Abstract
The ability to target DNA methylation toward a single, user-designated CpG site in vivo may have wide applicability for basic biological and biomedical research. A tool for targeting methylation toward single sites could be used to study the effects of individual methylation events on transcription, protein recruitment to DNA, and the dynamics of such epigenetic alterations. Although various tools for directing methylation to promoters exist, none offers the ability to localize methylation solely to a single CpG site. In our ongoing research to create such a tool, we have pursued a strategy employing artificially bifurcated DNA methyltransferases; each methyltransferase fragment is fused to zinc finger proteins with affinity for sequences flanking a targeted CpG site for methylation. We sought to improve the targeting of these enzymes by reducing the methyltransferase activity at non-targeted sites while maintaining high levels of activity at a targeted site. Here we demonstrate an in vitro directed evolution selection strategy to improve methyltransferase specificity and use it to optimize an engineered zinc finger methyltransferase derived from M.SssI. The unusual restriction enzyme McrBC is a key component of this strategy and is used to select against methyltransferases that methylate multiple sites on a plasmid. This strategy allowed us to quickly identify mutants with high levels of methylation at the target site (up to \( \sim 80\% \)) and nearly unobservable levels of methylation at off-target sites (<1%), as assessed in E. coli. We also demonstrate that replacing the zinc finger domains with new zinc fingers redirects the methylation to a new target CpG site flanked by the corresponding zinc finger binding sequences.

Introduction
CpG methylation is one of the most extensively studied epigenetic modifications; it broadly regulates and maintains transcriptional repression. CpG methylation is involved in proper cellular differentiation, heterochromatin formation and in maintaining chromosomal stability [1]. Further, aberrant methylation patterns cause or are observed in numerous diseases. Imprinting defects lead to disorders such as Prader-Willi and Angelman syndromes [2]. Notably, global genomic hypomethylation and local hypermethylation of CpG islands (CGIs) commonly occur in cancer [3]. Though much has been learned about how methylation patterns are established and erased, the causes of aberrant methylation and the reestablishment of methylation patterns during development remain active areas of research. To study the effects and dynamics of DNA methylation, it would be generally useful to target methylation toward specific, user-defined sequences.

Several groups have engineered methyltransferases that directly methylate user-defined DNA sequences. The general strategy, pioneered by Xu and Bestor, involves fusion of a sequence specific DNA binding protein to a methyltransferase enzyme [4]. These constructs have been used to affect methylation, in vitro, in E. coli, and in cancer cell lines [5–10]. These directed methyltransferases have been shown to stably and heritably reduce the expression of Sox2 and Map1l genes [11]. Siddique et al. demonstrated that targeting methylation towards the VEGF-A promoter significantly reduced gene expression in SKOV3 cells [12]. A recent review summarizes much of the literature on targeted methylation [13]. However, the engineered enzymes mentioned above methylate multiple CpG sites adjacent to the targeted DNA sequence. Despite the successes of these studies in biasing methylation to a particular region, only a few studies have focused on targeting methylation to single CpG sites [14–17].

Though methylation at single sites in eukaryotes is not believed to be the main means of epigenetic transcriptional silencing, multiple studies suggest single methylation events can alter the expression levels of select genes. In vitro methylation of a single CpG site within the S100A2 promoter on a reporter plasmid resulted in significant downregulation of gene expression, upon transfection, relative to an unmethylated, transfected control [18]. Methylated oligonucleotides targeting an intronic region of
Evolving Improved Zinc Finger Methyltransferases

Materials and Methods

Enzymes, Oligonucleotides and Bacterial Strains

Restriction enzymes, T4 ligase, T4 kinase, and Phusion High Fidelity PCR MMX were purchased from New England Biolabs (Ipswich, MA). Boxl was purchased from ThermoFisher Scientific (Waltham, MA). Platinum Pfx DNA polymerase was purchased from Life Technologies (Carlsbad, CA). PhiTurbo Cx Hotstart DNA polymerase was purchased from Agilent Technologies (Santa Clara, CA). Plasmid-Safe-ATP-dependent DNAses was purchased from Epicentre (Madison, WI). pDIMN8 and pAR plasmids have been previously described [16,17]. All oligonucleotides and gBlocks were synthesized by Invitrogen (Carlsbad, CA) or Integrated DNA Technologies (Coraville, IA). Gel electrophoresis and PCR were performed essentially as previously described [27]. Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). DNA fragments were purified from agarose gels using QiAquick Gel Extraction Kit (Qiagen, Valencia, CA) or PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) and further concentrated using DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA).

