Functional impacts of 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine at a single hemi-modified CpG dinucleotide in a gene promoter

Nataliya Kitsera, Julia Allgayer, Edris Parsa, Nadine Geier, Martin Rossa, Thomas Carell and Andriy Khobta

SUPPLEMENTARY DATA

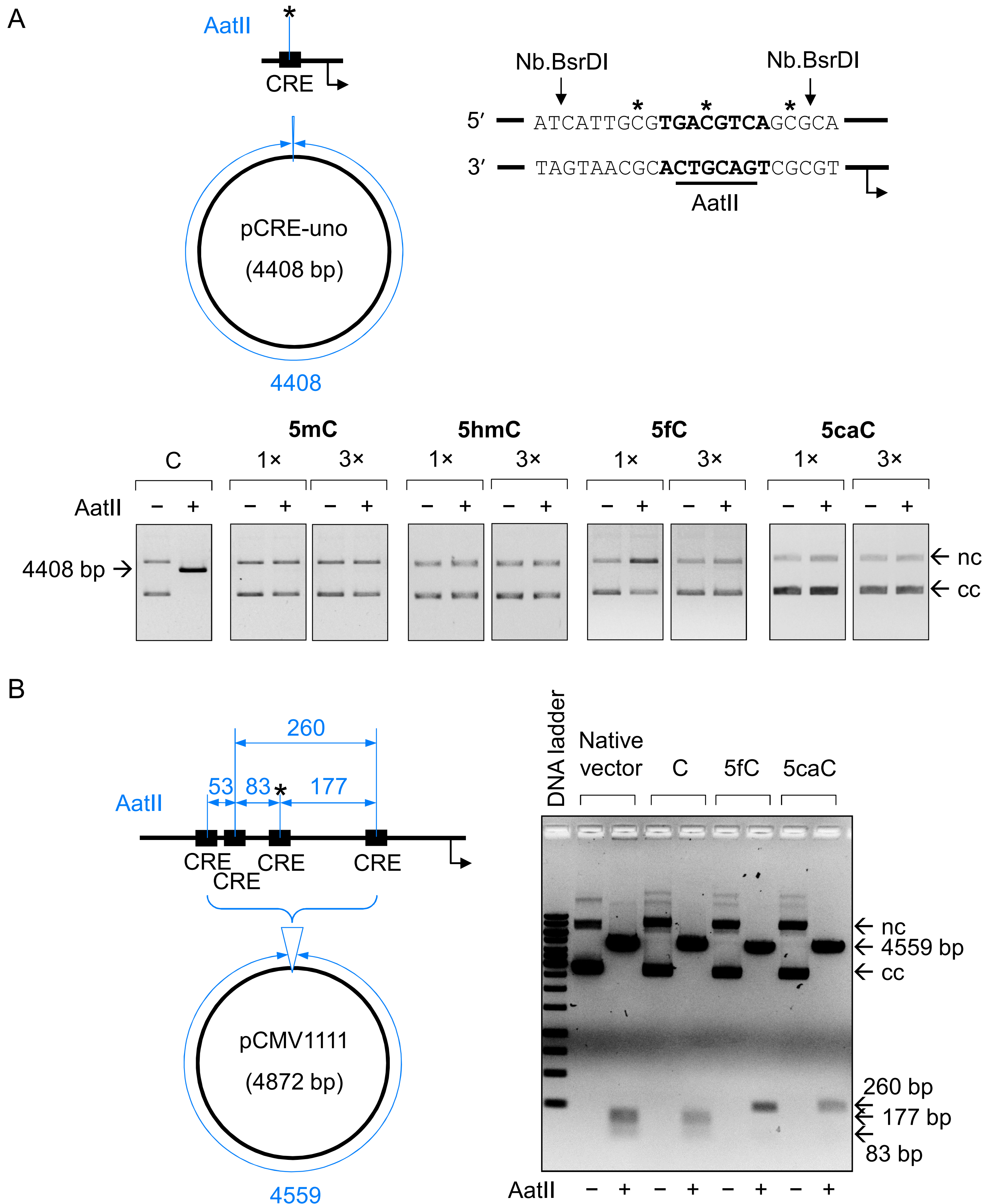


Figure S1. Representative agarose gels of pCRE-uno and pCMC1111 constructs containing 5-mC/5-hmC/5-fC/5-caC in the indicated CG-dinucleotides (asterisks). Incorporation of synthetic oligonucleotides containing the specified modifications was routinely confirmed by the inhibition of the AatII cleavage. (A) Detection of the single AatII-specific 4408 bp band in pCRE-uno. Other bands are covalently closed (cc) and nicked (nc) forms of circular vector DNA. (B) AatII cleavage pattern of the pCMV1111 vector: the 260 bp fragment is protected by 5-fC/5-caC. The gel image was intentionally overexposed to visualise the minor bands.

