Online Supplemental Data

Experimental Procedures

Plant material. The wild-type control in this study was the Columbia ecotype. *kyp-2* and *kyp-5* were isolated in the screen described in Jackson et al.[1], and *kyp-6* is a T-DNA insertion line obtained from the SALK Institute Genomic Analysis Laboratory (SALK_041474) [2]. The *met1-3* line was described in Saze et. al. [3]. The triple *drm1 drm2 cmt3* mutant was made by crossing the SALK T-DNA insertions lines (CMT3: SALK_148381; DRM1: SALK_095534: DRM2: SALK_150863). All plant material (DNA for bisulfite sequencing and tissue for ChIPs) was isolated from the first generation homozygous plants for both the *met1-3* line and the *drm1 drm2 cmt3* triple mutant.

Bisulfite Sequencing. DNA was isolated and treated with bisulfite as previously described [4]. In the *AtSN1* element there are a total of 4 CG, 7 CNG and 33 CNN residues. Ten to twelve independent clones were sequenced and the average number of methylated CG, CNG or CNN was determined (Table S1 for all primer sequences). At *AtCOPIA4* there are a total of 25 CG, 18 CNG and 116 CNN residues. Seven to twelve independent clones were sequenced, and the average number of methylated CG, CNG or CNN was determined.

Chromatin Immunoprecipitation. ChIPs were performed as described previously [5] with the following modifications. 0.3 gm of cross-linked tissue was ground by mortar and pestle and resuspended in 2 ml of lysis buffer plus inhibitors. Cells were disrupted for 12 minutes using a Dianode Bioruptor (30 seconds on, 30 seconds off; hi setting). H3K9me2-containing chromatin was immunoprecipitated using antibody from Upstate Biotechnology (#07-441). After reversal of cross-linking, the DNA was purified using a silica gel membrane (Qiagen) and analyzed by real-time PCR using Taqman probes (Primer 3 designed). The values represented are the average of two biological replicas done in duplicate with standard deviations shown.

Immunofluorescence. Interphase nuclei were isolated from leaves of 3 week old plants and immunolabeling was performed as previously described [6]. H3K9me2 was detected using polyclonal antisera from Upstate Biotechnology (#07-441, diluted 1/150) followed by incubation with rhodamine red conjugated secondary antibodies from Jackson Immunoresearch (#111 295 144, goat anti-rabbit diluted 1/200). After staining, vectashield mounting medium with DAPI was added and nuclei were visualized using a Zeiss Axioskop 2 with the Zeiss Axiocam HRC color digital camera system with the Zeiss Axiovision software.

Purification of GST-tagged SRA proteins. GST fusion proteins were made either using the Gateway cloning system with pDEST15 as the final vector, or using the vector pGEX2TK. Both of these vectors result in N-terminal GST fusion proteins. Protein expression in BL21-AI™ Chemically Competent Cells (Invitrogen) was induced by the addition of 2% arabinose and 1 mM IPTG (for pGEX2TK clones) and allowed to grow for 4 hours at room temperature or 15^{o}C overnight. Cells were resuspended in 50 mM Tris pH 7.5, 300 mM NaCl, 1% triton X-100, 10 mM EDTA, 10 mM DTT, 0.25 mg/ml lysozyme, 1 mM PMSF, 0.7 ug/ml pepstatin and complete protease inhibitor cocktail (Roche) and disrupted for 15 minutes with a Dianode Bioruptor (30 seconds on, 30 seconds off; hi setting). After removal of cell debris by centrifugation, immobilized glutathione beads were added and allowed to rotate for 1 hr at 4^{o}C. The beads were settled in a column and washed with 5 ml 50 mM Tris pH 7.6, 10% glycerol, 0.1% triton, 1M NaCl, 1 mM PMSF and then 10 mls of the same buffer with 0.1 M NaCl. Protein was eluted with 50 mM Tris pH 6.8, 10% glycerol, 0.1% triton, 0.3 M NaCl, 1 mM PMSF, 50 mM reduced glutathione and 1 mM DTT. Protein was then dialyzed into the same buffer with 40% glycerol and no glutathione.

