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Supplemental Information

In Embryonic Stem Cells, ZFP57/KAP1 Recognize a Methylated Hexanucleotide to Affect the Chromatin and DNA Methylation of Imprinting Control Regions

Simon Quenneville, Gaetano Verde, Andrea Corsinotti, Adamandia Kapopoulou, Johan Jakobsson, Sandra Offner, Ilaria Baglivo, Paolo V. Pedone, Giovanna Grimaldi, Andrea Riccio, and Didier Trono

Supplemental Experimental Procedures

Capillary Electrophoresis

Rasgrf1 ICR, was amplified and labeled by PCR using the forward primer conjugated by the HEX fluorochrome and then, subjected to capillary electrophoresis to separate the longest paternally allele to the shortest maternal one.

COBRA

The DNA methylation of multiple ICRs using wild-type (A3 cell line), *Zfp57*$^{-/-}$ and *Kap1* conditional knockout ES cells was analyzed by combined bisulfite restriction analysis (COBRA). After the treatment of 2 μg of genomic DNA with sodium bisulfite, each genomic region was amplified and radiolabeled (α^{32}PdCTP) by PCR and then digested with a restriction enzyme containing a CpG dinucleotide in its target sequence. The digested fragments were resolved on a non-denaturing polyacrylamide gel and quantified by using a PhoshorImager and ImageQuant software by Molecular Dynamics.

Lentiviral Vectors

Tetracyclin inducible vectors were based on previously described vectors (Barde et al., 2009). Vectors were produced by co-transfection of complementation and vector plasmids in 293T cells as already described, and titration was performed by qPCR analysis of transduced 293T cells (Barde et al., 2009). Vector titers were usually about 10^9 IP/ml and MOI of 50 IP/cell was used for ES cell transduction.

Antibodies

For ChIP PCR, H3K9me3 antibody from Abcam (ab8898), and the H3K9ac (Upstate, 06-942) were used. For the KAP1 ChIP we used an antibody recognizing the RBCC-domain of KAP1 (Sripathy et al., 2006) and HA ChIP were performed using anti-HA affinity matrix (Roche).
For allele-specific ChIPs, H3K9me3, H3K9Ac, H3K27me3, CTCF and HP1γ were captured with antibodies from Upstate (#07-442, #07-352, #07-449, #07-729, #05-690 respectively), for KAP1 and ZFP57 Abcam antibody were used: ab22553 ab45341 respectively, and SETDB1 with a rabbit polyclonal antibody IgG from Santacruz (sc-66884).

Validation of the Specificity of the Anti-ZFP57 Antibody

The specificity of the anti-ZFP57 antibody (ab45341) was tested by Western blot and ChIP analyses in wt and *Zfp57*$^{-/-}$ ESC (Suppl. Fig. 7A). Western blotting was performed using total

cell lysates prepared either from wild-type A3 or *Zfp57*$^{-/-}$ ESC. Cells were lysed in 10 mM Tris-HCl, pH,7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, supplemented with 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 20 mg/ml aprotinin, 4 mg/ml pepstatin, 10 mM leupeptin, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 25 mM glycerophosphate (Sigma-Aldrich). After 15 min incubation on ice, the lysates were cleared by centrifugation. Total cell extracts were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P transfer membranes (Millipore). After blocking with 5% nonfat milk, membranes were incubated with polyclonal anti-ZFP57 antibody (abcam, ab45341). The immune complexes were detected by the ECL Plus detection system according to the manufacturer's protocol (Amersham Bioscience). The ChIP analysis of ZFP57 binding at the *Snrpn* ICR (Suppl. Fig. 7B) was done as described above.

Statistical Analyses

Differences in methylated CpGs of immunoprecipitated DNAs vs input DNAs and of wt ESCs vs *Zfp57*$^{-/-}$ ESCs were analyzed with the Student's t-test by considering each sequenced clone as individuals. For Figure 6C, the test was made using each CpG as individual since all the input clones were methylated.