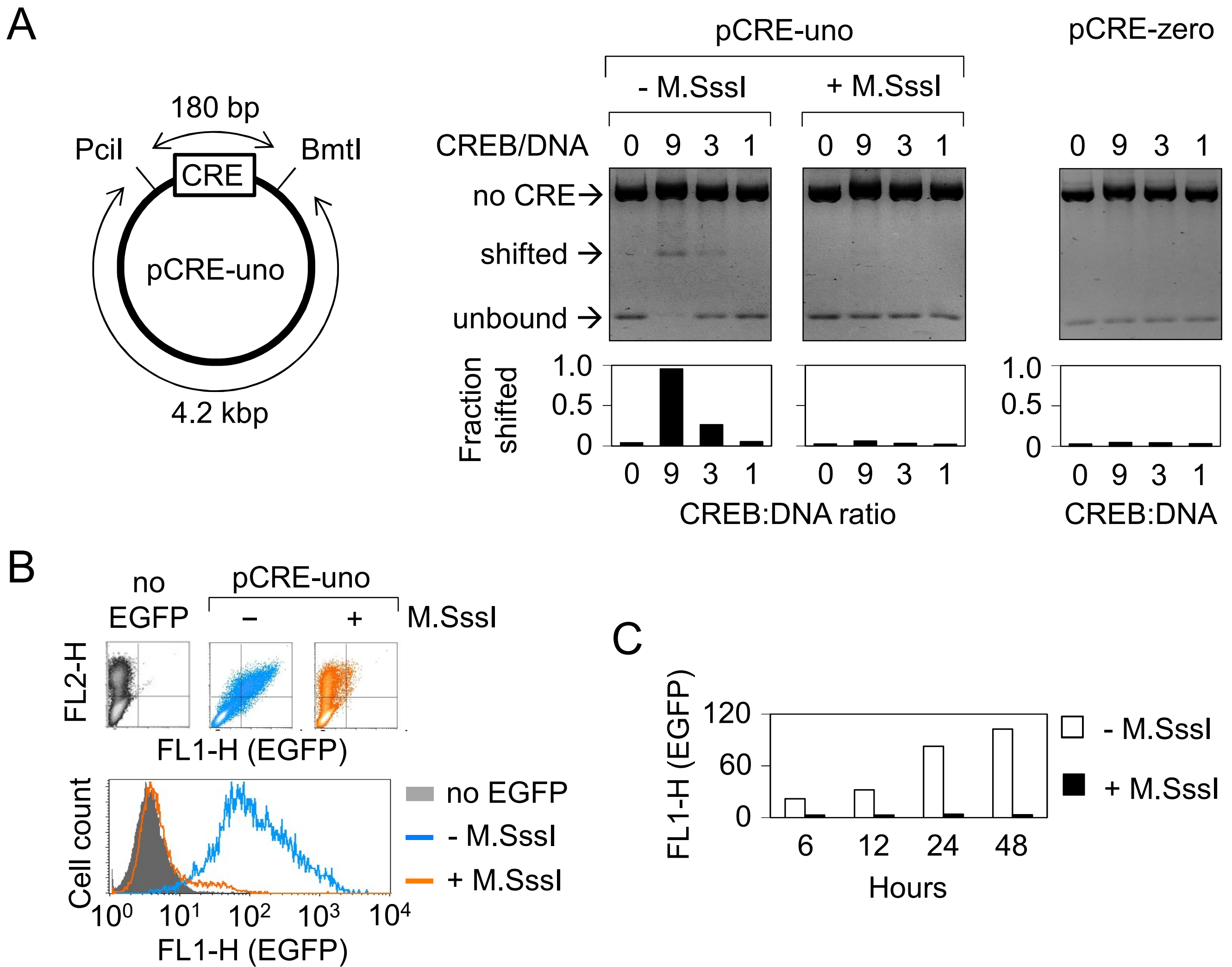


Figure S2. Impact of the M.SssI induced methylation of the pCRE-uno vector on the CREB binding and the reporter EGFP gene expression. (A) Electrophoretic mobility shift assay (EMSA) of the PciI/BmtI fragments of the pCRE-uno vector before and after methylation with M.SssI and of the reference CRE-less vector (pCRE-zero). (B) Representative fluorescence scatter plots and the correspondent overlaid fluorescence distribution plots of HeLa cells 