

Comprehensive characterisation of the underlying DNA methylation defects associated with all epimutated imprinting syndromes

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1. Project summary

DNA methylation is vital for the regulation of allelic expression of imprinted genes. Imprinted genes are the classic example of long-term epigenetic memory of parental origin since they are maintained throughout life (Ferguson-Smith. 2011). Methylation at these differentially methylated regions (DMRs) originates from the respective gametes that coordinate this parent-of-origin expression (Smallwood et al., 2011). These germline-derived DMRs retain the methylation on the maternally- or paternally-derived alleles resulting in one copy being heavily methylated and the other unmethylated. In humans, numerous complex disorders (including transient neonatal diabetes mellitus (TNDM), Beckwith-Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS) and pseudohypoparathyroidism (PHP1B)) are associated with loss of imprinting (LOI) at specific loci (reviewed in Eggermann et al., 2015). Over the past decade there has been increasing evidence that LOI is not only an isolated event occurring at a given disease associated locus, but that patients with imprinting disorders (IDs) may have multilocus imprinting defects affecting additional imprinted DMRs, which may influence the resulting phenotypes. To date, only mutations in the *trans*-acting factor ZFP57 have been associated with TNDM cases with multilocus imprinting defects (Mackey et al., 2008). This project aimed to fully characterise the methylation aberrations in imprinting disorders with an underlying epigenetic cause and to identify the underlying proteins involved in this mechanism.

2. Results

(1) Methylation profiling of imprinted DMRs to characterize multilocus methylation defects

Using initially custom designed Goldengate methylation arrays and subsequently commercial Illumina Infinium HumanMethylation450 Beadchip arrays we profiled all known imprinted DMRs in a large Spanish cohort of TNDM, BWS, SRS and

PHP1B patients. This revealed that multilocus methylation defects frequently occur in these individuals, suggesting a failure to maintain allelic methylation during embryonic epigenetic reprogramming (Nakabayashi et al., 2011; Court et al., 2013). Pyrosequencing of repeat element (LINE, SINE and ALU-Yb8) revealed normal methylation profiles suggesting that the epigenetic defects are restricted to imprinted loci (Court et al., 2013).

A mutation screen of *ZFP57*, *NLRP2*, *NLRP7* and *KHDC3L* revealed only one causative change, a 1bp deletion in *ZFP57*, in a TNDM patient with multilocus imprinting defects. All index case-parent trios associated with multilocus imprinting defects were subjected to exome sequencing to identify the causal mutations in these individuals. We are currently performing bioinformatics analysis for de novo, recessive and maternal effect modes of inheritance.

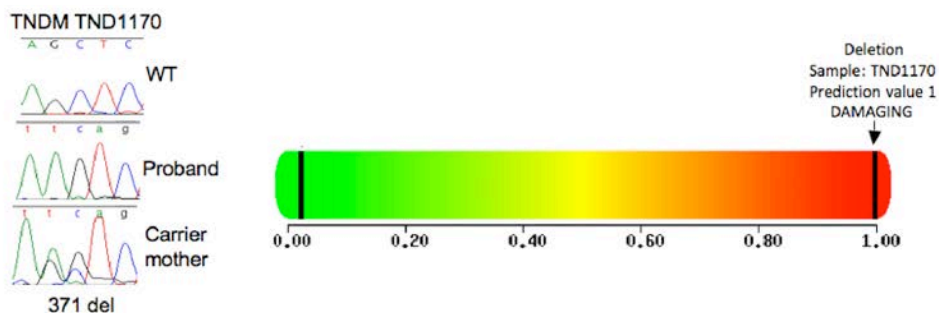


Figure 1. *ZFP57* mutation in a TNDM patient.