Escherichia coli K-12 strain ER2267 [F proA B lacD (lacZ)M15 zif::mini-Tn10 (KanR)/D(argF-lacZ)U169 glnV44 e14 (McrA) rfbD1? recA1 relA1? endA1 spoT1? thi-1 D(mcrC- mrr)114::IS10] was acquired from New England Biolabs (Ipswich, MA) and was used in selections, methylation assays and cloning. NEB 5-alpha Competent E. coli (High Efficiency) [phaK2 DiagF-lacZU169 phaA glnV44 phoA2(lacZ)M15 gcvK96 recA1 relA1 endA1 thi-1 hsdR17] were also used for cloning and were purchased from New England Biolabs (Ipswich, MA).

Plasmid Creation

pDIMN8 was used for library creation and testing of library variants [17]. pDIMN9 was constructed as a follow for use in golden gate cloning. Plasmid pDIMN3 was altered by silently mutating a BsaI site in the AmpR gene via pFunkel mutagenesis [28]. PCR, digestion and cloning removed a BsaI restriction site to create vector pDIMN9. Golden gate cloning was used to fuse new zinc fingers proteins to methyltransferase fragments. For the creation of plasmids used in golden gate cloning, regions encoding zinc finger proteins were replaced with BstI sites. pDIMN9 contained a M.SsaI [1–272]-BstI construct for the addition of zinc fingers to the N-terminal fragment. pAR contained BbsI-M.SsaI [273–306] construct for the addition of new zinc fingers to the C-terminal fragments [16]. gBlocks encoding zinc fingers and BbsI sites were purchased from Integrated DNA Technologies. Golden gate cloning to fuse zinc finger-encoding gBlocks to the above plasmids was performed essentially as described [29]. Zinc finger CDSs were designed using the zinc finger tools website and previously identified zinc finger domains [30–32]. As previously described, plasmids containing genes encoding individual C-terminal and N-terminal zinc finger-fused proteins were digested with EcoRI and SpeI and ligated together, in order to place these genes on a single large plasmid for characterization in E. coli [16]. Site 1 and site 2 on this plasmid refer to previously described cloning sites on this large plasmid [17] and were used to construct the various target and non-target sites described in the study.
Construction of Cassette Mutagenesis Library

An NNK cassette mutagenesis library of M.SssI [273–386] was constructed by overlap extension PCR. PCR was carried out using an oligonucleotide degenerate for a five amino acid region in the C-terminal fragment corresponding to amino acids 297–301 in the wild type enzyme. Fragments were digested with AgeI-HF and SpeI and ligated into pDIMN8 containing HS2 and the complete N-terminal fragment-HS1 fusion (Fig. 1 A,B). HS1 and HS2 have been described previously [33]. Site 1 contained a target site comprised of an internal CpG site nested within an FspI restriction site, and flanked by HS1 and HS2 recognition sequences (Fig. 1C). The non-target site used to assess off-target methylation. A target site contains an internal CpG site nested within an SnaBI restriction site (E) An overview of the selections used in this experiment. The schematic illustrates the fates of plasmids encoding inactive methyltransferase (digested by FspI, left), a desired targeting methyltransferase methylated at the target site (not digested, middle) and a nonspecific methyltransferase methylating multiple M.SssI (i.e CpG) sites (digested by McrBC, right).