Supplemental References

Barde, I., Laurenti, E., Verp, S., Groner, A.C., Towne, C., Padrun, V., Aebischer, P., Trumpp, A., and Trono, D. (2009). Regulation of episomal gene expression by KRAB/KAP1-mediated histone modifications. J Virol *83*, 5574-5580.

Bilodeau, S., Kagey, M.H., Frampton, G.M., Rahl, P.B., and Young, R.A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. Genes Dev *23*, 2484-2489.

Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M., and Tilghman, S.M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*, 486-489.

Sripathy, S.P., Stevens, J., and Schultz, D.C. (2006). The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. Mol Cell Biol *26*, 8623-8638.

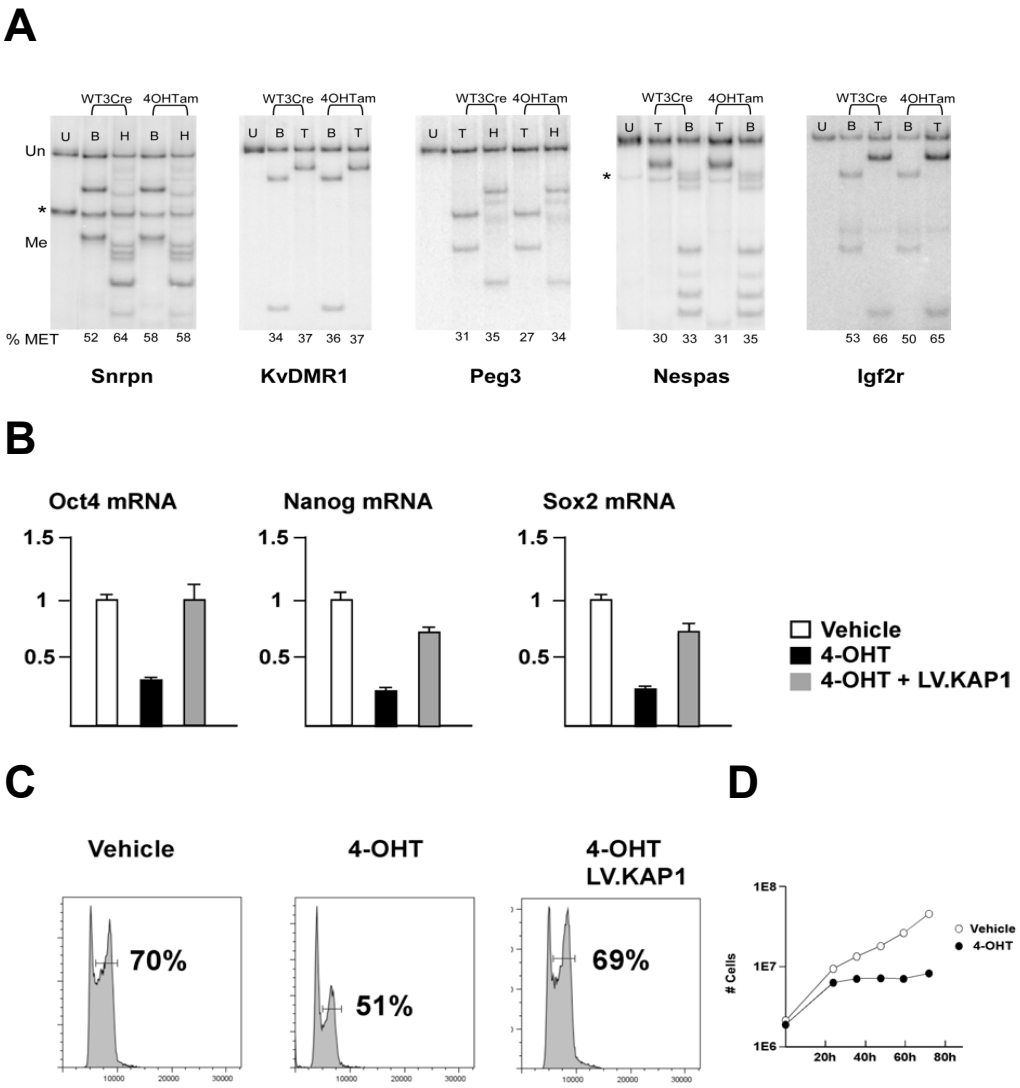


Figure S1. *Kap1* Removal Does Not Affect ICR DNA Methylation at Imprinting Control Regions But Blocks Cell Division, Related to Figure 1