24 hours after transfection with pDsRed alone (no EGFP) or in combination with pCRE-uno (+/- M.SssI). (C) Median EGFP expression for the experiment shown in (B) measured over the course of time.

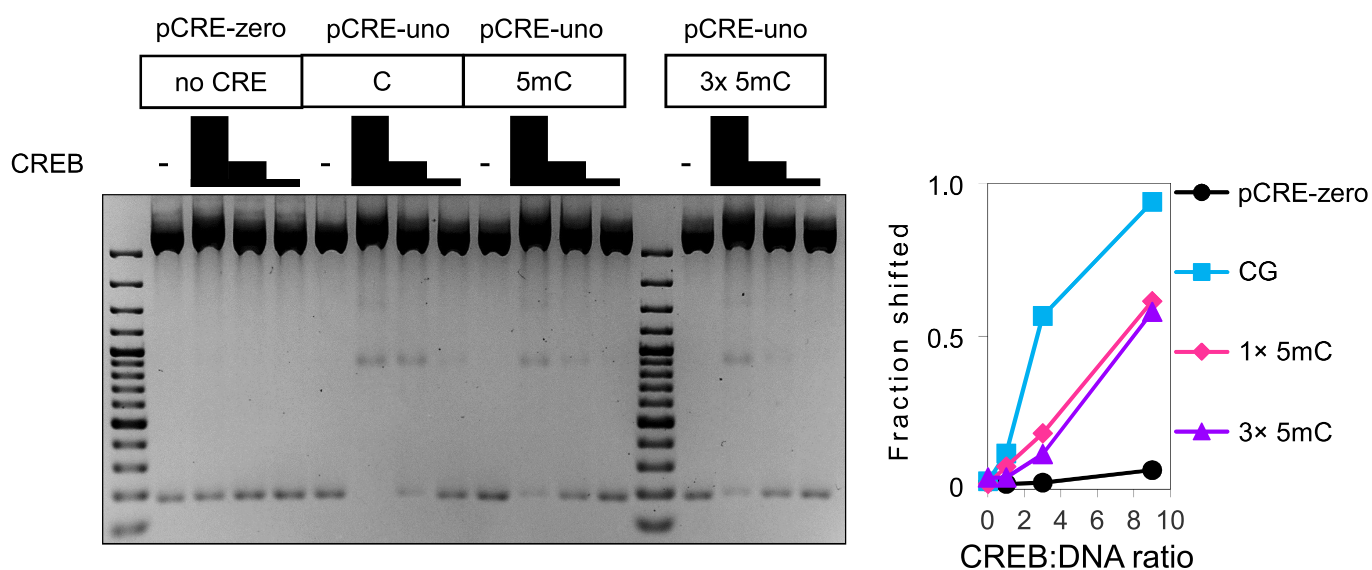


Figure S3. EMSA analyses of CREB binding to the pCRE-uno PciI/BmtI fragment containing one (in the central CG-dinucleotide) or three 5-mC. Representative agarose gel (on the left) and quantification of the specific band shifting (on the right).

