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Functional Reassembly of Split Enzymes On-Site: A Novel Approach for Highly Sequence-Specific Targeted DNA Methylation

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In mammalian genomes, a significant fraction of the cytosine residues is methylated at the 5-position (5-methylcytosine), and this modified nucleobase is found in 5′-CG-3′ sequences (CpG sites). The methylation pattern of the genome changes during ontogenesis, depends on the tissue and can substantially differ in several diseases, notably cancer. We are only at the beginning of understanding the biological role of DNA methylation in higher organisms, but the emerging view is that methylation of the promoter region of a substantial fraction of genes leads to transcriptional inactivation (gene silencing).[1]

Recognition of the importance of DNA methylation in gene regulation raised the possibility of silencing selected genes by exogenous, targeted methylation of their promoters. Directing DNA methylation to predetermined sites, besides being a promising research tool for silencing genes of interest and studying DNA methylation in higher eukaryotes, could lead to therapeutic applications in diseases characterised by aberrant expression of one or a small number of genes.[2] A potential advantage of DNA methylation-mediated gene silencing is that the de novo established methylation pattern is stably propagated by maintenance methylation through cycles of semiconservative replication. The silencing mechanism is likely to involve other epigenetic factors (e.g., histone methyltransferases, histone deacetylases) recruited by the DNA methylation marks.[1]

DNA methylation is catalysed by DNA methyltransferases (MTases), which transfer the activated methyl group from the ubiquitous cofactor *S*-adenosyl-L-methionine (AdoMet or SAM) to their target nucleobase within short DNA recognition sequences ranging from two to eight base pairs (bps). Directing DNA MTases to a preselected recognition sequence (targeted DNA methylation) was pioneered by Xu and Bestor, who genetically fused the bacterial DNA (cytosine-C5) MTase M.SssI (recognition sequence 5′-CG-3′) to a zinc finger protein (ZFP) that recognises a 9 bp DNA sequence with high specificity and serves as targeting domain.[3] The idea was that the chimeric protein would bind to the DNA sequence specific for the targeting domain, and the DNA MTase would selectively methylate CpG sequences located close to the binding site of the fusion partner. Indeed, preferential methylation of a CpG sequence located in the vicinity of the ZFP binding site was observed in in vitro experiments. Later research demonstrated that this approach can, in principle, also be applied in vivo for targeting chromosomal[4] as well as mitochondrial DNA.[5] In the most extensive study so far, the catalytic domains of the murine DNA MTases Dnmt3a and Dnmt3b were fused to sequence-specific DNA-binding proteins. In transient cotransfection experiments on human cells, dense methylation of the targeted promoter regions and silencing of the targeted genes was demonstrated.[6] In almost all such studies, ZFPs were used as targeting devices because customised ZFPs can now be engineered to bind with high affinity and sequence specificity to almost any DNA sequence.[7]

A key issue of targeted DNA methylation is specificity, that is, the difference between levels of exogenous methylation at the targeted site and nontargeted sites. Analysis of the methylation status of nontargeted sites revealed either methylation far from the target site,[4,8] or extensive methylation of regions flanking the binding site of the targeting domain.[6] Although efficient gene silencing might require methylation of many CpG sites in a promoter, and thus the latter phenomenon could be an advantage in many cases, high-resolution analysis of the effect of DNA methylation would require a method suitable for the methylation of single CpG sites. Methylation of nontargeted sites is not surprising owing to the inherent affinity of DNA MTases for their recognition sequences (Scheme 1 A). Nontargeted methylation limits the use of simple DNA MTase fusions as a research tool and would be a serious obstacle to therapeutic application. Therefore, novel strategies that allow highly sequence-specific targeted DNA methylation are of prime importance. One approach to improving targeting specificity employs DNA MTase variants with reduced DNA-binding affinity.[9] Here, DNA binding of the mutant DNA MTase-ZFP fusions is dominated by the targeting ZFP domain's improving the specificity.