(A) COBRA analysis showing DNA methylation levels at several ICRs in ESC 72 hours after KAP1 removal by addition of 4-hydroxy-tamoxifen (4OHTam). The results obtained on wild-type ESC (WT3cre) are shown as control. The numbers underneath each panel indicate the percent of DNA methylation relative to total (%MET). The molecular length of the undigested PCR product and the sites recognized by each restriction enzyme are indicated above each panel: B, BstuI; T, TaqI; U, Undigested; *, non-specific band; Me, Methylated DNA; Un, Unmethylated DNA.

(B) Pluripotency genes are maintained in HAKAP1-complemented cells after excision of the endogenous KAP1-allele (72Hrs post 4-OHT addition). Error bars represent SD (n=3).
(C and D) Cell division is stopped after *KAP1* excision. *KAP1*-excisable ES cells were plated after addition of 4OH-Tamoxifen and counted at 72 hours or indicated time points.

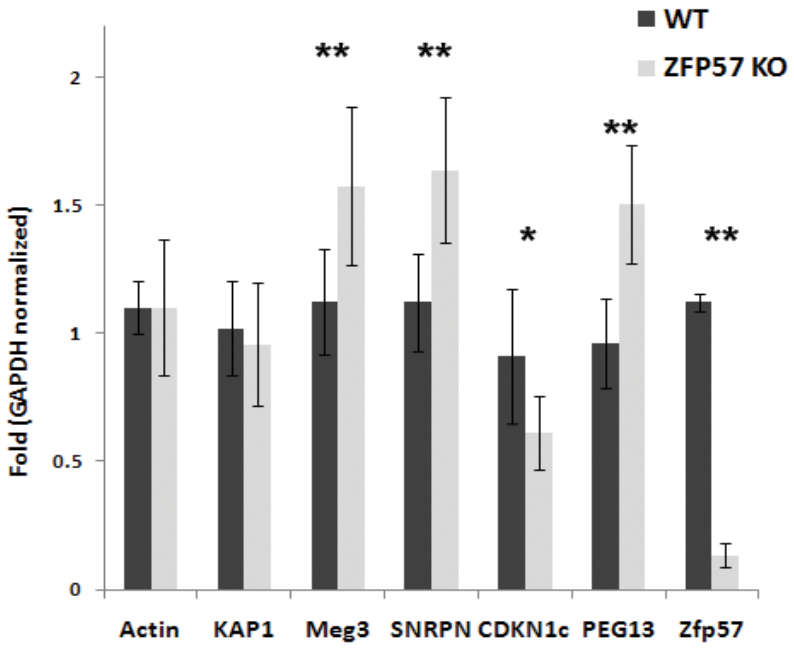


Figure S2. RT-qPCR Confirmation of the Expression Level Modification Induced by Zfp57-Knockout, Related to Figure 2

Relative expression is evaluated as following. The expression levels in WT and Zfp57$^{-/-}$ is normalized on GAPDH expression and then on WT levels. Error bars represent standard variation of biological triplicates. KAP1 and Actin expression showed no significantly difference, but the other tested genes were significantly altered (*: $p < 0.01$, **: $p < 0.001$)