DNA binding Assays. The oligonucleotides utilized in the binding assays are listed in Table S1, and were a gift from Sriharsa Pradhan. The complementary oligonucleotides were annealed and the double-stranded product purified by polyacrylamide gel electrophoresis. T4 polynucleotide kinase was used to label the oligonucleotides with ^{32}P. Binding reactions contained 15-73 pg

probe (2500-5000 cpm), 25 mM Tris pH 7.5, 5% glycerol, 60 mM NaCl, 0.4 mg/ml BSA (bovine serum albumin), 10 mM $MgCl_2$, 2 mM DTT and either 0.5 mg/ml polyglutamate or 2.5 μg/ml non-specific oligonucleotide as polyanion or 1.25 μg/ml lambda DNA digested with *HindIII* as non-specific competitor. Final protein concentration in the binding assay was between 25-350 nM, except for SUVH6-SRA where the concentration was 10 nM, and reactions were incubated at room temperature for 20 minutes. Binding was detected after electrophoresis in 6% acrylamide:bisacrylamide (40:1) gels containing 5% glycerol and Tris-borate-EDTA buffer by autoradiography.

Supplemental References

1. Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556-560.
2. Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science *301*, 653-657.
3. Saze, H., Mittelsten Scheid, O., and Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat Genet *34*, 65-69.
4. Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science *292*, 2077-2080.
5. Johnson, L., Cao, X., and Jacobsen, S. (2002). Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr Biol *12*, 1360-1367.
6. Jasencakova, Z., Soppe, W.J., Meister, A., Gernand, D., Turner, B.M., and Schubert, I. (2003). Histone modifications in Arabidopsis- high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. Plant J *33*, 471-480.