A recent paper by Nomura and Barbas[10] describes another approach that appears to be a significant step towards the goal of methylating single CpG sites within whole genomes. The authors capitalised on previous observations of protein fragment complementa-

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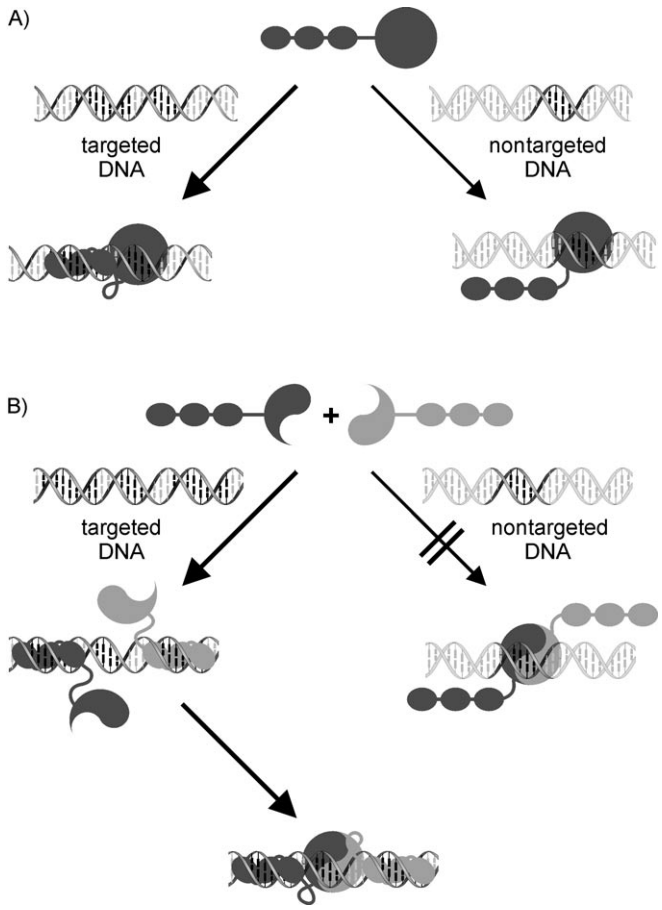

Scheme 1. Targeted DNA binding (and methylation) with zinc finger protein-directed DNA methyltransferases (DNA MTases). A) Fusion of a DNA MTase (dark grey circle) with a zinc finger protein (ZFP, dark grey ovals) leads to preferential binding to the targeted site (left) but, because of the inherent DNA-binding affinity of the DNA MTase, binding to nontargeted sites (right) also occurs, leading to background methylation. B) Splitting of the DNA MTase into two fragments (dark and light grey complementary shapes) and fusion of each fragment with a ZFP (dark and light grey ovals) leads to specific DNA binding of the ZFPs and assembly of the DNA MTase fragments on the targeted site (left pathway). In contrast, assembly and binding to nontargeted DNA is absent or at least strongly disfavoured (right) presumably because of the fused ZFPs.

tion described for some DNA (cytosine-C5) MTases. These enzymes generally act as monomers, but at least some of them can be split into two (preferably partially overlapping) fragments, which, while inactive by themselves, can assemble to form active enzyme when expressed in the same cell.[11,12] Nomura and Barbas fused an N-terminal segment of the DNA MTase M.HhaI, encompassing residues 2–240, to a ZFP engineered to recognise a 9 bp sequence. The C-terminal segment, spanning residues 210–327, was fused to another ZFP targeting a different 9 bp sequence. There were two reasons for selecting M.HhaI for this work; M.HhaI is one of those DNA MTases for which protein fragment complementation has been shown before,[12] and its 5′-GCGC-3′ methylation specificity (the methylated cytosine is underlined) makes the approach, in principle, directly applicable to targeted methylation of CpG sites flanked by a 5′-G and a 3′-C residue. When the two DNA MTase-ZFP fusion proteins were expressed in the same *E. coli* cell, the targeted M.HhaI recognition site, which was flanked by the two closely spaced ZFP binding sites, became methylated, whereas the other M.HhaI recognition sites on the same plasmid stayed unmethylated, as shown by fragmentation analysis with the cognate restriction endonuclease R.HhaI and bisulfite DNA sequencing.

