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Epigenetic Modification of the Repair Donor Regulates Targeted Gene Correction

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Optimizing design of vectors is critical to effective gene therapy. In targeted gene correction (TGC), cleavage of chromosomal DNA near a mutation stimulates homology-directed repair of a target gene using a donor provided *in trans*. We have systematically addressed epigenetic parameters of donor design, using a flow-based assay to quantify correction frequencies and expression levels of a green fluorescent protein (GFP) reporter gene in a human cell line. We show that active transcription of the donor increased correction frequency by threefold, establishing that a proximal promoter enhances donor use. Conversely, CpG methylation of the donor diminished correction frequency and reduced expression of the repaired gene. However, bisulfite sequencing of the target revealed no transfer of methylation marks during repair with a methylated donor. Treatment with histone deacetylase (HDAC) inhibitors can partially compensate for epigenetic inactivation, suggesting a role for class I and II HDACs in regulation of donor use. These results establish that epigenetic status of a *trans*-donor determines both the efficiency and outcome of gene correction, and identify and clarify parameters that should guide donor design for targeted gene therapy.

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Introduction

Targeted gene correction (TGC) is a potentially powerful approach to gene therapy because it corrects the mutant gene *in situ*, in its physiological location and under control of its natural promoter, thereby avoiding many of the problems associated with integration of therapeutic transgenes in the cellular genome. An important challenge will be to establish the critical parameters of TGC to guide vector design and to optimize the outcome and efficiency of gene correction. TGC is initiated by cleavage of the chromosomal DNA at a site close to a disease-associated mutation by expression of a rare-cutting endonuclease.[1] This stimulates the cell's own repair machinery to repair the target gene using a donor provided in *trans*, thereby erasing the mutation.

The conserved pathways that promote homology-directed repair (HDR) have been studied in considerable detail.[2] HDR pathways repair DNA double-strand breaks (DSBs) that occur as a result of DNA damage or replication fork collapse. HDR is also critical to physiological processes of gene diversification, including mating type switching in yeasts and immunoglobulin gene conversion in fowl and other vertebrates. In contrast to gene correction, HDR pathways depend upon endogenous sequences to template repair. A sister chromatid is typically used to repair DSBs and collapsed replication forks; and HDR is rare but sufficiently frequent to be evident as loss-of-heterozygosity in tumor cells and aging.[3,4] HDR may also involve donors in *cis*, for example in regulated gene diversification, where an adjacent gene or gene segment serves as templates for DNA repair.

TGC repair donors are supplied in *trans*, either by transfection or by transduction with a viral vector carrying the repair template. These exogenous donors undergo rapid chromatinization upon entry into the cell nucleus,[5] determined in part by the specific regulatory elements present in the donor DNA. Two chief epigenetic regulatory mechanisms in mammalian cells are post-translational modification of histones and DNA methylation at CpG dinucleotides.[6] Activating histone modifications, especially acetylation, promote accessibility of the DNA duplex and characterize actively transcribed genes. CpG methylation is typically a repressive modification, which downregulates gene expression by preventing binding by some transcriptional activators or by recruiting chromatin modifiers that induce compaction of the DNA.

Epigenetic status of a repair donor in *cis* has previously been shown to determine the efficiency of HDR in vertebrate cells.[7–10] However, the efficiency of HDR has not been systematically and rigorously correlated with the epigenetic status of a donor in *trans*. For example, the presence of a promoter on the donor is widely believed to enhance TGC frequencies, but this is based on a very early experiment that documented only a modest effect.[11] Conversely, it has been reported that donor methylation can be transferred to the target, potentially providing an approach for downregulation of a target gene,[12] but the mechanism underlying this process is unknown.

Optimization of repair donor vectors is important to the success of TGC, especially now that the field is moving so rapidly to translational applications. Here, we systematically investigate the effect of donor epigenetic status on gene correction initiated by a DSB at a single target cleavage site. We use a

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streamlined, flow-based assay that enables quantification of both gene correction and expression of a single green fluorescent protein (GFP) reporter gene in individual human cells. We show that an actively transcribed donor is three times more effective than a nontranscribed donor, as measured by the relative frequencies of corrected cells. Conversely, we show that donor CpG methylation significantly reduces correction frequency, and causes a subtle (60%) but reproducible decrease in expression of the target gene. Both of these effects are due to donor CpG methylation, as they are completely overcome by pretreatment of cells with 5-aza-2′-deoxycytidine (5-azadC), which inhibits maintenance methyltransferase activity. Treatments with class I and class II histone deacetylase (HDAC) inhibitors partially reverse the effect of CpG methylation. These results provide a clear experimental basis for design of vectors for TGC and their use in gene therapy.