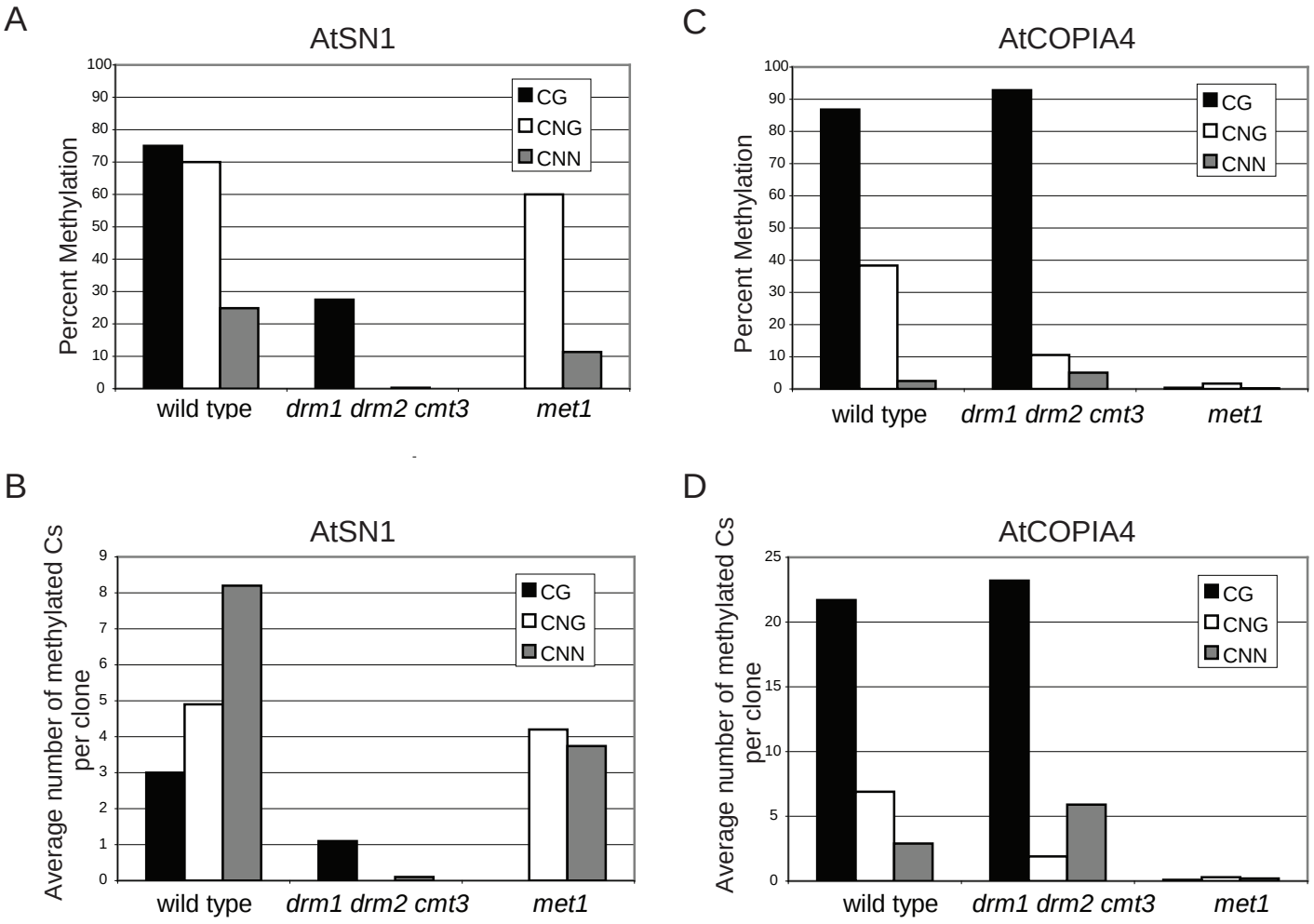

Figure S1. Bisulfite sequence data at AtSN1 and AtCOPIA4 represented in two different ways.
A and C: data represented as the percent of CG, CNG or CNN sites that are methylated.
B and D: data represented as the average number of methylated CG, CNG or CNNs per clone.