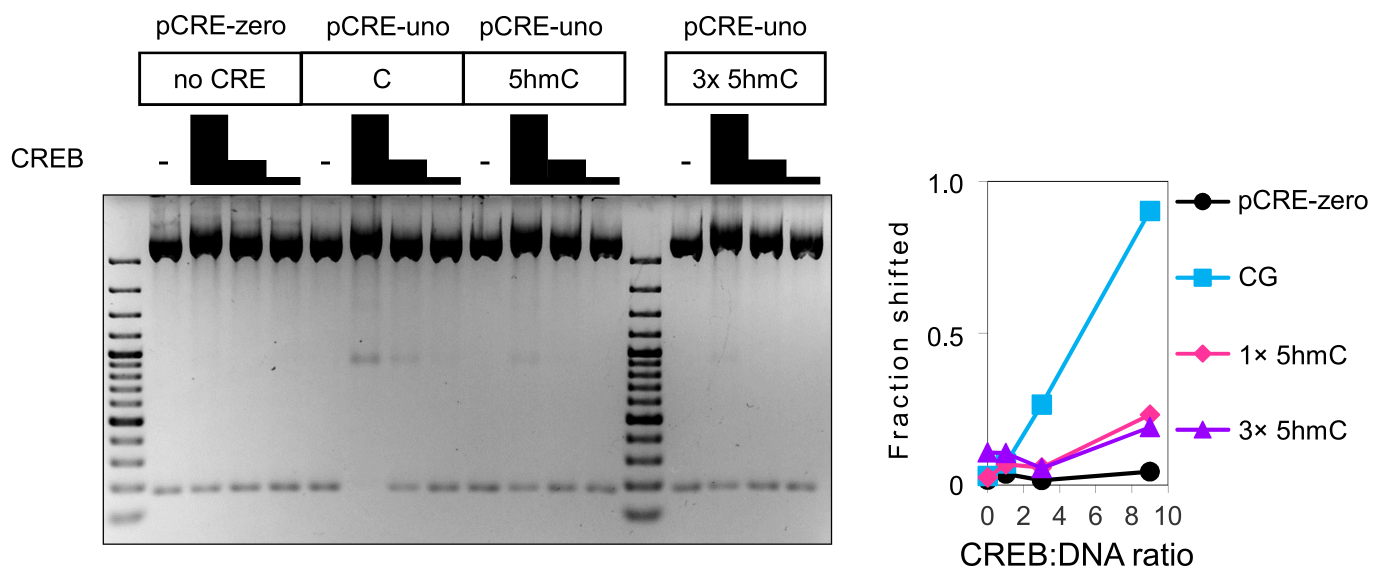


Figure S4. EMSA analyses of CREB binding to the pCRE-uno PciI/BmtI fragment containing one (in the central CG-dinucleotide) or three 5-hmC. Representative agarose gel (on the left) and quantification of the specific band shifting (on the right).