Results

TGC is stimulated by a repair donor with a fully active promoter

TGC depends on the interaction between a repair donor provided in *trans* and its target. It is therefore intuitively plausible that activation of chromatin structure of the donor promotes efficient HDR, but the magnitude of the effect remains undetermined. We therefore asked if donor transcription stimulates TGC initiated by a DSB in experiments that modulate transcription in two different ways.

We first compared TGC by a donor DNA carrying an intact or truncated promoter from the phosphoglycerol kinase (PGK) gene (P_{PGK} or $P_{PGK\text{-}\Delta}$). In control experiments, we verified that the promoter truncation effectively impaired transcription, by comparing expression of a GFP gene driven by either the intact or truncated promoter (**Figure 1a**, above). Linear DNAs were used to avoid the possibility that read-through transcription could activate a promoterless gene. The promoter truncation clearly diminished GFP expression, as evidenced by a clear reduction in GFP intensity (**Figure 1a**, below). Thus the intact and truncated P_{PGK} promoters differ significantly in their ability to activate gene expression.

Donors consisted of linear duplex DNA molecules carrying either the intact or truncated promoter upstream of a defective GFP gene, which had been inactivated by deletion of 14 residues from the 3′-end (GFPΔ) (**Figure 1b**). The repair target was a GFP gene bearing two in-frame N-terminal stop codons to prevent GFP expression (GFP–) (**Figure 1b**), integrated in the chromosome of HEK293T cells to generate the cell line "293T-GFP15". The target gene was driven by an intact P_{PGK} promoter, and the P_{PGK} and $P_{PGK\text{-}\Delta}$ repair donors differ in 5′-homology with the target (790 and 100 bp, respectively), but not 3′-homology (865 bp). TGC between the donor and chromosomal target generates GFP+ cells that can be readily quantified by flow cytometry. TGC was initiated by transfection with a construct that expresses the rare-cutting nuclease, I-AniI, joined by a T2A translational linker to mTagBFP, to permit identification of cells expressing I-AniI