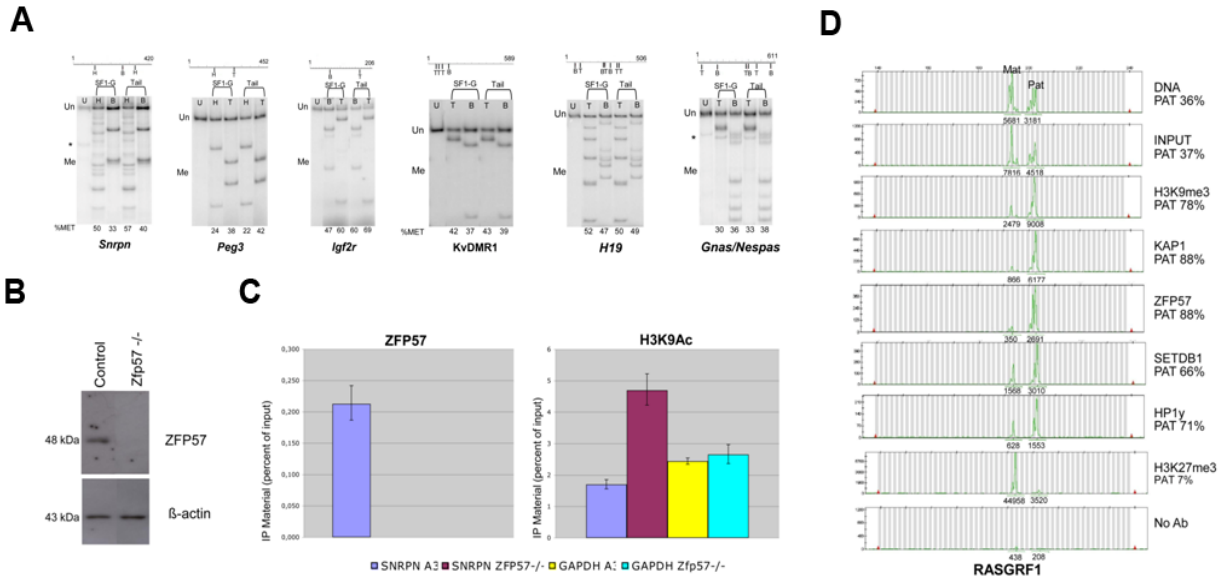


Figure S3. DNA Methylation Patterns at Imprinting Control Regions in SF1-G Embryonic Stem Cells, ZFP57 Antibody Validation, and *Rasgrf1* Locus Evaluation, Related to Figure 3

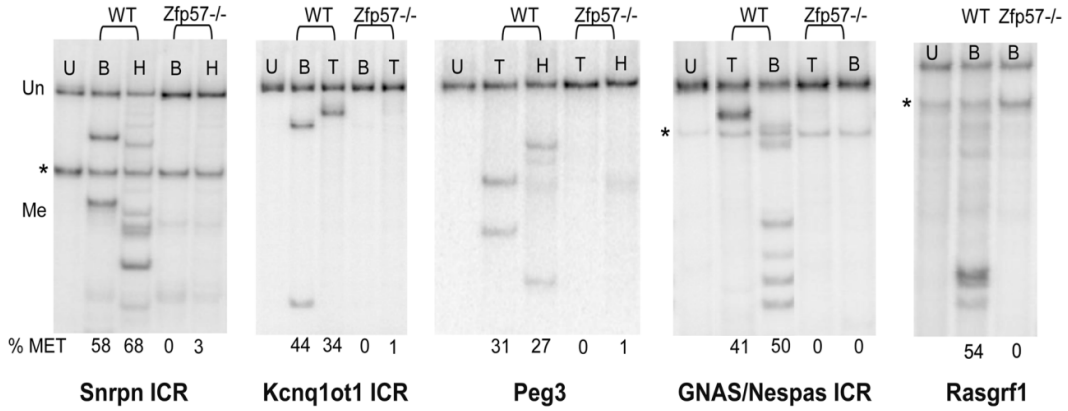
(A) COBRA analysis showing DNA methylation levels at ICRs in SF1-G ESC. Mouse tail DNA was used as a control. Numbers underneath each panel indicate the percentage of DNA methylation relative to total (%MET). Length of undigested PCR product and sites recognized by each restriction enzyme are indicated above each panel. B, BstuI; H, HpyCH4IV; T, TaqI; U, undigested PCR product; *non-specific band; Me, methylated DNA; Un, unmethylated DNA.