(2) Identification of novel imprinted loci using genome-wide methylation screening

During the course of this project we identified a BWS patient with paternal uniparental diploidy (all chromosome are of paternal origin, in a mosaic state) (Romanelli et al., 2011). As a result of our case report, five additional patients with similar chromosome complements were either reported in literature or referred to our laboratories. In combination with a maternal uniparental diploidy samples from a Silver-Russell syndrome patient (the phenotypic opposite to BWS) (Yamazawa et al., 2010) we performed genome-wide methylation screens using the Illumina Infinium HumanMethylation450 Beadchip arrays and methyl-seq (whole

genome bisulphite sequencing). The high-density of array probes mapping to CpG islands allowed for the extent of allelic methylation at imprinted DMRs to be precisely mapped, and also gave a unique opportunity for novel imprinted loci to be identified. As a result we identified 8 DMRs with known imprinted domains and seven novel imprinted loci, *PPIEL*, *WDR27*, *HTR5A*, *ERLIN2*, *TRAPPC9* (also known as *PEG13*), *WRB* and *NHP2L1*. A similar approach was used comparing term placenta samples and hydatidiform mole biopsies, which identified 15 placenta-specific maternally methylated domains not present in somatic tissues (Court et al., 2014). By extending the number of imprinted regions previously identified in humans, we have identified loci which are often subject to hypomethylation in BWS, SRS and TDNM patients with multilocus methylation defects. These results have recently been confirmed by Docherty and colleagues in a UK based ID cohort in which they observe *WRB* and *NH2PL1* being frequently affected in these rare individuals. Our genome-wide approach also confirmed the LOM signature associated with recessive ZPF57 mutations, with only a small subset of DMRs (*PLAGL1*, *GRB10* and invariably *PEG3*, *NAP1L5*, *GNAS* and *NHP2L1*) showing methylation defects (Mackay et al., 2008; Court et al., 2013, Docherty et al., 2014).