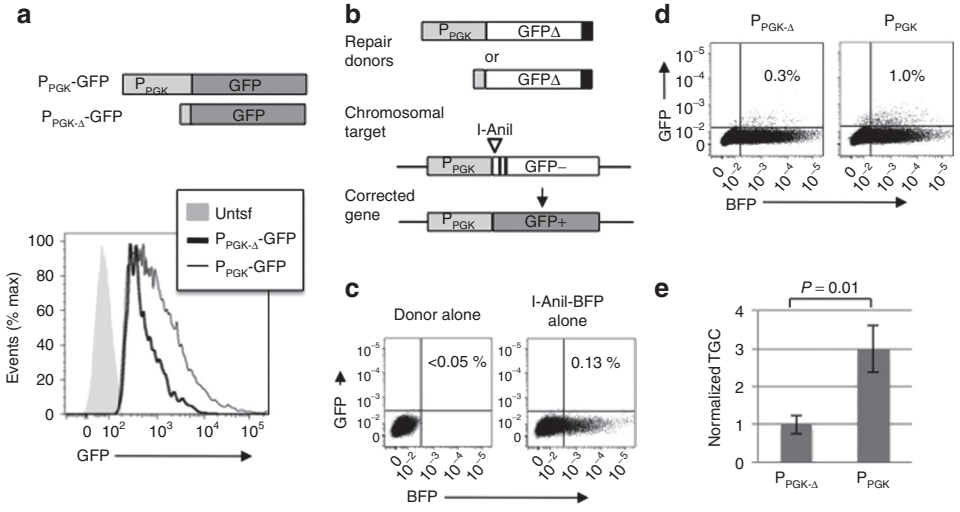

Figure 1 TGC is stimulated by a repair donor with a fully active promoter. (**a**) Above, diagram of linear DNA driving GFP expression by intact (P_{PGK}-GFP) or truncated ($P_{PGK\text{-}\Delta}$-GFP) PGK promoters. Below, representative histogram of GFP expression at 48 hours post-transfection in untransfected 293T cells (untsf) or 293T cells transfected with P_{PGK}-GFP or $P_{PGK\text{-}\Delta}$-GFP linear DNA. GFP fluorescence intensity of GFP+ gated cells is shown relative to the number of events analyzed. (**b**) Reporter assay to measure TGC. Repair donors carry a GFP gene that is nonfunctional due to deletion (black box) of 14 residues from the 3′-end (GFPΔ), driven by an intact or truncated P_{PGK} promoter. The chromosomal target carries a GFP gene in which two in frame N-terminal stop codons (black lines) prevent GFP expression (GFP–). Expression of the rare-cutting endonuclease, I-AniI, initiates TGC by generating a DSB at its target site (open triangle). Homologous recombination generates a functional chromosomal GFP gene and GFP+ cells are quantified by flow cytometry. (**c**) Representative FACS profiles of TGC in 293T-GFP15 cells transfected with the P_{PGK}-GFPΔ donor or I-AniI-BFP alone. Profiles quantify TGC (GFP, y-axis) relative to I-AniI expression (BFP, x-axis). Absolute TGC frequencies are shown in upper right sector of each profile. (**d**) Representative FACS profiles of TGC in 293T-GFP15 cells using donor linear duplex DNA containing either an intact or truncated PGK promoter. Notations as in **c**. (**e**) Quantification of mean TGC efficiencies supported by P_{PGK} and $P_{PGK\text{-}\Delta}$ donors in eight independent experiments. TGC was normalized relative to the truncated PGK donor. On average, $P_{PGK\text{-}\Delta}$ resulted in 0.19% TGC (n = 8), whereas P_{PGK} resulted in 0.56% TGC (n = 9). BFP, blue fluorescent protein; DSB, double-strand break; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; PGK, phosphoglycerol kinase; TGC, targeted gene correction; untsf, untransfected.

