ZFP57 and the Targeted Maintenance of Postfertilization Genomic Imprints

NOZOMI TAKAHASHI,1 DIONNE GRAY,1 RUSLAN STROGANTSEV,2 ANGELA NOON,1 CELIA DELAHAYE,1 WILLIAM C. SKARNES,3 PERI H. TATE,3 AND ANNE C. FERGUSON-SMITH1

1Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom
2Babraham Institute, Cambridge CB22 3AT, United Kingdom
3Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, United Kingdom

Correspondence: afsmith@mole.bio.cam.ac.uk

Epigenetic modifications play an important role in modulating genome function. In mammals, inappropriate epigenetic states can cause embryonic lethality and various acquired and inherited diseases; hence, it is important to understand how such states are formed and maintained in particular genomic contexts. Genomic imprinting is a process in which epigenetic states provide a sustained memory of parental origin and cause gene expression/repression from only one of the two parental chromosomes. Genomic imprinting is therefore a valuable model to decipher the principles and processes associated with the targeting and maintenance of epigenetic states in general. Kruppel-associated box zinc finger proteins (KRAB-ZFPs) are proteins that have the potential to mediate this. ZFP57, one of the best characterized proteins in this family, has been shown to target and maintain epigenetic states at imprinting control regions after fertilization. Its role in imprinting through the use of ZFP57 mutants in mouse and the wider implications of KRAB-ZFPs for the targeted maintenance of epigenetic states are discussed here.

Genomic imprinting is an epigenetically regulated process that results in the functional nonequivalence of maternally and paternally inherited genomes (Surani 2001). Imprinting is regulated by parental origin–specific DNA methylation at differentially methylated regions (DMRs) that is acquired during germ cell development. In mice, many CpG sites are differentially methylated in oocyte and sperm (Kobayashi et al. 2012); however, most of them, with the exception of the imprinted DMRs, lose their germline-specific methylation differences during a process of global demethylation after fertilization often followed by remethylation after the blastocyst stage (Proudhon et al. 2012). Hence, the stage of preimplantation methylation maintenance during global genome-wide reprogramming is the critical period for the successful establishment of a functional imprint. Similar to mouse imprinted loci, differential methylation at human imprinted DMRs seems to be established in germ cells and maintained during global demethylation (Okae et al. 2014). Although most imprinted genes are conserved between human and mouse, there are reports of species-specific imprinted genes (Morison et al. 2005; Babak et al. 2015) and species-specific acquisition of methylation imprints. In the nonhuman primate macaque, post-oocyte acquisition of methylation has been observed at three imprinted DMRs, suggesting that mechanisms might exist to acquire maternal allele–specific DNA methylation after fertilization (Cheong et al. 2015). Imprinted genes can be exquisitely dosage-sensitive and highly regulated; hence, the ability to maintain the methylation memory from the germline is essential. Here we consider how these epigenetic marks are acquired and maintained during development and explore the role of the Kruppel-associated box (KRAB) zinc finger protein ZFP57 in the process.

DNA METHYLATION DYNAMICS AND KEY PLAYERS IN METHYLATION IMPRINTING

In the last decade, researchers studying genomic imprinting have identified the timing of establishment, maintenance, and erasure of epigenetic marks, as well as characterized key players involved (Fig. 1; Table 1). In the female germline, DNA methylation is established in an oocyte size–dependent manner after birth (Hiura et al. 2006), and de novo DNA methyltransferases Dnmt3a and Dnmt3l are involved in this process (Bourc’his et al. 2001; Hata et al. 2002; Hirasawa et al. 2008). Whole-genome bisulfite sequencing (WGBS) of oocytes from knockout (KO) mice shows that Dnmt3a, Dnmt3b, and the cofactor Dnmt3l are involved in this process (Bourc’his et al. 2001; Hata et al. 2002; Hirasawa et al. 2008). Whole-genome bisulfite sequencing (WGBS) of oocytes from knockout (KO) mice shows that Dnmt3a and Dnmt3l are necessary for the acquisition of methylation at all known maternally derived imprinted DMRs (Shirane et al. 2013). The oocyte methylation targeting mechanism is poorly understood, but it is thought that the machinery may differ in a region-dependent manner. For example, demethylation of histone H3 on lysine 4 (H3K4) by KDM1B histone demethylase is required for the acquisition of some DNA methylation imprints in the mouse germline (Table 1; Ciccone et al. 2009; Stewart et al. 2015). It is unknown whether the removal of H3K4 methylation is required for only a specific subset of imprinted loci or whether other H3K4
demethylases are involved in DNA methylation acquisition independent of KDM1B. At some loci, including nonimprinted regions, transcription in the female germline is required for the establishment of DNA methylation at loci downstream of the transcriptional start site (Kelsey and Feil 2013). Methylation at the imprinted Gnas locus is acquired in this manner (Chotalia et al. 2009). These germline-established marks are present in the oocyte at the time of fertilization and represent a maternally derived germline methylation state.