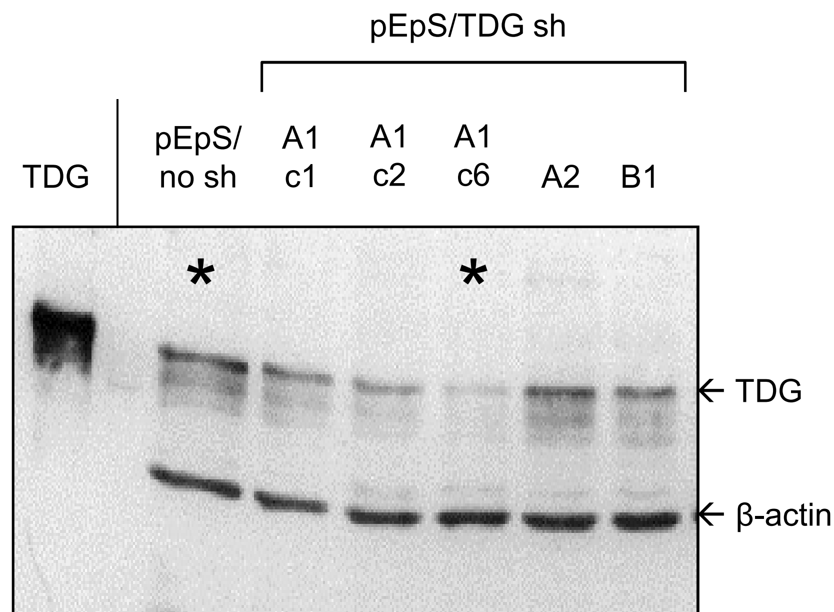


Figure S5. Verification of the TDG knockdown in HeLa cells by Western blotting with PA5-29140 antibody (Thermo Fischer Scientific). Generation of the short hairpin RNA constructs by cloning into the pEntr/pSuper+ vector (Addgene) and stable transfection of cells with three different TDG-specific shRNA expression constructs (A1, A2 and B1) was descrifed previously (Lühnsdorf er al., Ref. 29). Here, TDG knockdown in selected single clones (three different clones for the A1 shRNA sequence) was re-assessed to pick the cell lines suitable for the expression analyses of the reporter constructs containing 5-fC and 5-caC (data shown in Figure 6). The selected clonal cell line transfected with TDG-specific shRNA expression construct (pEpS/TDG sh A1-c6) and the reference clonal cell line transfected with pEntr/pSuper+ expression vector which carries an irrelevant DNA sequence downstream from the human H1 promoter are marked with asterisks.