(B)Western blot for A3 (Control) or *ZFP57*$^{-/-}$ ESC using a rabbit polyclonal antibody raised against the endogenous ZFP57 to demonstrate antibody specificity.

(C) ZFP57 binding and H3K9Ac at the *Snrpn* ICR were assessed by ChIP combined with quantitative PCR in wt and *ZFP57*$^{-/-}$ ESC. GAPDH was used as control. Error bars indicate standard deviation. Note that the *Snrpn* ICR was amplified from IP material from wt but not *ZFP57*$^{-/-}$ ESC with the ZFP57 antibody and in both wt and *ZFP57*$^{-/-}$ ESC with the H3K9Ac antibody.

(D) Length polymorphism analysis of KAP1-, ZFP57-, SETDB1- and HP1-bound material for *Rasgrf.*

A



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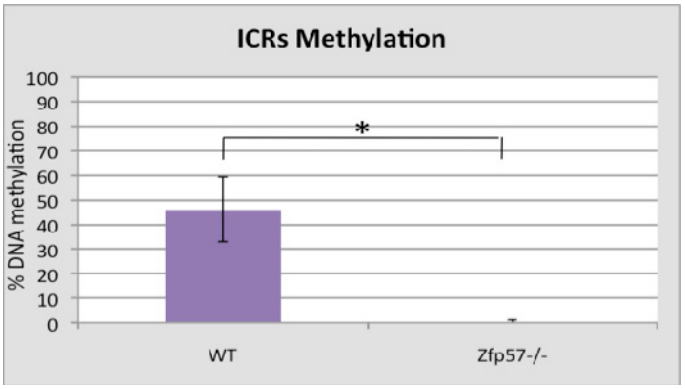
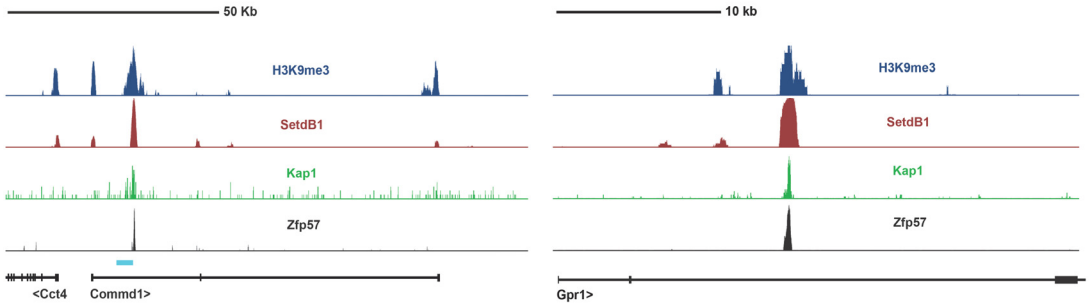


Figure S4. ZFP57 Removal Leads to a Loss of DNA Methylation at Imprinting Control Regions, Related to Figure 5

(A) COBRA analysis demonstrating loss of DNA methylation at several ICRs in *ZFP57*$^{-/-}$ ESC. Wild-type ES cells are used as control (WT). Numbers underneath each panel indicate the percentage of DNA methylation relative to the total (%MET). U: undigested ;H: HpyCH4IV; B: BstUI; T, TaqI; Me, methylated DNA; Un: unmethylated DNA; *, non-specific PCR band.