In the male germline, de novo methylation occurs around embryonic day (E) 16–17.5 before meiosis (Singh et al. 2013). Like in the female germline, Dnmt3a, Dnmt3b, and Dnmt3l are responsible for the acquisition of DNA methylation during spermatogenesis (Kaneda et al. 2004; Kato et al. 2007). The deletion of Dnmt3a and Dnmt3l in the male germ cells causes meiotic arrest at the pachytene stage as a consequence of transposon activation (Bourc’his and Bestor 2004; Kaneda et al. 2004). Although KDM1B is not necessary to establish paternal imprints (Ciccone et al. 2009), it is highly possible that H3K4 methylation modulates de novo methylation in the male germline because H3K4me2-enriched regions (which include those maternally imprinted DMRs) are resistant to methylation during spermatogenesis (Singh et al. 2013) and may therefore be protected from methylation in the male germline. There are three known paternally derived methylation imprints: H19, Rasgrf1 DMRs and the IG-DMR at the Dlk1-Dio3-imprinted domain. Rasgrf1 DMR contains a transposon-derived sequence which requires the PIWI-interacting RNA (piRNA) pathway for its de novo methylation (Watanabe et al. 2011). Deletions of genes involved in piRNA processing affect this one imprint but also result in the failure of spermatogenesis.

A global genome-wide demethylation occurs after fertilization and is caused by both passive and active mechanisms. Passive demethylation is a replication-dependent pathway occurring as a result of failure to methylate CpGs on the newly synthesized DNA strand. Levels of the maintenance DNA methyltransferase Dnmt1 are low in the nucleus during preimplantation embryo development (Hirasawa et al. 2008), resulting
### Table 1. List of genes associated with methylation imprints, which are confirmed by knockout (KO) mouse studies