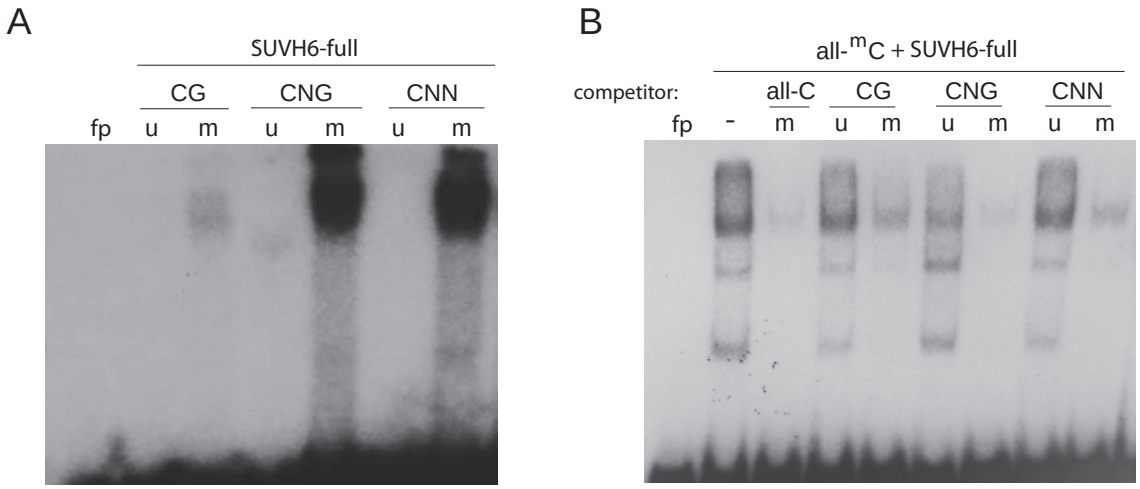

Figure S2. SUVH6 binds preferentially to methylated DNA. (A) Mobility shift assays where GST-SUVH6-full (amino acids 1-781) was added to a binding reaction with either an unmethylated (u) or methylated (m) CG, CNG, or CNN double stranded oligonucleotide probe. (B) GST-SUVH6-full was bound to oligonucleotides with cytosines methylated in all sequences contexts (all-mC) and competed with 1000x excess double stranded oligonucleotides that were unmethylated (u) or methylated (m) in CG, CNG, or CNN context. Free probe is indicated by fp and no competitor by -. The lower bands are due to SUVH6 degradation products binding to DNA.

