non-synonymous variants confer genetic susceptibility to recurrent reproductive wastage. *J Med Genet*. 48, 540-548.

80. Slim R and Wallace E.P. (2013) NLRP7 and the Genetics of Hydatidiform Moles: Recent Advances and New Challenges. *Front Immunol*. 4, 242.

81. Wang C.M. *et al*. (2009) Identification of 13 novel NLRP7 mutations in 20 families with recurrent hydatidiform mole; missense mutations cluster in the leucine-rich region. *J Med Genet*. 46, 569-575.

82. Akoury E. *et al*. (2015) NLRP7 and KHDC3L, the two maternal-effect proteins responsible for recurrent hydatidiform moles, co-localize to the oocyte cytoskeleton. *Hum Reprod*. 30, 159-169.

83. Petrussa L. *et al*. (2014) Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies. *Mol Hum Reprod*. 20, 861-874.

84. Mahadevan S. *et al*. (2014) NLRP7 affects trophoblast lineage differentiation, binds to

overexpressed YY1 and alters CpG methylation. *Hum Mol Genet*. 23, 706-716.

85. Singer H. *et al*. (2015) NLRP7, Involved in Hydatidiform Molar Pregnancy (HYDM1), Interacts with the Transcriptional Repressor ZBTB16. *PLoS One*. 10, e0130416.