| Genes   | Mating Genotype                        | Sample                  | IG-DMR | H19-DMR | Rasgrf1 | Snrpn | Peg3   | Igf2r | Impact | Zac1 | Mest | Grb10 | KvDMR | Naat | Peg10 | Nespas | Gnas 1A | Reference(s)          |
|---------|----------------------------------------|-------------------------|--------|---------|---------|-------|--------|-------|--------|------|------|-------|--------|------|-------|--------|---------|----------------------|
| Dnmts   | Dnmt3a(+/f) and Zp3-Cre                 | Oocyte                  | Normal | Hypo    | partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         | Kaneda et al. 2010; Shirane et al. 2013 |
|         |                                        |                         | Normal | Hypo    | partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3a(b+/f) and Zp3-Cre                 |                         | Normal | Normal  | Normal  | Partial | hypo  |       | hypo  |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3b(+/f) and Zp3-Cre                 |                         | Normal | Normal  | Normal  | Partial | hypo  |       | hypo  |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3L(+/−)                             |                         | Normal | Hypo    | partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3a(+/−) and TNAP-Cre                |                         | Partial | hypo   | Partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3b(+/−) and TNAP-Cre                |                         | Partial | hypo   | Partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3L(+/−)                             |                         | Partial | hypo   | Partial | hypo  |       | hypo  |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt3a(b+/f) and Zp3-Cre × Dnmt3a(b+/−) | E9.5 embryo            | Normal | Partial | hypo   |       |       |       |        |      |      |       |        |      |       |        |         | Kato et al. 2007             |
|         | Dnmt1(M−/+)                             |                         | Partial | hypo   | Partial | hypo  |       |       |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt1(MZ−/−)                            |                         | Hypo    | Hypo    | Hypo    |       |       |       |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt1o(−/−) × Dnmt1o(M+/−)              |                         | Partial | hypo   | Partial | hypo  |       |       |        |      |      |       |        |      |       |        |         | Howell et al. 2001            |
|         | Dnmt1o(+/−)                             |                         | Partial | hypo   | Partial | hypo  |       |       |        |      |      |       |        |      |       |        |         |                      |
|         | Dnmt1o(+/−)                             |                         | Partial | hypo   | Partial | hypo  |       |       |        |      |      |       |        |      |       |        |         | Cirio et al. 2008             |
|         | Dnmt1o(Z−/−)                            |                         | Partial | hypo   | Partial | hypo  |       |       |        |      |      |       |        |      |       |        |         |                      |
| NP95    | Np95(Z−/−) × Np95(Z+/−)                 | E9.5 embryo            | Hypo    |       |       |       |        |        |        |      |      |       |        |      |       |        |         | Sharif et al. 2007          |
| KDM1A   | KDM1A(+/f) and Vasa-Cre × (++/+)        | Perinatal pup          | Normal  | Normal  | Hyper   |       |       |       |        |      |      |       |        |      |       |        |         | Wasson et al. 2016          |
|         | KDM1A(M−/+)                             |                         | Normal  | Normal  | Normal  | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Partial hypo |
|         | KDM1A(+/f)                              |                         | Normal  | Normal  | Normal  | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Partial hypo |
|         | KDM1B(+/−)                              | MII oocyte              | Normal  | Normal  | Normal  | Partial | hypo  | Partial | Partial | Hypo  | Partial | Partial | Partial | Partial | Partial | Partial | Kacere et al. 2009 |
|         | KDM1B(+/−)                              | MII oocyte              | Normal  | Normal  | Normal  | Partial | hypo  | Partial | Partial | Hypo  | Partial | Partial | Partial | Partial | Partial | Partial |               |
| Genes       | Mating                  | Genotype                        | Sample     | IG-DMR | H19-DMR | Rasgrf1 | Snrpn | Peg3 | Igf2r | Impact | Zac1 | Mest | Grb10 | KvDMR | Nnat | Peg10 | Nespal | Gnas | IA       | Reference(s) |
|-------------|-------------------------|---------------------------------|------------|--------|---------|---------|-------|------|-------|--------|------|------|-------|-------|-------|-------|--------|-------|----------|---------------|
| PGC7        | PGC7(−/−) x              | PGC7(M−/−)                      | Oocyte     | Normal | Partial | Hypo    | Normal| Normal| Normal| Hypo   |     |     | Normal| Normal| Normal| Normal| Normal | Nakamura et al. 2007 |
|             | (+/+)                   |                                 | Zygote     | Normal | Partial | hypo   |       |      |       |        |     |     |       |       |       |       |        |       |          |               |
| KAP1        | KAP1(+/f) and Zp3-cre x  | KAP1(M−/+)+                     | Oocyte     | Normal | Normal  | normal | Normal| Normal| Normal| Normal |     |     | Normal| Normal| Normal| Normal | Lorthongpanich et al. 2013 |
|             | (+/+)                   |                                 | 8-cell     | Normal | Normal  | hypo   |       |      |       |        |     |     |       |       |       |       |        |       |          |               |
|             |                         |                                 | E4.5       | Normal | Normal  | hypo   |       |      |       |        |     |     |       |       |       |       |        |       |          |               |
|             |                         |                                 | E12.5      | Normal | Normal  | hypo   |       |      |       |        |     |     |       |       |       |       |        |       |          |               |
| Rexl        | (+/+) ×                 | Rexl−/−×                       | Sperm      | Normal | Normal  | Normal | Normal| Normal| Normal| Normal |     |     | Normal| Normal| Normal| Hyper |       | Kim et al. 2011 |
|             | Rexl(+/−) ×             | Rexl−/−×                       | Blastocyst | Normal | Normal  | Normal | Normal| Normal| Normal| Normal |     |     | Normal| Normal| Normal| Hyper |       |           |               |
|             | MinI−/− ×               | MinI−/−×                       | Blasto×    | Normal | Normal  | Normal | Partial| hypo  |       |       |     |     |       |       |       |       |        |       |          |               |
|             | MinI+/− ×               | MinI+−×                       | Neonatal liver | Normal | Normal  | Normal | Partial| hypo  |       |       |     |     |       |       |       |       |        |       |          |               |
| CTCF        | H19-DMR CTCF site       | H19-DMR CTCF site              | Oocyte     | Normal |       |       |       |       |       |       |     |     |       |       |       |       |        | Schoenherr et al. 2003 |
|             | (mut/mut) ×             | (mut/mut)×                     | Blastocyst | Normal | Normal  | Normal | Partial| hypo  |       |       |     |     |       |       |       |       |        |       |          |               |
| Mili        | Mili−/− ×               | Spermatogonia                  | Normal     | Normal | Partial | hypo  |       |       |       |       |     |     |       |       |       |       |        | Watanabe et al. 2011 |
| MitollD     | MitollD−/− ×            |                                 | Normal     | Normal | Partial | hypo  |       |       |       |       |     |     |       |       |       |       |        |           |               |
| Miwi2       | Miwi2−/− ×              |                                 | Normal     | Normal | Partial | hypo  |       |       |       |       |     |     |       |       |       |       |        |           |               |
| Aid         | Aid−/− ×                | E13.5 PGCs                     | Partial   |       |       |       |       |       |       |       |     |     |       |       |       |       |        | Popp et al. 2010 |
| Tet         | Tet1−/− ×               | E13.5 PGCs                     | Partial   |       |       |       |       |       |       |       |     |     |       |       |       |       |        | Yamaguchi et al. 2013 |
|             | (+/+) ×                 |                                 | E19.5 PGCs | Partial |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1+/P− ×              |                                 | E9.5 embryo | Partial |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1−/− ×               |                                 | E19.5 placenta |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | (+/+) ×                 |                                 | Tail       | Partial |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1−/− ×               |                                 | Hyper      | Partial |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | (+/+) ×                 |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1−/− ×               |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | (+/+) ×                 |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1−/− ×               |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | (+/+) ×                 |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |
|             | Tet1−/− ×               |                                 | Partial |       |       |       |       |       |       |       |     |     |       |       |       |       |        |           |               |