(B) Average of DNA methylation at the five ICRs analyzed in wt and *Zfp57*$^{-/-}$ ESCs. Data are presented as mean +/- SD of three biological replicates. Asterisk indicates Student's t-test *P value<0.05.* Differences are statistically significant (P-values < 0.001)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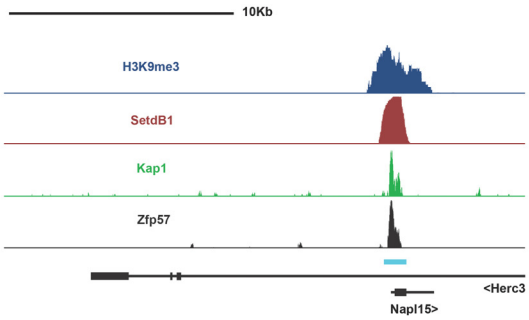
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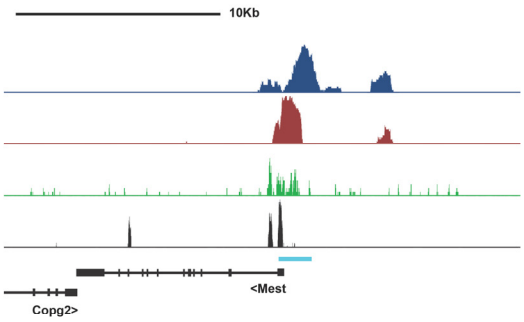
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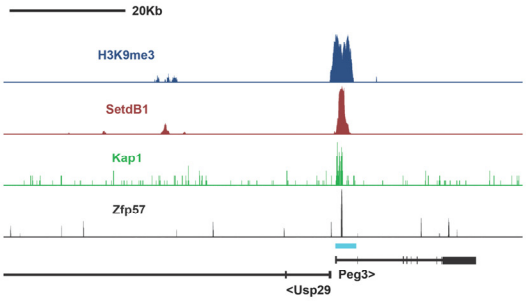
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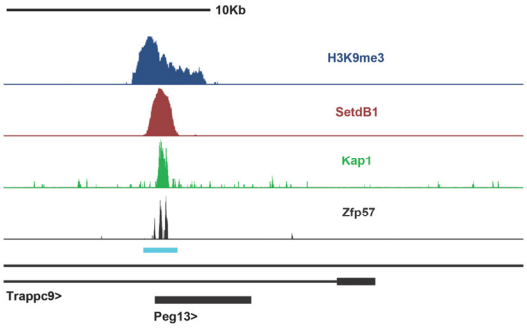
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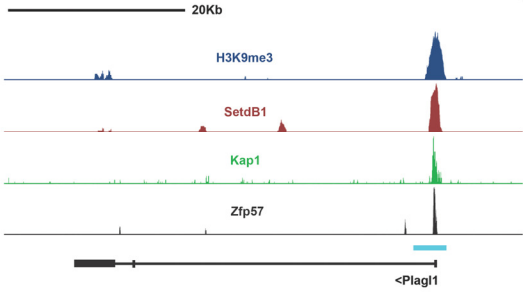
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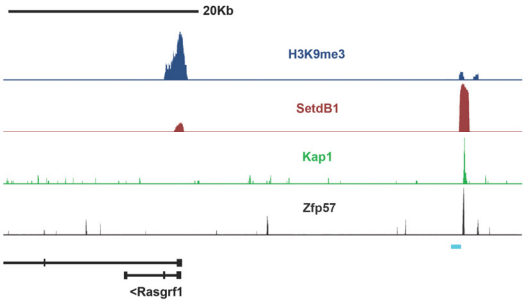
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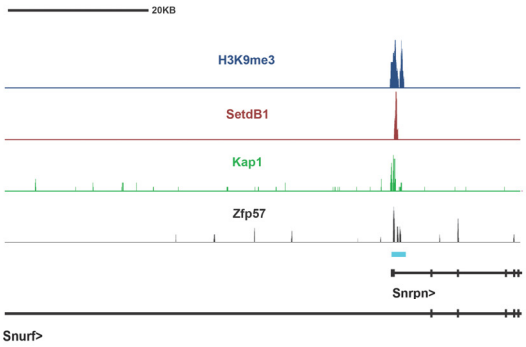
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Figure S5. ZFP57/KAP1, SETDB1, and H3K9me3 at Imprinting Control Regions and Imprinted Genes, Related to Figure 6

ChIP-Seq analyses with antibodies agasint indicated proteins (Kap1 (green), Zfp57 (Black). SETDB1 (Red) and H3K9me3 (Dark blue) (Bilodeau et al., 2009).ICR are light blue, between mRNAs and ZFP57.