doi:10.1371/journal.pone.0096931.g001

Library Selection

Plated library variants were recovered from the plate in lysogeny broth supplemented with 15% v/v glycerol and 2% w/v glucose and stored at −80° C. Aliquots were thawed and used to inoculate 10 ml of lysogeny broth supplemented with 100 μg/ml ampicillin salt, 0.2% w/v glucose, 1 mM IPTG, and 0.0167% w/v arabinose. These cultures were incubated overnight at 37°C and 250 rpm. Plasmid DNA was isolated via QIAprep Spin Miniprep Kit and digested for 3 hours at 37°C with McrBC (10 units/μg DNA). FspI (2.5–5 units/μg DNA) in 1X NEBuffer 2 supplemented with 100 μg/ml BSA and 1 mM GTP. Reactions were halted by incubation at 65° C for over 20 min to which ExoIII (30 units/μg DNA) was added and the solution incubated at 37°C for 1 hour. Plates were incubated overnight at 37°C. The naive library contained 2×10^6 transformants.
60 min. ExoIII digestion was halted by incubation at 80°C for over 30 min and the DNA was desalted using Zymo Clean and Concentrator-5 kits per manufacturer’s instructions. DNA was transformed into ER2267 electrocompetent cells and plated on agar supplemented with 2% w/v glucose and 100 mg/ml ampicillin salt.

Cells were recovered from the plate as before and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit. The DNA was digested with FspI (2–2.8 units/mg DNA) in 1X NEBuffer 4 and linear DNA was isolated via gel electrophoresis. PCR was used to amplify the portion of the linear plasmid containing genes encoding for the N-terminal and C-terminal fragments fused to zinc fingers. Purified PCR products were subcloned into the selection plasmid for an additional round of selection.

Restriction Endonuclease Protection Assays

Cultures from colonies were incubated overnight at 37°C and 250 rpm in lysogeny broth supplemented with 0.2% w/v glucose and 100 μg/ml ampicillin salt and stored as glycerol stocks.

Glycerol stocks were used to inoculate 10 ml of lysogeny broth supplemented with 100 μg/ml ampicillin salt, 0.2% w/v glucose, 1 mM IPTG, and 0.0167% w/v arabinose. After growth overnight at 37°C and 250 rpm, plasmid DNA was purified from the cultures with a QIAprep Spin Miniprep Kit. Plasmid DNA (500 ng) was digested with NcoI-HF (20 Units/µg DNA) in 1X CutSmart Buffer. Linear plasmids were purified using DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA). Linearized plasmids (500 ng) were treated with bisulfite reagent using the EZ-DNA Methylation Gold Kit (Zymo Research, Irvine, CA). Touchdown PCR, using PhTurbo Cx Hotstart DNA polymerase was used to amplify regions encoding the target and non-target sites and was modified from [34]. An initial cycle of 95°C for 3 min was
followed by a touchdown PCR (95°C for 1 min, annealing temperature for 1 minute, 72°C for 2 minutes). The annealing temperature started at 64°C and was dropped 2°C degrees after two cycles and then decreased 1°C after every other cycle until the annealing temperature reached 52°C. After the touchdown PCR, an additional 30 cycles were carried out with the parameters above and an annealing temperature of 51°C. A final extension was carried out at 72°C for 10 min. The antisense strand at the target site was amplified with primers 5’-AAG ACA GAG CTC AAA CTA AAT AAC GTT CCC CAT TAT AAT TCT TCT’(Fw) and 5’-CCG TAG CCA TGG TAT ATT TTT AAT AAA TTT TTT AGG GAA ATA GGT TAG GTT TTT AT-3’(Rev). The antisense strand at the non-target site was amplified with primers 5’-AAG ACA GAG CTC CTC TAC TAA TCC TAT TAC CAA TAA CTA CTA CCA ATA A-3’(Fw) and 5’-CCG TAG CCA TGG GTA AAG TTT GGG GTG TTT AAT GAG TGA GTT AAT TTA TAT TAA TTA TTT-3’ (Rev). PCR amplified products were purified by gel electrophoresis as above digested with SacI-HF and NcoI-HF, ligated into pDIMN9 and transformed into NEB 5-alpha Competent E. coli (High Efficiency). Individual colonies were sequenced and analyzed using quantification tool for methylation analysis (QUMA) [35]. Low quality sequences were excluded if they had more than five unconverted CpH sites or if less than 95% of all CpH sites were converted. Sequences were also excluded if they either had over 10 alignment mismatches or less than 90% percent identity to the reference sequence.