*aWGBS data.
*bSingle-cell analysis.
*cVariation among KOs.
*dReduced representation bisulfite sequencing (RRBS) data.
ZFP57 AND IMPRINT METHYLATION

in global hypomethylation. Importantly, imprinted DMRs are resistant to passive DNA demethylation; however, in the Dnmt1 maternal effect mutant (KO M−/+), the maternal-zygotic mutant (KO MZ−/−), methylation imprints are respectively partially or completely lost, indicating a specific role for Dnmt1 in maintaining the methylation memory of parental-origin at imprints (Table 1; Howell et al. 2001; Cirio et al. 2008; Hirasawa et al. 2008). Imprints are also resistant to active DNA demethylation that occurs independently of DNA replication, via base excision repair (BER) and/or ten-eleven translocation 3 (Tet3)-mediated oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in the fertilized zygotes (Gu et al. 2011; Santos et al. 2013). The resistance of imprinted methylation to both active and passive DNA demethylation events is also dependent on PGC7/Stella. Oocyte-derived PGC7 prevents Tet3-induced active demethylation (Wossidlo et al. 2011), and its deletion causes loss of methylation (LOM) at a subset of imprints (Peg3, Peg1, Peg10, Rasgfr1, and H19 DMRs) but not at others (Surpn DMR and IG-DMR) in zygotes (Table 1; Nakamura et al. 2007). The specificity likely arises from the localization of H3K9me2; H3K9me2 is abundant at H19 and Rasgfr1 DMRs but not at the IG-DMR in mature sperm (Nakamura et al. 2012).

Erasure of methylation imprints must take place in the germline prior to the establishment of new parental origin specific imprints. This erasure, which generally encompasses the whole genome, occurs by both passive and active demethylation in primordial germ cells (PGCs) between E11.5 and E13.5 (Hajkova et al. 2002). The cytidine deaminase AID and also Tet1 contribute to the active demethylation (Popp et al. 2010; Dawlaty et al. 2013; Yamaguchi et al. 2013). Reduced litter sizes are observed with crosses involving either Tet1 homozygous males or homozygous females with genetically wild-type mice with growth phenotypes observed upon paternal transmission of the deletion and placental abnormalities upon maternal transmission (Yamaguchi et al. 2013). Reduced representative bisulfite sequencing (RRBS) in the Tet1 KO E13.5 PGCs shows hypermethylation at several imprinted DMRs (Table 1), indicating that the phenotypic variation is likely caused by their incomplete demethylation during PGC development.