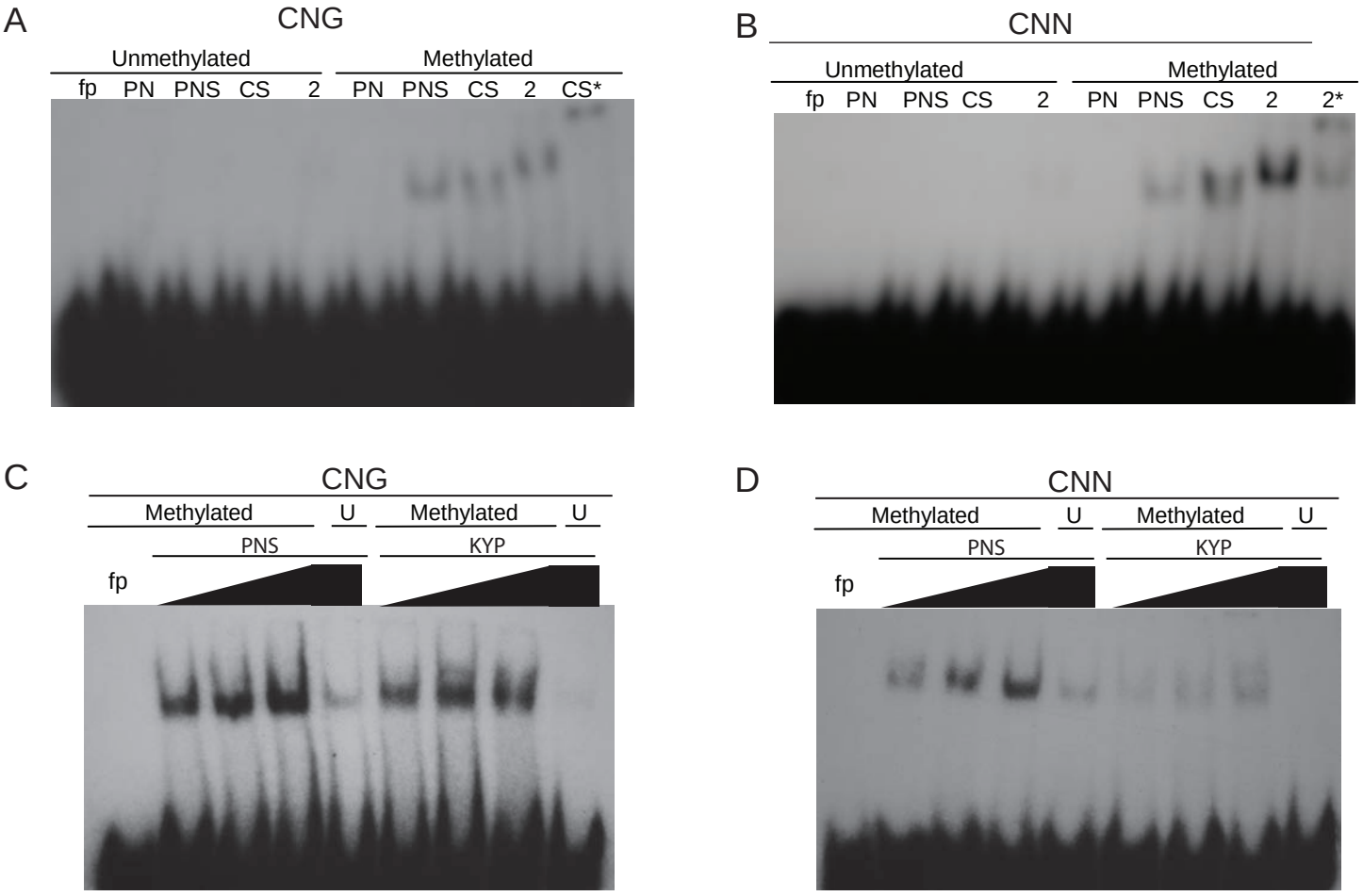

Figure S3. Specificity of DNA binding by ORTH and KYP proteins.
Panels A and B show that all ORTH1 and ORTH2 constructs containing the SRA domain can bind to CNG and CNN methylated substrates in addition to CG methylated substrates (see Figure 4B). Mobility shift assays with no protein (fp), ORTH1 with the amino-terminal PHD and RING domains (PN); PHD, amino-terminal RING, and SRA domains (PNS); carboxy-terminal RING and SRA domain (CS); and full-length ORTH2 (2) binding to unmethylated (lanes 1-5) or methylated (lanes 6-9), CNG (A) or CNN (B) oligonucleotides. Lane 10 contains binding of either CS (A) or 2 (B) to methylated CG supershifted by addition of an anti-GST antibody (*). Panels C and D compare ORTH1 PNS and KYP in their ability to bind CNG and CNN methylated substrates (see also Figure 4D for CG methylated substrates). Mobility shift assays with increasing amounts (100, 200, and 400 ng) of PNS (lanes 2-5) or KYP (lanes 6-9) binding to CNG (C) or CNN (D), either methylated (lanes 2-4, 6-8) or unmethylated (lanes 5, 9).

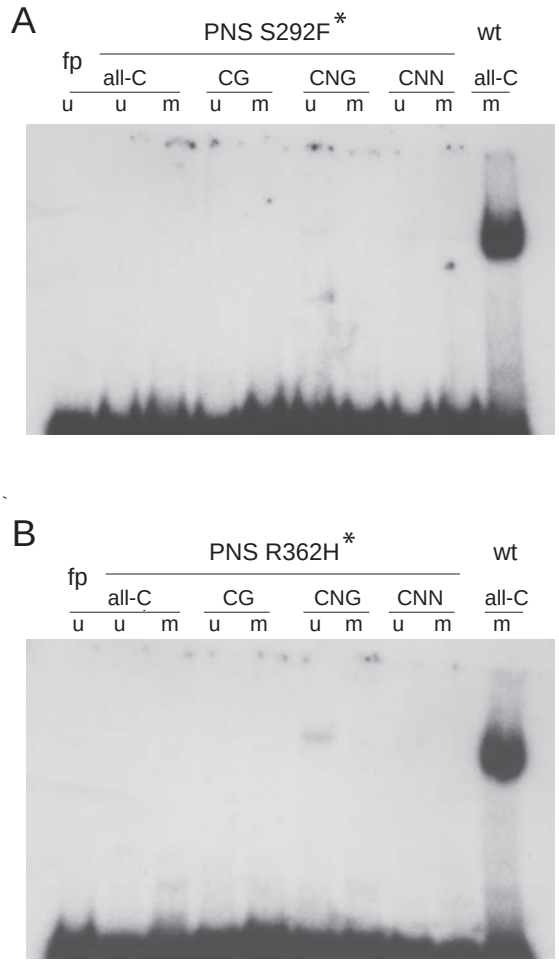

Figure S4. Point mutations within the SRA domain disrupt binding of ORTH1 to methylated DNA. The binding of the N-terminus of ORTH1 (PNS) containing a serine mutated to phenylalanine, S292F (A), or an arginine mutated to histidine, R362H (B). See Figure 2 for positions of mutations. Last lane in each panel shows wild type ORTH1 PNS as a positive control. Oligonucleotides utilized contained cytosines in all sequence contexts (all-C), or a CG context (CG), CNG context (CNG), or CNN context (CNN), which were either methylated (m) or unmethylated (u). The first lane in each panel represents oligonucleotide with no added protein, or free probe (fp).
* In ORTH1 the serine mutated corresponding to S200F in KYP is at position 292 and the arginine mutated corresponding to R260H in KYP is at position 362.