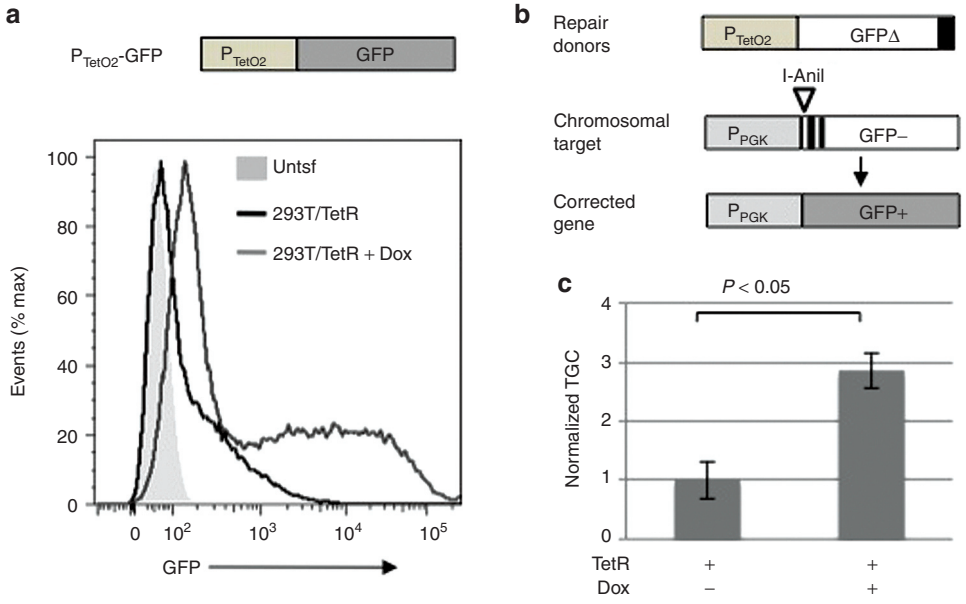

Figure 2 Active transcription of the repair donor enhances TGC. (**a**) Above, diagram of plasmid driving GFP expression by the inducible P_{TetO2} promoter. Below, representative histogram of GFP expression, in untransfected 293T cells (untsf) or 293T/TetR cells transfected with the P_{TetO2}-GFP and untreated or treated with doxycycline (Dox, 10 ng/ml). GFP fluorescence intensity is shown relative to the number of cells analyzed. (**b**) Reporter assay for TGC using the repair donor containing a tetracycline-inducible promoter (P_{TetO2}). (**c**) Quantification of mean TGC efficiencies from three independent experiments in 293T-GFP15/TetR cells, untreated or cultured with Dox (10 ng/ml). TGC was normalized to efficiencies in untreated 293T-GFP15/TetR cells. GFP, green fluorescent protein; PGK, phosphoglycerol kinase; TGC, targeted gene correction; untsf, untransfected.

as blue fluorescent protein (BFP+). In control experiments (**Figure 1c**), we showed that very few GFP+ cells (<0.05%) were observed following transfection of 293T-GFP15 cells with the donor alone, or with I-AniI-BFP alone (0.13%). Similar controls were run in all our experiments.

We compared TGC frequencies following transfection of 293T-GFP15 cells with I-AniI-BFP and linear donors carrying either the intact or truncated P_{PGK} promoter. The intact promoter supported a higher frequency of gene correction, as shown by a representative fluorescence-activated cell sorting profile (**Figure 1d**). Quantification of eight independent transfections showed that there was a threefold difference between the levels of TGC supported by the intact and truncated promoters (**Figure 1e**).

Active transcription of the repair donor enhances TGC

To confirm that the results documented above (**Figure 1**) did not reflect differences in target homology lengths of the donor DNAs tested, we next assayed repair donors in which transcription is inducible. Donors carried the $TetO_2$ promoter (P_{TetO2}), in which tandem tetracycline operators (TetO) confer negative regulation by Tet repressor (TetR), and inducibility by the tetracycline analog, doxycycline, which binds TetR to release it from TetO. We verified transcriptional regulation by analyzing expression of the P_{TetO2}-GFP construct, in which P_{TetO2} drives a functional GFP gene (**Figure 2a**, above). As predicted, P_{TetO2}-GFP expression was downregulated in 293T cells transiently transfected with TetR (293T/TetR), and doxycycline strongly induced GFP expression (**Figure 2a**, below).

We then assayed TGC by a repair donor regulated by the P_{TetO2} promoter (**Figure 2b**) in 293T-GFP15/TetR cells, which were either untreated or treated with doxycycline. TGC was quantified and normalized relative to untreated 293T-GFP15/TetR cells (**Figure 2c**). Culture of 293T-GFP15/TetR transfectants in the presence of doxycycline increased TGC frequency almost threefold (**Figure 2c**). Together with the results presented in **Figure 1**, we conclude that active transcription of the repair donor stimulates TGC threefold.

CpG methylation of the repair donor inhibits TGC

Exogenous DNA may undergo rapid chromatinization and *de novo* methylation upon entry into the cell nucleus.[13–15] We therefore asked how methylation affects TGC, by comparing repair by methylated and unmethylated donors. The donor repair templates were carried on an episomal DNA containing an SV40 origin of replication to allow replication in 293T cells. Methylation was carried out *in vitro* using *M. SssI* methyltransferase, which specifically methylates cytosines in CpG dinucleotides.

CpG methylation is predicted to promote repressive DNA structure, and may inhibit TGC either by rendering the repair substrate less accessible for recombination or by preventing transcription (**Figures 1** and **2**). We showed that CpG methylation impaired transcription by assaying GFP fluorescence in 293T cells transfected with CpG-methylated or unmethylated pP_{PGK}-GFP plasmids (**Figure 3a**). To establish that reduced GFP gene expression was not due to diminished transfection or replication of the methylated construct in 293T cells, we recovered low molecular weight supercoiled DNA from transfectants, and quantified recovered plasmids by measuring transformation frequency in *Escherichia coli*. Results from four independent experiments showed that there was no significant difference in recovery of methylated and unmethylated plasmids (**Figure 3b**).