**KRAB-ZFPs**

ZFPs are one of the largest groups of transcription factors in mammals and are now known to be present in all kingdoms of life including eubacteria and archaeabacteria, protists, fungi, animals, and plants (Looman et al. 2002; Urrutia 2003). ZFPs can contain up to 30 zinc fingers that mediate sequence-specific DNA or RNA binding. The canonical C2H2 zinc finger is composed of a short β hairpin and α helix structure that coordinates a single zinc atom, held in place by two highly conserved histidine and cysteine residues (Lupo et al. 2013). DNA-binding specificity is achieved through hydrogen bond interactions mediated by three amino acids within the zinc finger motif that contact three consecutive bases on one strand of DNA (Klug 2010).

In humans, one-third of the approximately 800 zinc finger proteins contain a highly conserved KRAB domain and are known as KRAB zinc finger proteins (Bellefroid et al. 1991; Huntley et al. 2006). The KRAB domain is restricted to tetrapods and KRAB-ZFPs are found in all mammalian genomes (Emerson and Thomas 2009). The conserved 50–75 amino acid sequence consists of one or both KRAB A and B boxes. The A box has been shown to play a key role in transcriptional repression by binding the potent corepressor protein KAP1, also known as TIF1β or TRIM28 (Friedman et al. 1996). The B box is thought to enhance repression mediated by the A box (Urrutia 2003).

KAP1 has been shown to initiate heterochromatin formation through interaction with histone H3K9 methyltransferase SETDB1/ESET; heterochromatin protein 1 (HP1), and the nucleosome remodeling and deacetylation (NuRD) histone deacetylase complex (Lechner et al. 2000; Schultz et al. 2002). Expression of KAP1 is high in embryonic stem (ES) cells where it has been shown to repress endogenous retroviral elements (ERVs) (Rowe et al. 2010). KAP1 has no DNA- or chromatin-binding activity itself and must be tethered to the genome by interaction with members of the KRAB-ZFP family. The large number of KRAB-ZFPs present in the mammalian genome gives rise to a complex, locus-specific regulatory network that targets genes for silencing during development. The modular structure of ZFPs makes them well suited to rapid adaptive evolution. Indeed, KRAB-ZFPs are thought to have emerged and evolved in parallel with endogenous retroviruses as a way of suppressing their expression and retrotransposition to preserve and protect transcriptional dynamics (Emerson and Thomas 2009).

Recently, ZFP809 has been shown to repress murine leukemia viruses in ES cells and to initiate endogenous retroviral silencing during embryonic development (Wolf and Goff 2009; Silva et al. 2015). Human ZNF91 and ZNF93 rapidly evolved throughout the primate lineage to silence SVA and L1 retroelements (Jacobs et al. 2014). In addition to controlling transposable elements, a number of KRAB-ZFPs are thought to have been co-opted to function in host regulatory gene pathways (Rowe et al. 2013).

**ZFP57—ROLE IN GENOMIC IMPRINTING**

In 2008, Li et al. (2008) found that ZFP57 is necessary for the postfertilization maintenance of methylation at several imprinted DMRs and for the establishment of methylation at the Surpn DMR. The methylation analysis was conducted by combined bisulfite restriction analysis (COBRA) and bisulfite Sanger sequencing standard techniques of the time. To elucidate the extent of demethylation with greater precision, we generated a new ZFP57 KO mouse model harboring a deletion from exon 4 to exon 5, including the KRAB domain and five zinc fingers (Fig. 2). As the targeted ES cell was on a C57BL6N genetic background, the mouse was backcrossed to...
not all imprinted DMRs were equally influenced by ZFP57. In particular, the ZFP57 MZ(+/−) mutants died prenatally, whereas the ZFP57 zygotic KO (Z−/−) mutants showed partially perinatal lethality and growth retardation. The milder phenotype in the ZFP57 Z(+/−) mutants could be explained by a functional role for maternal ZFP57 accumulated during oogenesis, contributing to the protection of methylation imprints early after fertilization.