Supplementary Table 1. Primer and probe sequences. R represents a mix of G and A, Y represents a mix of C and T and M represents 5-methyl cytosine.

Primer Number	Primer Name	Primer Sequence (5'-3')
JP1595	*ACTIN* – Real-time	CGTTTCGCTTTCCTTAGTGTTAGCT
JP1596	*ACTIN* – Real-time	AGCGAACGGATCTAGAGACTCACCTTG
M-actin	*Actin* - probe	TCATCTTCTTCTTCTTCAAGGTGA
JP1821	*AtSN1*- Bisulfite	CAATATACRATCCAAAAAACARTTAAAATAATATCTTAA
JP1822	*AtSN1*- Bisulfite	GTTGTATAAGTTTAGTTTTAATTTTAYGGATYAGTATTAATTT
JP2669	*AtSN1*- Real-time	GTTGGCCCAGTGGTAAATCTGTTGGCCCAGTGGTAAATCT
JP2683	*AtSN1*- Real-time	TGGTGGTTGTACAAGCCTAGTT
M2	*AtSN1*- probe	ATCTCCCAGAGGCGGGACCC
JP3100	*AtCOPIA4* – Bisulfite	GGTTGTYTGTGTTTTTTATGGTTYAGATTTTATA
JP3101	*AtCOPIA4* – Bisulfite	ATAACTRAACCACARATTCARACCCATTTTCATTT
JP3067	*AtCOPIA4* – Real-time	CTTGTTTGTCTTCCCCGTGT
JP3068	*AtCOPIA4* – Real-time	TGACGAAGAGCGTACCTGTG
M1	*AtCOPIA4* - probe	CATTCATCACAGCCGACAAC
JP3010	all-C	AACGCAGCATGCGCTGCTAGCGCAGCTAGCGCTGCATG
JP3011	all-C	AACGCAGCATGCGCTGCTAGCGCAGCTAGCGCTGCATG
JP3018	all-^{m}C	AAMGMAGMATGMGMTGMTAGMGMAGMTAGMGMTGMATG
JP3019	all-^{m}C	AAMATGMAGMGMTAGMTGMGMTAGMAGMGMATGMTGMG
JP3524	CG	CGCGACGACGCACGACGACGCACGACGCGAACGCGCGAA
JP3525	CG	TTCGCGCGTTCGCGTCGTGCGTCGTCGTGCGTCGTCGCG
JP3536	^{m}CG	MGMGAMGAMGCAMGAMGAMGCAMGAMGMGAAMGMGMGAA
JP3537	^{m}CG	TTMGMGMGTTMGMGTMGTGMGTMGTMGTGMGTMGTMGMG
JP3441	CNG	CAGCAGACAGTCAGCAGTTCAGCAGACAGCAGCCAGCAG
JP3442	CNG	CTGCTGGCTGCTGTCTGCTGAACTGCTGACTGTCTGCTG
JP3443	^{m}CNG	MAGMAGAMAGTMAGMAGTTMAGMAGAMAGMAGCMAGMAG
JP3444	^{m}CNG	MTGMTGGMTGMTGTMTGMTGAAMTGMTGAMTGTMTGMTG
JP3445	CNN	CACTCCCCCACTCTCCCACCCACTCACTCCCTCCCACTT
JP3446	CNN	AAGTGGGAGGGAGTGAGTGGGTGGGAGAGTGGGGGAGTG
JP3447	^{m}CNN	MAMTMCMCMAMTMTMCMAMCMAMTMAMTMCMTMCMAMTT