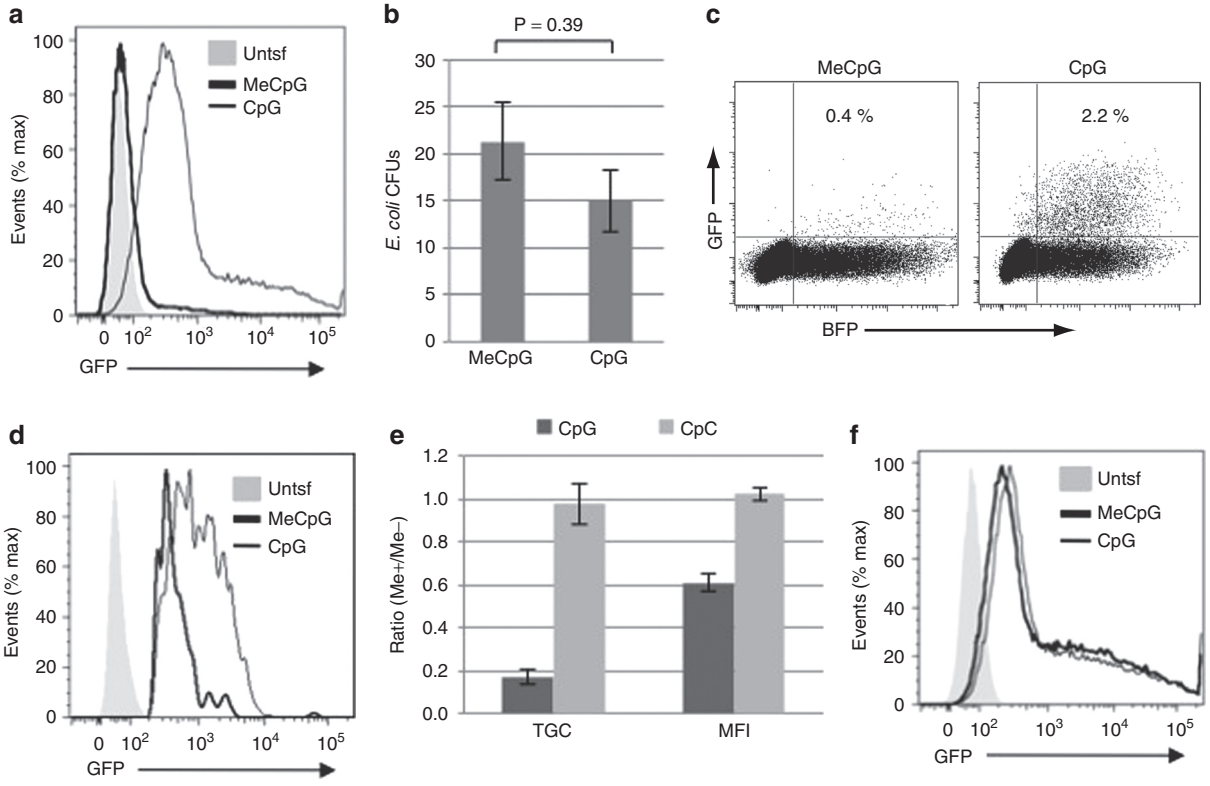

Figure 3 CpG methylation of the repair donor inhibits TGC. (**a**) Representative histogram of GFP expression in untransfected 293T cells (untsf) or 293T cells transfected with CpG-methylated (MeCpG) or unmethylated (CpG) pP_{PGK}-GFP plasmids. GFP fluorescence intensity of GFP+ gated cells is shown relative to the number of events analyzed. (**b**) Quantification of plasmid recovery by transformation efficiency. Mean number of colony-forming units (CFUs) from four independent experiments is shown. (**c**) Representative FACS profiles of TGC in 293T-GFP15 cells by methylated or unmethylated pP_{PGK}-GFPΔ repair donors. (**d**) Representative histogram of GFP expression of 293T-GFP15 cells repaired by CpG methylated or unmethylated donors. GFP fluorescence intensity of GFP+ gated cells is shown relative to the number of events analyzed. (**e**) Quantification of TGC efficiency (left) and median fluorescence intensity (MFI, right) of 293T-GFP15 cells repaired by methylated relative to unmethylated (Me+/Me–) donors. Donor DNA was methylated on CpG or CpC dinucleotides. Mean TGC or MFI was calculated from four (CpG) or two (CpC) independent experiments. (**f**) Representative histogram of GFP expression by 293T cells transfected with CpC methylated (MeCpC) or unmethylated (CpC) pP_{PGK}-GFP plasmid. GFP fluorescence intensity of GFP+ gated cells is shown relative to the number of events analyzed. BFP, blue fluorescent protein; GFP, green fluorescent protein; TGC, targeted gene correction; untsf, untransfected