Because ZFP57 levels are high in oocytes, we set out to confirm in more detail, whether ZFP57 may be necessary for the establishment of methylation imprints in oocytes. We analyzed methylation of multiple imprinting control regions including the Snrpn, Peg3, Zac1/PLAGL1, Nespas, H19 DMRs, and the IG-DMR and KvDMR in the ZFP57 deleted germinal vesicle (GV) and metaphase II (MII) oocytes using bisulfite pyrosequencing (Fig. 3A). All of the imprinted DMRs showed normal methylation in the ZFP57 deleted oocytes, indicating that ZFP57 is not involved in de novo methylation of the imprinted DMRs, including Snrpn, in oocytes.

Subsequently, we examined methylation at the nine representative imprinted DMRs in the brain, liver, and placenta of the ZFP57 MZ(+/−), Z(−/−), and M(−/+). E12.5 embryos (Fig. 3B–J). We found that not all imprinted DMRs were equally influenced by ZFP57 loss. For example, whereas Snrpn and Zac1 DMRs lost methylation completely in the brain, liver, and placenta of the ZFP57 MZ(+/−) mutants, Peg3, Nespas, Igf2r, Rasgrf1 DMRs, and IG-DMR showed greater hypomethylation in the embryonic tissues than in the placenta in the ZFP57 MZ(+/−). This suggests that inner cell mass (ICM) derivatives are more easily demethylated in the absence of ZFP57 than trophoectoderm (TE) derivatives. Tet1 may contribute to this susceptibility because Tet1 is necessary for demethylation at imprinted DMRs during PGC development and is expressed in the ICM but not the TE (Ito et al. 2010; Yamaguchi et al. 2013). In general, methylation imprints are maintained across tissues (Babak et al. 2015); therefore, it was expected that there would be no difference in the methylation levels of brain compared to liver in the ZFP57 mutants. However, methylation of Rasgrf1 DMR was almost completely lost in the brain (<20%) but partially lost in the liver (20%–40%) in the ZFP57 MZ(−/−) and Z(−/−) mutants (Fig. 3I). Interestingly, among the paternally imprinted DMRs, only Rasgrf1 DMR shows reduction in methylation in the Dnmt3a/3b MZ(+/−) embryos (Table 1; Hirasawa et al. 2008). Therefore, it is likely that Dnmt3a and/or Dnmt3b are required to maintain the methylation state at this paternally derived imprint in contrast to the maintenance of the H19 and IG-DMRs and consistent with its different mechanism of germline imprint acquisition. Its unique tissue-specific behavior in the ZFP57 mutants (Fig. 3I) indicates a role for ZFP57 and also may reflect the methyltransferase activity of these de novo enzymes and the different acquisition and maintenance mechanisms acting at this germline DMR.

One of the most interesting findings in analyzing ZFP57 mutants is that each imprinted DMR has a different demethylation pattern in the mutants (Fig. 3). The variation could be caused by a combination of the timing of and susceptibility to demethylation. For instance, the Snrpn DMR seems highly dependent on ZFP57 and, in its absence, loses methylation quickly and completely in the ZFP57 MZ(−/−). It also consistently loses some methylation in ZFP57 M(−/+). mutants, which have no oocyte-derived ZFP57 but can express ZFP57 from the paternal chromosome after fertilization (Fig. 3B). In contrast, the Rasgrf1 DMR is less sensitive than Snrpn and is likely to lose methylation in later-stage preimplantation embryos. This is suggested because the level of methylation loss is similar between the ZFP57 MZ(−/−) and Z(−/−) mutants (Fig. 3I); the latter having normal oocyte-derived ZFP57. Further analysis in preimplantation embryos is necessary to understand the dynamics and mechanism of loss of methylation memory after fertilization.
Figure 3. Quantitative analysis of methylation at nine germline imprinted DMRs using bisulfite pyrosequencing in the ZFP57 mutants. (A) The GV and MII oocytes were collected after hormone injection from the ZFP57Z(−/−) and ZFP57 heterozygous female mice on the 129aa/C57BL6J mixed background whose ages were >8 wk. Each tube includes 43–55 GV oocytes or 101–158 MII oocytes. (B–J) The four genotypes of embryos were generated by the mating of the ZFP57 mutants on the 129aa genetic background. The ZFP57 heterozygous and M(−/+ ) embryos were collected from two separate litters, and the ZFP57 Z(−/−) and MZ(−/−) embryos were obtained from three and four litters, respectively. Each tissue was collected at E12.5, and DNA was isolated using standard phenol/chloroform extraction method. Bisulfite treatment was performed using the Imprint DNA Modification Kit (Sigma-Aldrich). The vertical axis represents methylation level, and the horizontal axis shows individual CpG sites analyzed. The lines represent the mean ± standard deviation (SD). The statistical analysis was conducted by unpaired t-test with Welch’s correction. (*) P < 0.05; (**) P < 0.01 versus control.
**ZFP57 IN VITRO AND IN VIVO**