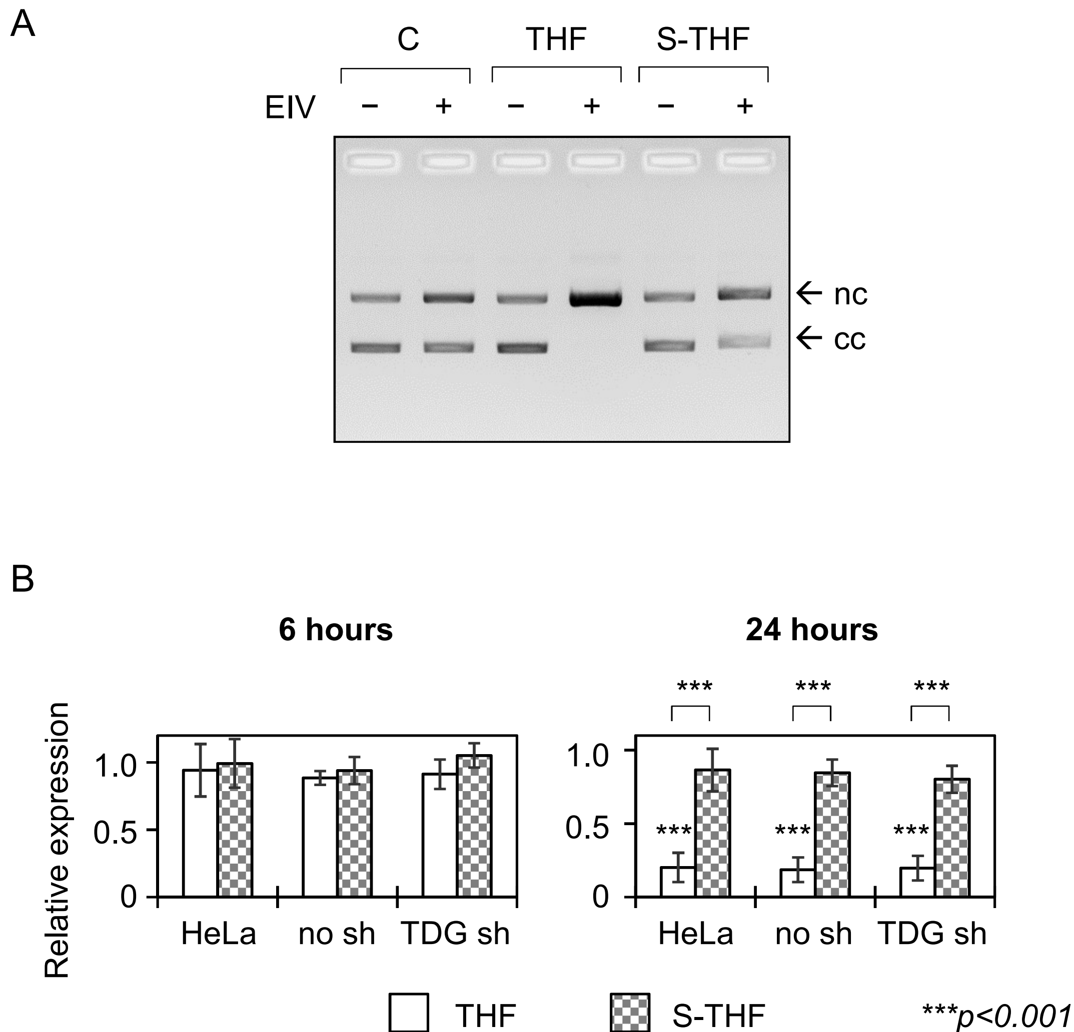


Figure S6. Effects of synthetic abasic sites in the minimal CRE promoter (pCRE-uno vector) on the EGFP expression (relative to the "C" construct). Expression constructs contained no modification (C), a stable abasic site analog tetrahydrofurane (THF) or THF with a nuclease resistant phosphorothioate 5′-linkage (S-THF). (A) Assessment of incorporation of synthetic oligonucleotides into vector DNA. The presence of cleavable AP-site was confirmed by the incubation with E. coli endonuclease IV (EIV). (B) Quantification of expression (relative to the "C" construct) in the isogenic cell lines with different TDG statuses (no sh, TDH sh) and in the parental HeLa cell line (mean ±SD, n=5). Both inhibition of the gene expression by THF and the rescue by S-THF are highly significant (Student's t-test)