To evaluate the effect of CpG methylation of the donor DNA on TGC, 293T-GFP15 cells were transfected with methylated or unmethylated pP_{PGK}-GFPΔ repair plasmid and the I-AniI expression construct, and GFP+ cells were quantified. As shown by the representative flow profiles (**Figure 3c**), the CpG-methylated donor was considerably less efficient than the unmethylated donor. Methylation affected not only TGC frequency, but also the outcome of repair, as the level of GFP expression per corrected cell was reduced following TGC with a methylated donor (**Figure 3d**). Quantification of four independent transfections showed that methylation of the repair donor diminished the efficiency of TGC more than five-fold (**Figure 3e**, left), and decreased expression of the corrected reporter genes to 60% of that of the genes corrected by unmethylated donors (**Figure 3e**, right).

To address the possibility that TGC by a methylated donor occurred at reduced frequency due to effects of DNA methylation unrelated to altered chromatin structure, we carried out analogous experiments with plasmids methylated by *M. MspI* methyltransferase, which methylates the 5′-C in the sequence 5′-CCGG-3′. Methylation at CpC dinucleotides had no impact on gene expression from the pP_{PGK}-GFP expression vector (**Figure 3f**). Moreover, CpC methylation did not affect TGC efficiency by the pP_{PGK}-GFPΔ repair donor (**Figure 3e**, left) or expression of the corrected gene (**Figure 3e**, right). Thus the inhibitory effects of DNA methylation on TGC are specific to CpG-methylated DNA and are not simply due to the covalent modification of the repair donor.

5-azadC counteracts the repressive effects of CpG methylation of the repair donor

In mammalian cells, DNA methyltransferases maintain CpG methylation patterns of the daughter strand following DNA replication. 5-azadC inhibits DNA methyltransferase activity and prevents methylation of newly replicated DNA. 5-azadC would therefore be predicted to counteract the repressive effect of CpG methylation of the pP_{PGK}-GFPΔ repair template on TGC, by allowing unmethylated repair templates to emerge following replication. We tested this by comparing the frequency of TGC by methylated and unmethylated repair donors in cells treated with 1 μmol/l 5-azadC 24 hours before transfection. TGC efficiency was comparable in 5-azadC-treated cells transfected

with CpG-methylated or unmethylated repair donors, as illustrated by a representative fluorescence-activated cell sorting profile (**Figure 4a**). Moreover, pretreatment of cells with 5-azadC-resulted in indistinguishable levels of GFP expression for cells corrected by methylated and unmethylated donors (**Figure 4b**). Four independent experiments showed that 5-azadC treatment relieved the repressive effect of CpG methylation of the repair donor on both TGC efficiency (**Figure 4c**, left) and expression of the corrected gene (**Figure 4c**, right).

Bisulfite sequencing reveals no transfer of methylation marks from the repair donor to the chromosomal target

TGC by methylated repair donors gave rise to GFP+ cell populations with lower GFP expression than populations corrected by unmethylated repair donors (**Figure 3d,e**). One possible explanation might be that CpG methylation marks are transferred from the repair donor to the chromosomal target. This could, in principle, occur during the course of heteroduplex formation in HDR. If so, the result would be a genetically corrected but silenced chromosomal gene.

To test this, we carried out bisulfite sequencing of corrected clones from both GFP+ and GFP– cell populations. We simultaneously assayed genetic correction and methylation in a short (250-bp) region spanning the I-AniI recognition site and stop codons on the chromosomal target, amplifying bisulfite-treated genomic DNA with primers designed to distinguish the chromosomal target from the repair donor (primers 2F/1R, **Figure 5a**). Bisulfite mapping and sequencing of the chromosomal target region in GFP+ cells corrected by methylated or unmethylated donor confirmed that over 90% of the clones analyzed had undergone correction, as evidenced by loss of the I-AniI site and stop codons. Moreover, most clones were hypomethylated regardless of whether methylated or unmethylated donor was used (**Figure 5b**), suggesting that the lower levels of GFP expression cannot be attributed to CpG methylation.