ZFP57 is highly expressed in ES cells in vitro, and is down-regulated upon differentiation into neural stem cells (Ahn et al. 2004). Intriguingly, despite having variable demethylation effects in the ZFP57 mutant mice in vivo, ZFP57 protein binds at all known imprinted DMRs in ES cells in a parental origin-specific and methylation-sensitive manner. Intriguingly the binding in each case was found to be restricted to the germline-imprinted DMRs and not secondary somatically methylated DMRs (Strogantsev et al. 2015). Furthermore, ZFP57 null ES cells show LOM at all tested imprinted DMRs including KvDMR and H19 DMR (Quenneville et al. 2011), which are not affected in the ZFP57 MZ(+/−) mutant mice (Fig. 3G,J). These findings suggest that in the mouse mutants there may be functional redundancy perhaps with another KRAB-ZFP or indeed that there are other mechanisms involved in maintenance of methylation at some imprinted DMRs in vivo. Redundancy of ZFP57 with other KRAB-ZFP(s) is supported by the finding that methylation at the H19 DMR, which is resistant to demethylation in the ZFP57 mutants, is partially lost in the KAP1 M(−/+ ) embryos lacking the maternally derived interacting partner of KRAB-ZFPs (Table 1; Messerschmidt et al. 2012; Lorthongpanich et al. 2013).

ZFP57 binds methylated CpGs in a sequence-specific manner within the TGCCGC/CR consensus sequence, and recruits Dnmt3a and Setd1a via KAP1 in ES cells (Quenneville et al. 2011; Liu et al. 2012; Strogantsev et al. 2015). H3K9me3 is lost at the imprinted DMRs in both the Dmnt3a/3b triple KO and ZFP57 KO ES cells, indicating that methylated CpGs within the motif are required for H3K9me3 at imprints. On the other hand, DNA methylation is only minimally affected in the Setdb1 KO ES cells; however, because these ES cells can survive for only 6–7 d, it is unclear whether this effect on methylation is directly dependent on H3K9me3 (Leung et al. 2014). KAP1 and Setdb1 are required for the silencing of ERVs (Rowe et al. 2010; Leung et al. 2014) and embryos with zygotic deletion of those genes die at ~E7.5 with severe growth defects (Cammas et al. 2000; Dodge et al. 2004). As expected, the phenotype of the zygotic ZFP57 mutants is less severe than those of the KAP1 and Setdb1 zygotic mutants. Our recent data using ES cells indicates that ZFP57 is also involved in regulating a subset of ERVs and nonimprinted genes (Strogantsev et al. 2015; R Strogantsev, H Shi, and A Ferguson-Smith, unpubl.). Interestingly, by mapping ZFP57 genomic binding sites in reciprocal hybrid (C57BL/6–Cast/Eij) ES cells, we found a large number of genetically determined monoallelic binding sites, which are frequently coincident with skewed allelic expression of neighboring genes (Strogantsev et al. 2015). Hence, ZFP57 may have evolved functions in addition to its role in the targeted maintenance of imprints and may play a role in the regulation of non-imprinted unique regions and in the silencing of repetitive elements consistent with established roles for some other KRAB-ZFPs. To date, no other KRAB-ZFP has been found to be DNA methylation-sensitive, and further studies are required to determine which KRAB-ZFPs may have this property.