Results and Discussion

Design of the Selection System

Our in vitro selection system preferentially enriches variants from a mutagenesis library that possess the ability to methylate a target site, but also lack the ability to methylate other non-targeted M.SssI sites on the plasmid. In vitro selection strategies have been used to enrich for methyltransferases with relaxed or altered specificity. Most strategies rely on methylation-dependent protection from restriction endonuclease digestion to positively select for DNA encoding a methyltransferase with altered specificity [36–40]. Our selection scheme differs from previous studies as it additionally employs McrBC as a negative selection against unwanted methylation activity. In our system for altering methyltransferase specificity, a single plasmid contains both genes encoding the zinc finger-fused M.SssI fragments as well as a targeted M.SssI CpG site that is nested within an FspI restriction site and flanked by zinc finger binding sequences (Fig. 1A–C). The plasmid also has over 400 other M.SssI (i.e. CpG) sites and a non-target site, comprised of a SnaBI restriction site, for the assessment of off-target methylation (Fig. 1D). Once transformed into E. coli, the methyltransferase fragments encoded by the plasmid are expressed, resulting in methylation of the same plasmid. The
plasmid DNA is isolated and subjected to in vitro digestions with endonucleases FspI and McrBC (Fig. 1E). Since FspI digestion is blocked by methylation, FspI digestion serves to select for methylation at the targeted CpG site. McrBC is an endonuclease that recognizes and cleaves DNA with two distally methylated sites [41,42]. McrBC will not digest a single site that is methylated or hemimethylated unless there is a second methylated site on the same DNA within about 40–3000 bp [43]. We therefore expect that most plasmids methylated at multiple M.SssI sites will be digested by McrBC. Thus, McrBC digestion selects against off-target methylation. The DNA is then incubated with ExoIII to degrade any plasmid that is digested at least once, ideally leaving the same DNA within about 40–3000 bp [43]. We therefore expect that most plasmids methylated at multiple M.SssI sites will be digested by McrBC. Thus, McrBC digestion selects against off-target methylation. 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sequence logo using weblogo 3.3 [48,49]. This sequence logo indicated that a functional heterodimeric methyltransferase strongly preferred certain residues at positions 299 and 300 (Fig. 3). Position 296 (wildtype phenylalanine) was almost exclusively composed of aromatic residues. Position 300 (wildtype serine) was almost exclusively composed of small residues (defined as an amino acid with an R side chain containing 1–3 heavy atoms). The observed conservation at these residues is consistent with sequence alignments showing these two residues are relatively well-conserved among methyltransferases of different species [45]. In contrast, positions 297, 299 and 301 exhibited little preference for specific amino acids. This finding is consistent with the mutational study discussed above [46]. Our study reveals that there are numerous solutions for improving the specificity of our methyltransferase. As seen in the sequence logo using using weblogo 3.3 [48,49]. This sequence logo strongly preferred certain residues at positions 298 and 300 with sequence alignments showing these two residues are relatively conserved among methyltransferases of different species [45].