Although a detailed comparison of the approaches employing the MTase mutants with low DNA-binding affinity[9] and the split MTase fragments[10] is not yet available, the latter method seems to produce even less off-target methylation. Part of the reason could be the entropic cost of assembling the fragments, whereas ZFP-mediated binding of both DNA MTase fragments to the targeted site will assist assembly by locally increasing the effective fragment concentrations (Scheme 1 B). However, the observed level of targeted methylation specificity is still surprising because co-expression of the same, but unfused M.HhaI fragments, resulted in at least 75% methylation of 5′-GCGC-3′ sequences of the test plasmid;[12] this indicated that the split M.HhaI fragments by themselves can reassemble to form an active enzyme. Apparently, fusion of the M.HhaI fragments with ZFPs had a pronounced negative effect on nontargeted assembly. It will be interesting to perform detailed binding and kinetic experiments to identify the underlying biophysical principle for the observed highly sequence-specific DNA methylation.

Future research will tell whether the split DNA MTase strategy can also work in eukaryotic cells, against the background of a eukaryotic genome. The paper by Nomura and Barbas will undoubtedly stimulate investigations exploring whether the split DNA MTase approach is applicable to CpG-specific MTases (M.SssI, Dnmt3a, Dnmt3b), which can, in principle, target all CpG sites, not just those in the 5′-GCGC-3′ context. Such split DNA MTases might also be valuable tools for highly specific DNA labelling by using modified AdoMet analogues.[13,14]

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[1] R. J. Klose, A. P. Bird, *Trends Biochem. Sci.* **2006**, *31*, 89–97.

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[2] A. Jeltsch, R. Z. Jurkowska, T. P. Jurkowski, K. Liebert, P. Rathert, M. Schlickenrieder, *Appl. Microbiol. Biotechnol.* **2007**, *75*, 1233–1240.
[3] G.-L. Xu, T. H. Bestor, *Nat. Genet.* **1997**, *17*, 376–378.
[4] C. D. Carvin, R. D. Parr, M. P. Kladde, *Nucleic Acids Res.* **2003**, *31*, 6493–6501.
[5] M. Minczuk, M. A. Papworth, P. Kolasinska, M. P. Murphy, A. Klug, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 19689–19694.
[6] F. Li, M. Papworth, M. Minczuk, C. Rohde, Y. Zhang, S. Ragozin, A. Jeltsch, *Nucleic Acids Res.* **2007**, *35*, 100–112.
[7] J. G. Mandell, C. F. Barbas III, *Nucleic Acids Res.* **2006**, *34*, W516–W523.
[8] A. R. McNamara, P. J. Hurd, A. E. Smith, K. G. Ford, *Nucleic Acids Res.* **2002**, *30*, 3818–3830.
[9] A. E. Smith, K. G. Ford, *Nucleic Acids Res.* **2007**, *35*, 740–754.
[10] W. Nomura, C. F. Barbas III, *J. Am. Chem. Soc.* **2007**, *129*, 8676–8677.
[11] G. Pósfai, S. C. Kim, L. Szilák, A. Kovács, P. Venetianer, *Nucleic Acids Res.* **1991**, *19*, 4843–4847.
[12] W. Choe, S. Chandrasegaran, M. Ostermeier, *Biochem. Biophys. Res. Commun.* **2005**, *334*, 1233–1240.
[13] G. Pljevaljčić, F. Schmidt, A. J. Scheidig, R. Lurz, E. Weinhold, *ChemBioChem* **2007**, *8*, 1516–1519.
[14] G. Lukinavicius, V. Lapiene, Z. Stasevskij, C. Dalhoff, E. Weinhold, S. Klimasauskas, *J. Am. Chem. Soc.* **2007**, *129*, 2758–2759.

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