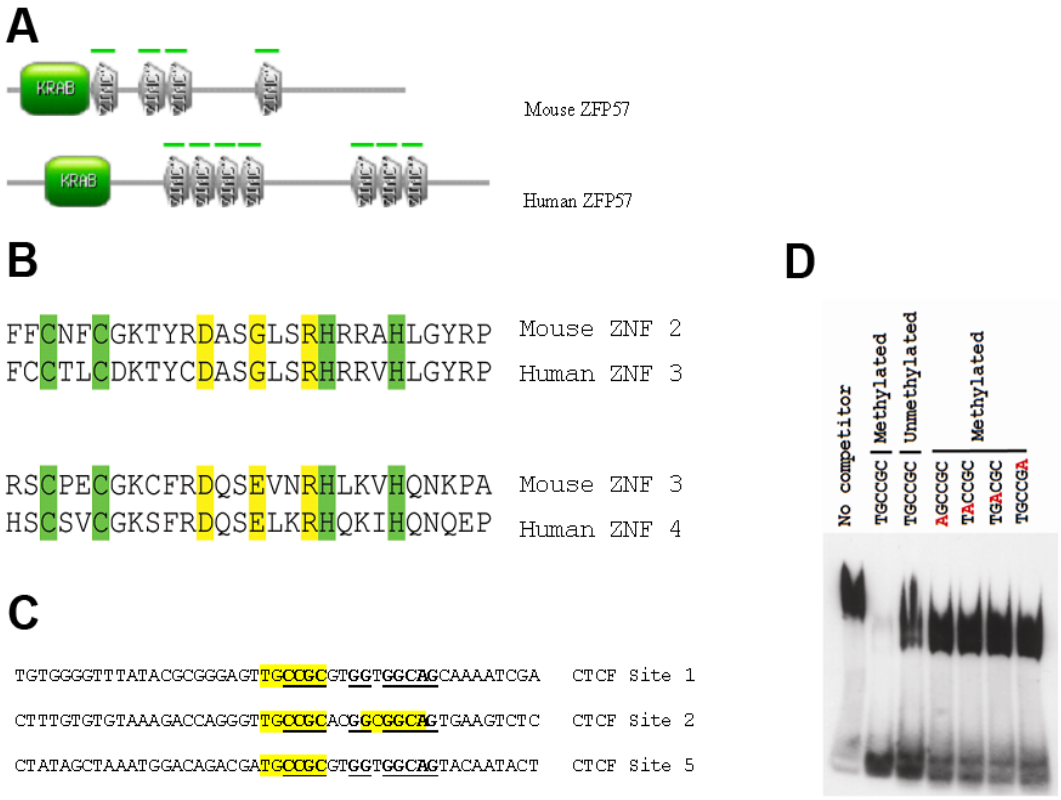

Figure S6. DNA and Protein Sequences Implicated in ZFP57/DNA Interaction, Related to Figure 7

(A) Prosite prediction reveals 4 C2H2 zinc fingers for mouse ZFP57 and 7 for its human orthologue.

(B) Sequence comparison of Mouse C2H2 zinc fingers (Znf) 2 and 3 with human Znf 3 and 4 four (with canonical cysteine and histidine residues in green) shows conservation of the DNA interacting residues (yellow).

(C) ZFP57 and CTCF binding sites in H19 ICR are often overlapping. CTCF binding sites are underlined in bold (Hark et al., 2000). ZFP57 motifs are in yellow.

(D) ZF2-3 EMSA with point mutants as competitors. EMSA was performed with GST.ZF2-3 and DIG labeled WT methylated probe. Lane's names indicate the competitor (20X) that was used. First lane is the optimal shift and lane 2, 3 are with WT competitor with and without methylation. The last 4 lanes are using methylated competitors that bear point mutations outside the CpG dinucleotide.