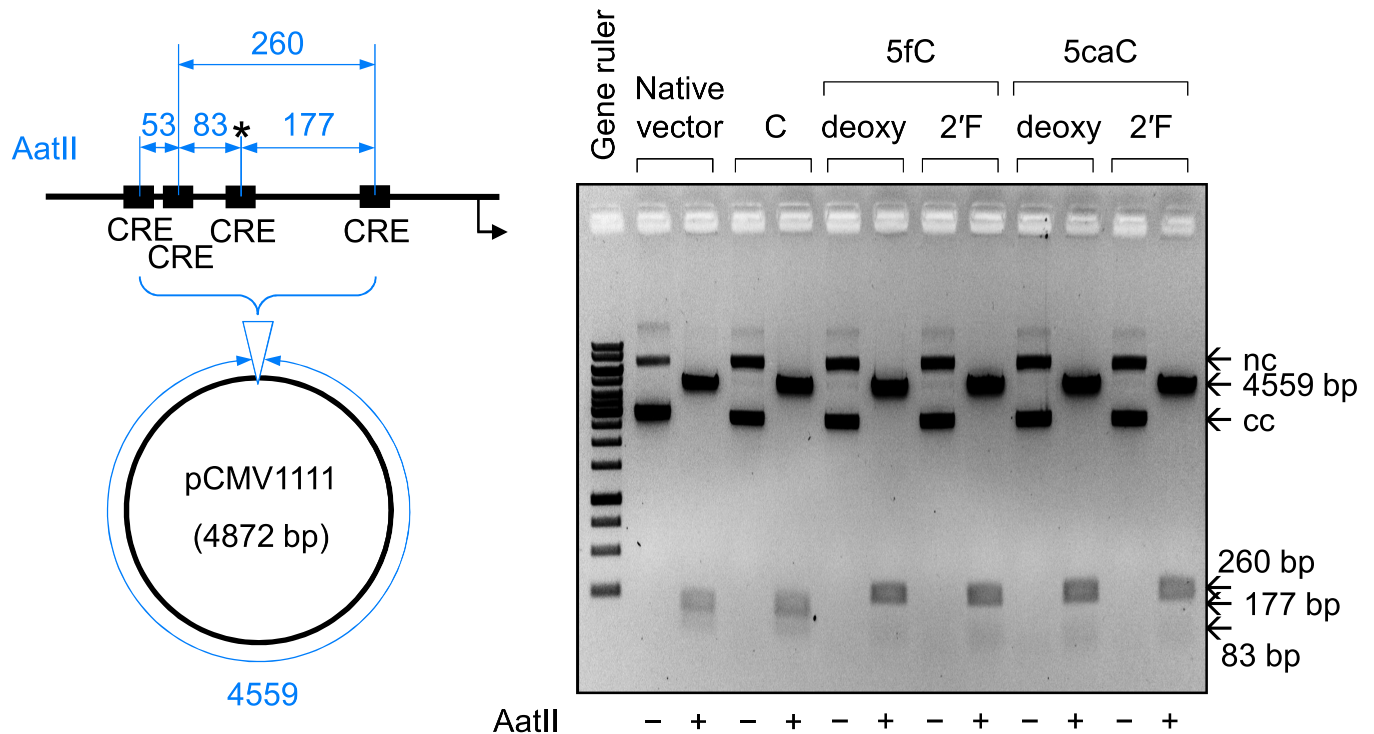
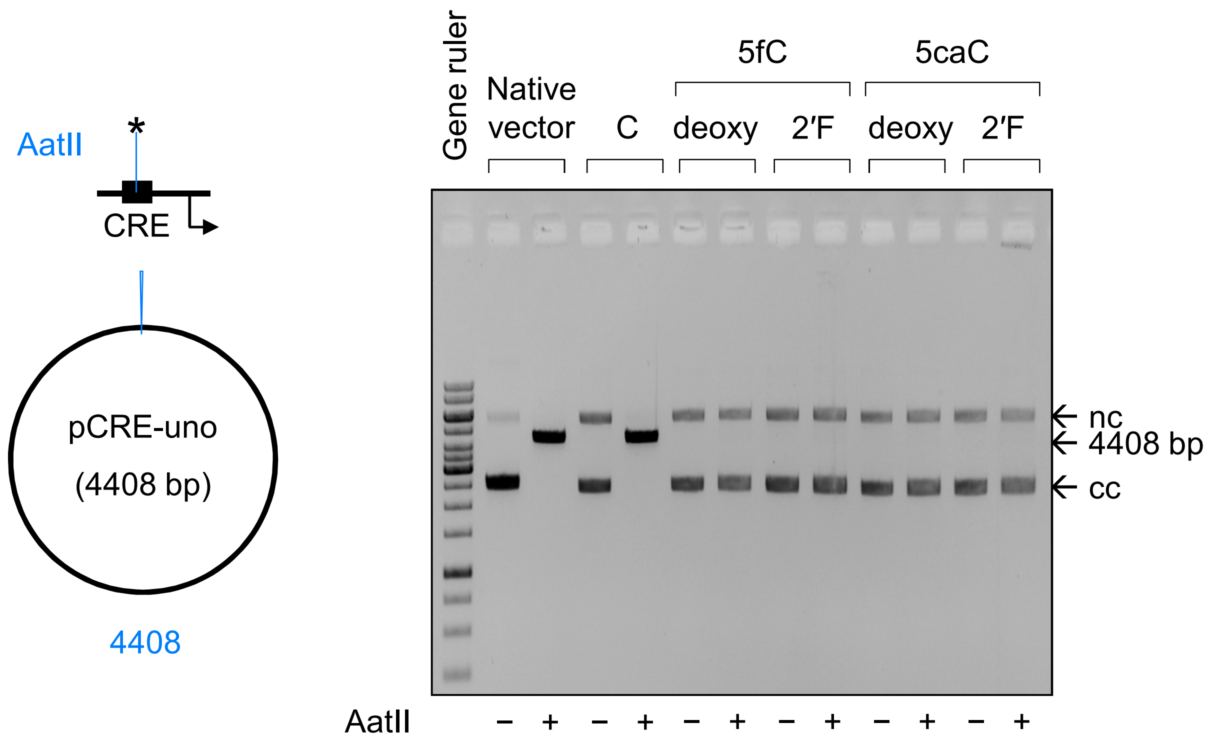