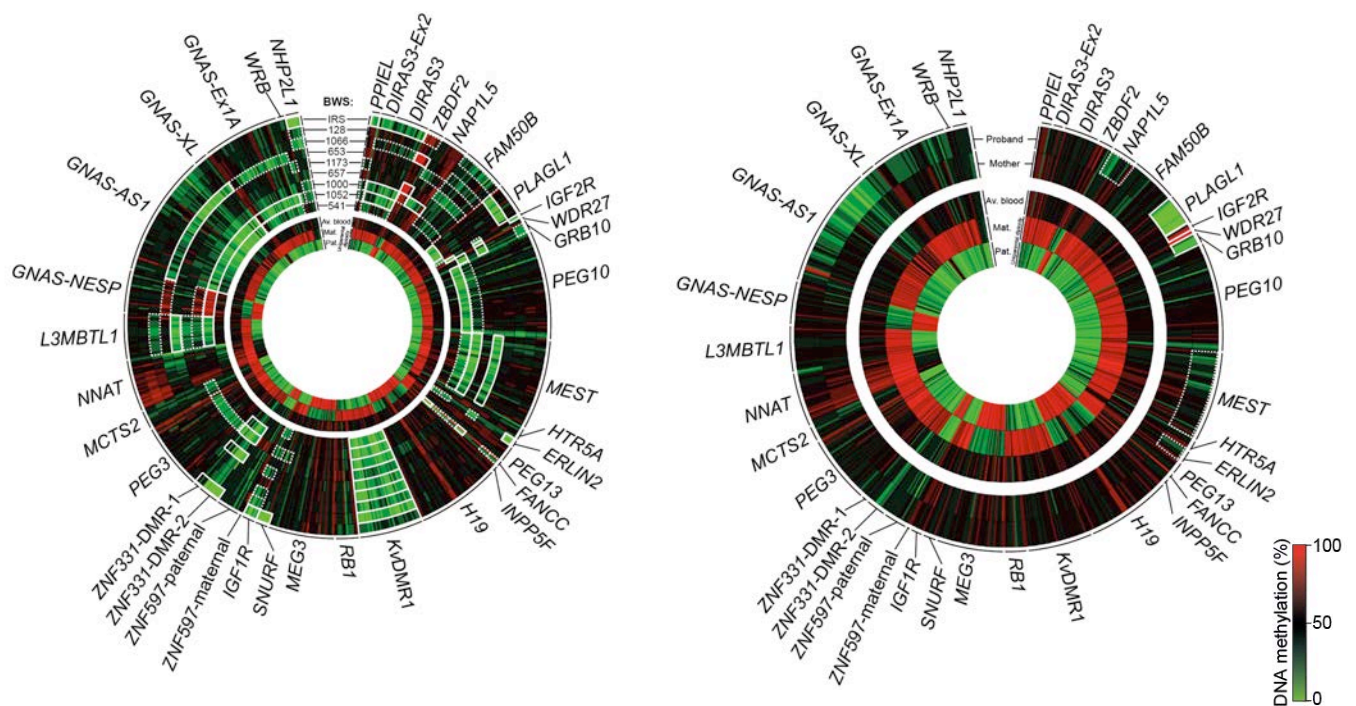


Figure 2. Circular heatmap of the characterisation of the human “imprintome” in BWS and the TNDM patient with ZFP57 mutation. Severely hypomethylated regions highlighted by white box, partially affected regions surrounded by dashed box.

(3) Characterization of brain-specific chromatin interactions and imprinting within the 8q24 intellectual disability risk locus

One of the novel maternally methylated regions, *TRAPPC9/PEG13* mapped to a locus implicated in developmental delay (both autism and intellectual disabilities). We therefore performed an extensive epigenetic characterization of this domain. We observed that the maternally methylated *PEG13* DMR binds CTCF-cohesin, possesses enhancer-blocker activity, which we hypothesize dictates mutually exclusive chromatin looping between a novel enhancer region and the promoters of the reciprocally imprinted *PEG13* and *KCNK9* transcripts (Court et al., 2014). We followed up these mechanistic experiments with a mutation screen in a large cohort of patients with idiopathic intellectual disabilities, but this failed to identify any pathological changes (Sanchez-Delgado et al., 2014).

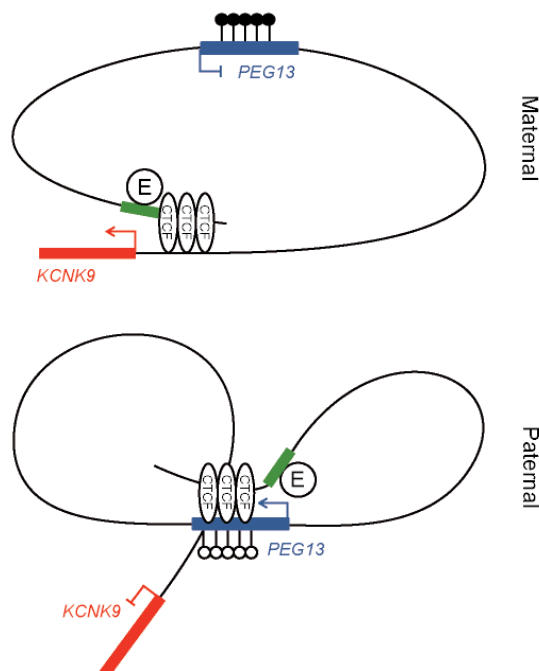


Figure 3. Chromatin looping at the *PEG13* locus between the maternally expressed *KCNK9* gene and brain-specific enhancer 58-500 kb upstream.

(4) Absence of maternal methylation in biparental hydatidiform moles with *NLRP7* maternal-effect mutations.

Familial recurrent hydatidiform mole (RHM) is a maternal-effect autosomal recessive disorder associated with mutations of the *NLRP7* gene (Judson et al., 2002; Murdoch et al., 2006). It is characterized by HM with excessive trophoblastic proliferation, which mimics the appearance of androgenetic molar conceptuses despite their diploid biparental constitution. It has been proposed that the phenotypes of both types of mole are associated with aberrant genomic imprinting. To characterize the extent of aberrant imprinting in RHM samples we performed genome-wide methylation profiling of both spontaneous androgenetic and biparental *NLRP7* defective molar tissues using Illumina Infinium HumanMethylation450 Beadchip arrays. We observe total paternalization of all ubiquitous and placental-specific DMRs in four androgenetic moles; namely the gains of methylation at paternally methylated loci and the absence of methylation at maternally methylated regions. The methylation defects observed in four RHM biopsies from *NLRP7* defective patients are restricted to loss-of-methylation at maternal DMRs. Surprisingly, RHMs from two sisters with the same missense mutations show subtle differences, with some DMRs maintaining allelic methylation suggesting inter-individual variation. These epigenotypes are consistent with *NLRP7* being a maternal-effect gene and involved in imprint acquisition in the oocyte. In addition, bioinformatics screening of the resulting methylation datasets identified over sixty loci with methylation profiles consistent with imprinting in the placenta, of which we confirm 20 as novel maternally methylated loci. These observations strongly suggest that the molar phenotypes are due to defective placental-specific imprinting and over-expression of paternally expressed transcripts, highlighting that maternal-effect mutations of *NLRP7* are associated with the most severe form of multilocus imprinting defects in humans.