To further characterize our engineered methyltransferases, we subjected plasmids containing optimized variants, PFCSY, CFESY (named for the sequence at residues 297–301), and the un-optimized ‘WT’ variant to bisulfite analysis at both the target and non-target sites. These plasmids were isolated from cultures grown under conditions known to induce the expression of the plasmid’s methyltransferase fragment fusion genes. In addition to assessing levels of methylation at the target and non-target CpG sites, the regions subjected to bisulfite sequencing assessed the methylation status of 47 and 59 additional CpG sites around the target and non-target sites, respectively (covering over 25% of the total CpG sites present on the plasmid). We sequenced ≥15 clones for each variant to quantify the frequency of methylation at all CpG sequences around both sites (Fig. 4A,B). Based on this sequencing, the PFCSY variant methylated the target site at a frequency of 78.9%. In contrast, only fifteen off-target methylation events were observed in the 34 sequence reads (out of a total of 1793 possible off-target methylation events), which corresponds to an off-target methylation frequency of 0.84%. The PFCSY variant’s specificity for the target site is a marked improvement over the un-optimized, ‘WT’ variant, which methylated the target site at a frequency of 94.1% and off-target sites at a frequency of 49.5%. Thus, for this variant, our selections resulted in the identification of a variant with an almost 60-fold reduction in off-target methylation and a minimal decrease in methylation at the target site. The CFESY variant methylated the target site at a lower frequency compared to the PFCSY variant, but exhibited a similar low frequency of methylation at other CpG sites (target frequency of 42.1% and a 0.71% frequency at all other CpG sites).

The Targeted Heterodimeric Methyltransferases are Modular

To test whether our targeted M.SssI methyltransferases are modular with respect to the zinc finger domains, we replaced zinc finger HS1 and HS2 with two zinc fingers designed to target a specific site in the promoter of intercellular adhesion molecule 1 (ICAM1). The previously designed zinc finger CD54-31Opt [50] is adjacent to a CpG site in this promoter. To generate a pair of zinc fingers capable of flanking this CpG site, we designed a second zinc finger, CD54a, to bind downstream from the recognition sequence of CD54-31Opt and adjacent CpG site (Fig. 3A). The two zinc fingers were fused to fragments comprising un-optimized bifurcated M.SssI fragments (residues KFNSE at positions 297–301) and to two selected variants (CFESY and SYSSS at positions 297–301), replacing the HS1 and HS2 zinc fingers (Fig. 3A). These two optimized variants (CFESY and SYSSS) were chosen because preliminary experiments (preformed essentially as described in [17]) suggested that methylation at the target site (containing both zinc finger binding sites) was greater than the additive amount of methylation levels observed at “half-sites” composed of only one or the other of the zinc finger binding sequences.

We assessed the methyltransferase activity and specificity of these constructs in E. coli with a restriction endonuclease protection assay at the target and non-target sites (Fig. 3A-D). Notably, the ‘non-target’ site assessed in this experiment contained the zinc finger sequences recognized by HS1 and HS2 zinc fingers (compare Fig. 3B and 1C). Although all three constructs methylated the target site derived from the ICAM1 promoter, the CFESY and SYSSS constructs targeted methylation to the desired site with little to no observable methylation at the non-target site (Fig. 3D).

The CD54-31Opt was chosen because it was shown to effectively target the ICAM1 promoter, altering transcription levels when fused to transcriptional activators or repressors [50,51]. Additionally, fusion of CD54-31Opt to Ten-Eleven Translocation 2 enzyme resulted in a small, observable amount of demethylation around the target site, correlating with a 2-fold upregulation in ICAM1 transcription [52]. Our construct may potentially enable assessment of the biological affects of targeted methylation at this site.

Supporting Information

Figure S1 The DNA and amino acid sequences for the (A) N-terminal and (B) C-terminal M.SssI fragments fused to CD54-31Opt and CD54a respectively. The methyltransferase fragments (cyan), amino acid linkers (yellow), and zinc finger domains (red) are shown along with the ‘wildtype’ sequence from 297-301 (KFNSE) shown in magenta.

Table S1 Variants from the selected library. Sequenced library variants are shown. Aromatic amino acids at position 298 are highlighted in yellow and small amino acids (defined as an amino acid with an R side chain containing 1–3 heavy atoms) at position 300 are highlighted in cyan. Stop codons are denoted by a *.

Acknowledgments

We would like to thank Dr. Tim Bestor for suggesting McrBC as a way to select against non-targeted methylation.

Author Contributions

Conceived and designed the experiments: BC MO. Performed the experiments: BC. Analyzed the data: BC MO. Wrote the paper: BC MO.
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