Figure S7. Incorporation of synthetic oligonucleotides containing C or 5-fC/5-caC as deoxyribinucleotides (deoxy) or as the respective 2′-fluorinated analogs (2′F) into CRE sequence of the pCMV1111 vector. The presence of modifications was verified by the inhibition of the AatII cleavage (a representative agarose gel). The gel image was intentionally overexposed to visualise the minor bands.



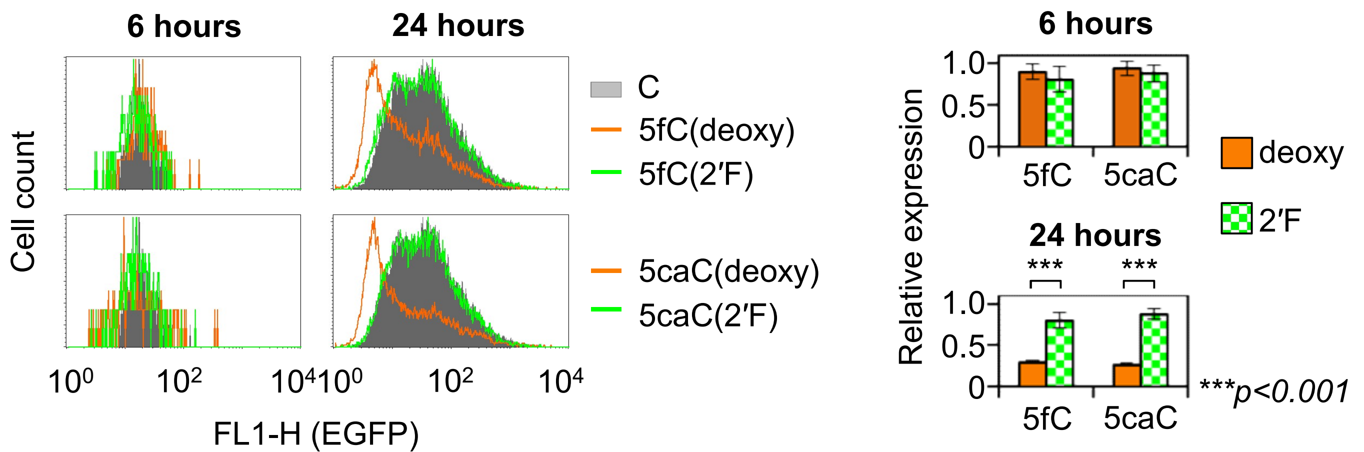


Figure S8. Impact of deoxyribose 2′-(R)-fluorination of the 5-fC/5-caC nucleotides in CRE on expression of pCRE-uno reporter constructs in HeLa cells (A) Incorporation of synthetic oligonucleotides containing C or 5-fC/5-caC as deoxyribinucleotides (deoxy) or as the respective 2′-fluorinated analogs (2′F) into pCRE-uno vector (a representative agarose gel). (B) EGFP expression at the specified times after transfection with pCRE-uno containing single 5-fC or 5-caC as deoxyribonuceotides (deoxy) or the respective 2′-(R)-fluorinated derivatives (2′F). Representative FACS data and relative EGFP expression values (mean ±SD, n=5, p-values calculated by the Student's t-test).