**ZFP57 FUNCTION IN HUMAN**

Human ZFP57 has KRAB A and B domains and seven zinc fingers. Homozygous zygotic effect mutations in human ZFP57 cause transient neonatal diabetes (TND) (Mackay et al. 2008; Boonen et al. 2013). Interestingly, all individuals homozygous for a ZFP57 mutation show LOM at ZAC1, PEG3, and GRB10 DMRs, and additionally some of the individuals have LOM at PEG1, NESPAS DMRs, and KVDPR in the peripheral blood leukocytes (Table 2). One case of a maternal-zygotic effect mutation has been reported, whose phenotype and degree of LOM is not more severe than the individuals with zygotic effect mutation (Boonen et al. 2013). It is unknown whether this is because of the nature of the ZFP57 missense mutation or the function of ZFP57 in humans. Unlike the mouse, single-cell RNA sequencing data show that ZFP57 does not appear to be expressed in human oocytes (Yan et al. 2013; Okae et al. 2014); hence, only zygotic ZFP57 seems to be required to maintain imprint methylation in humans. Further molecular studies are needed to understand which regions of the genome are targeted by human ZFP57 and the extent to which targeted regions lose methylation in patients with ZFP57 mutations.

**CONCLUSION**

Since the first imprinted genes were identified in 1991, exploitation of imprinting as a paradigm for elucidating epigenetic mechanisms involved in mammalian transcriptional control has been extensive (for review, see Ferguson-Smith 2011). However, our knowledge of the relationship between the DNA sequence and the establishment and maintenance of functionally important epigenetic modifications remains limited. The identification and characterization of the function of KRAB-ZFPs, key sequence-specific players in these important epigenetic processes, will provide major insights into the regulation and modulation of both unique and repetitive sequences in mammalian genomes. For example, their role in the mechanisms targeting and maintaining epigenetic marks in vivo and in vitro based on the underlying DNA sequence will provide insights into the recruitment of DNA and chromatin modifiers, the genomic properties required for these interactions, and the hierarchical steps involved in the dynamic temporal events that regulate chromatin states at unique and repetitive sequences. This is not only of basic biological interest but also suggests therapeutic potential, perhaps leading to the development of methods for epigenetic manipulation at specific loci and contributions and to epigenetic therapies for human diseases in the future.

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Table 2. Comparison of imprinted differentially methylated region’s (DMR’s) methylation in the ZFP57-mutated human and ZFP57 knockout (KO) mouse

| Genotype | Sample | IG-DMR | H19-DMR | Rasgrf1 | Snrpn | Peg3 | Igf2r | Zac1 | Peg1(Mest) | Grb10 | KvDMR | Nespas | Reference |
|----------|--------|--------|---------|---------|-------|------|-------|------|------------|-------|--------|--------|------------|
| Mouse    | E12.5 Brain | Normal | Normal | Hypo    | Partial hypo | Slightly hypo | Normal | Partial hypo | Partial hypo | Normal | Normal | This study |
|          | E12.5 Liver  | Normal | Normal | Partial hypo | Partial hypo | Slightly hypo | Normal | Partial hypo | Partial hypo | Normal | Normal |          |
|          | E12.5 Placenta | Normal | Normal | Slightly hypo | Partial hypo | Normal | Normal | Partial hypo | Partial hypo | Normal | Normal |          |
| ZFP57(Z−/−) | E12.5 Brain  | Hypo   | Normal | Hypo    | Hypo | Partial hypo | Hypo | Normal | Partial hypo | Normal | Partial hypo |          |
|          | E12.5 Liver  | Hypo   | Normal | Partial hypo | Hypo | Partial hypo | Hypo | Normal | Partial hypo | Normal | Partial hypo |          |
|          | E12.5 Placenta | Partial hypo | Normal | Slightly hypo | Hypo | Partial hypo | Slightly hypo | Hypo | Normal | Partial hypo | Normal | Partial hypo |          |
| ZFP57(MZ−/−) | E12.5 Brain  | Normal | Normal | Normal | Partial hypo | Normal | Normal | Normal | Normal | Normal | Normal |          |
|          | E12.5 Liver  | Normal | Normal | Normal | Partial hypo | Normal | Normal | Normal | Normal | Normal | Normal |          |
|          | E12.5 Placenta | Normal | Normal | Normal | Partial hypo | Normal | Normal | Normal | Normal | Normal | Normal |          |
| Human    | Peripheral blood leukocytes | Normal | Normal | Normal | Partial hypo | Hypo | Normal/ partial hypo | Hypo/ partial hypo | Normal/ partial hypo | Normal/ partial hypo | Normal/ partial hypo | Boonen et al. 2013 |

*aVariation among patients.*
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