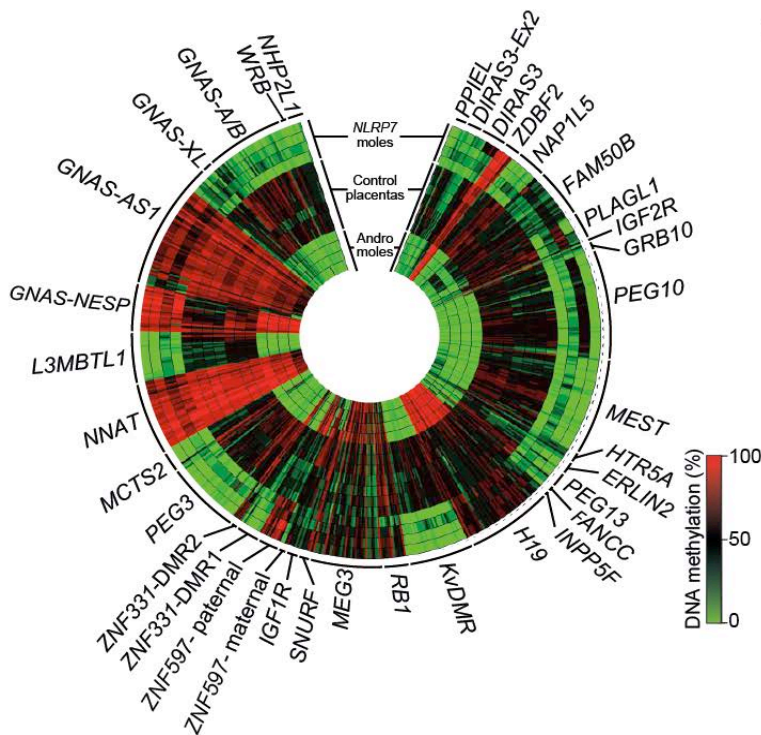


Figure 4. Circular heatmap revealing the methylation profiles of ubiquitously imprinted DMRs in RHM samples.

3. Implications

The genome-wide description of multilocus imprinting defects has enabled a list of frequently affected loci to be described for each imprinting disorder. In some cases hypomethylation of genes with known involvement in diabetes (*PLAGL1*) and cancer (*IGF2* and *RB1*) suggests individuals with these loci affected should be subject to frequent clinical examinations to survey for early onset of these comorbidities. Our results have increased the list of known imprinted loci in humans, many of which have show abnormal methylation in patients with multilocus imprinting defects. As a direct result of our work, the European COST-action for congenital imprinting disorders (<http://www.imprinting-disorders.eu>) has recommended a list of loci that should be analysed for methylation anomalies, including *PPIEL*, *WRB* and *NHP2L1*, three of the novel imprinted loci identified in this project. These new imprinted loci are now routinely assessed by laboratories

performing ID diagnosis and have been submitted for regulatory Locus Reference Genomic (LRG) status approval.

Our work has shown that maternal effect mutations of *NLRP7* result in catastrophic hypomethylation of maternally methylated imprinted DMRs in familial recurrent hydatidiform moles. As a direct result of our work several laboratories are determining the methylation of imprinted DMRs to differentiate between sporadic androgenetic and RHMs, since both look similar histologically but require different management and genetic counselling.

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