To focus on cells that had undergone I-AniI cleavage and correction with methylated donor but did not express GFP, we enriched for loss of the region containing the I-AniI site by nuclease digestion of DNA before bisulfite treatment and PCR amplification and cloning (see details in Materials and Methods). No genetically corrected clones were identified among 17 nuclease-resistant clones analyzed with target-specific primers 2F/1R from the GFP– population. Instead, sequences showed disrupted I-AniI sites and intact stop codons in the GFP gene (**Figure 5c**, left), and appear to result from repair by nonhomologous end-joining pathway rather than HDR following DNA cleavage. Bisulfite mapping showed that those clones were unmethylated (**Figure 5c**, right). The absence of genetically corrected clones in the GFP– population argues against silencing of the target by CpG-methylated repair template.

We validated the ability of this bisulfite sequencing approach to identify methylated DNA by amplifying the DNA from GFP– cells with a primer pair that did not distinguish the donor and the chromosomal target (primers 1F/2R, **Figure 5a**). Sequence analysis identified nine clones that had lost the I-AniI site and the stop codons (**Figure 5d**, left). Of these, four were heavily methylated while the others were largely unmethylated (**Figure 5d**, right). These DNA sequences are probably

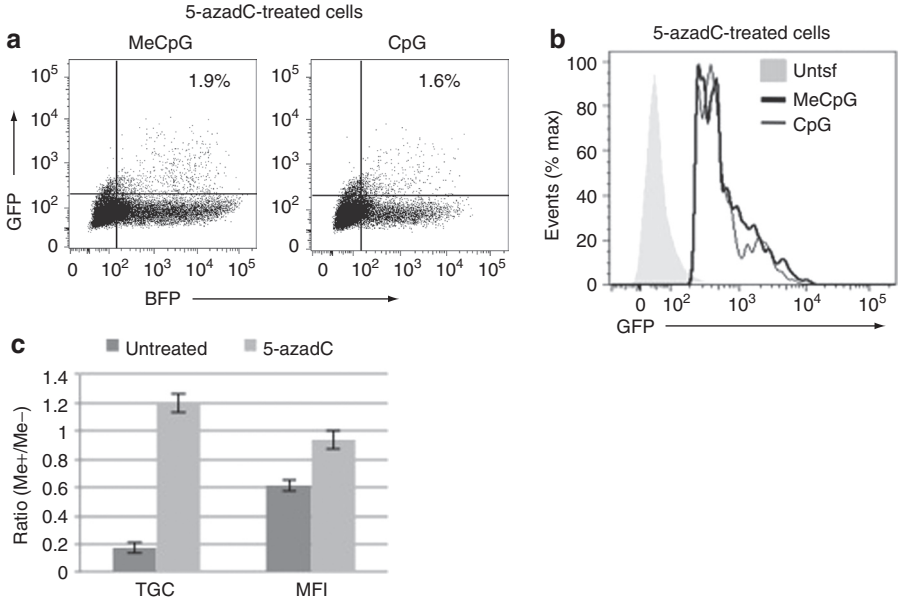

Figure 4 5-azadC counteracts the repressive effect of CpG methylation of the repair donor. (**a**) Representative FACS profiles of TGC by methylated (MeCpG) or umethylated (CpG) pP_{PGK}-GFPΔ repair donors in 293T-GFP15 cells treated with 1 µmol/l 5-azadC at 24 hours before transfection. (**b**) Representative histogram of GFP expression in 293T-GFP15 cells treated with 1 µmol/l 5-azadC at 24 hours before correction with methylated or unmethylated repair donors. GFP fluorescence intensity of GFP+ gated cells is shown relative to the number of events analyzed. (**c**) Quantification of TGC efficiency (left) and median fluorescence intensity (MFI, right) of 293T-GFP15 cells repaired by CpG methylated relative to unmethylated (Me+/Me–) donors. Cells were untreated or treated with 1 µmol/l 5-azadC starting at 24 hours before transfection. Mean TGC or MFI was calculated from four independent experiments. 5-azadC, 5-aza-2′-deoxycytidine; BFP, blue fluorescent protein; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; PGK, phosphoglycerol kinase; TGC, targeted gene correction; untsf, untransfected.

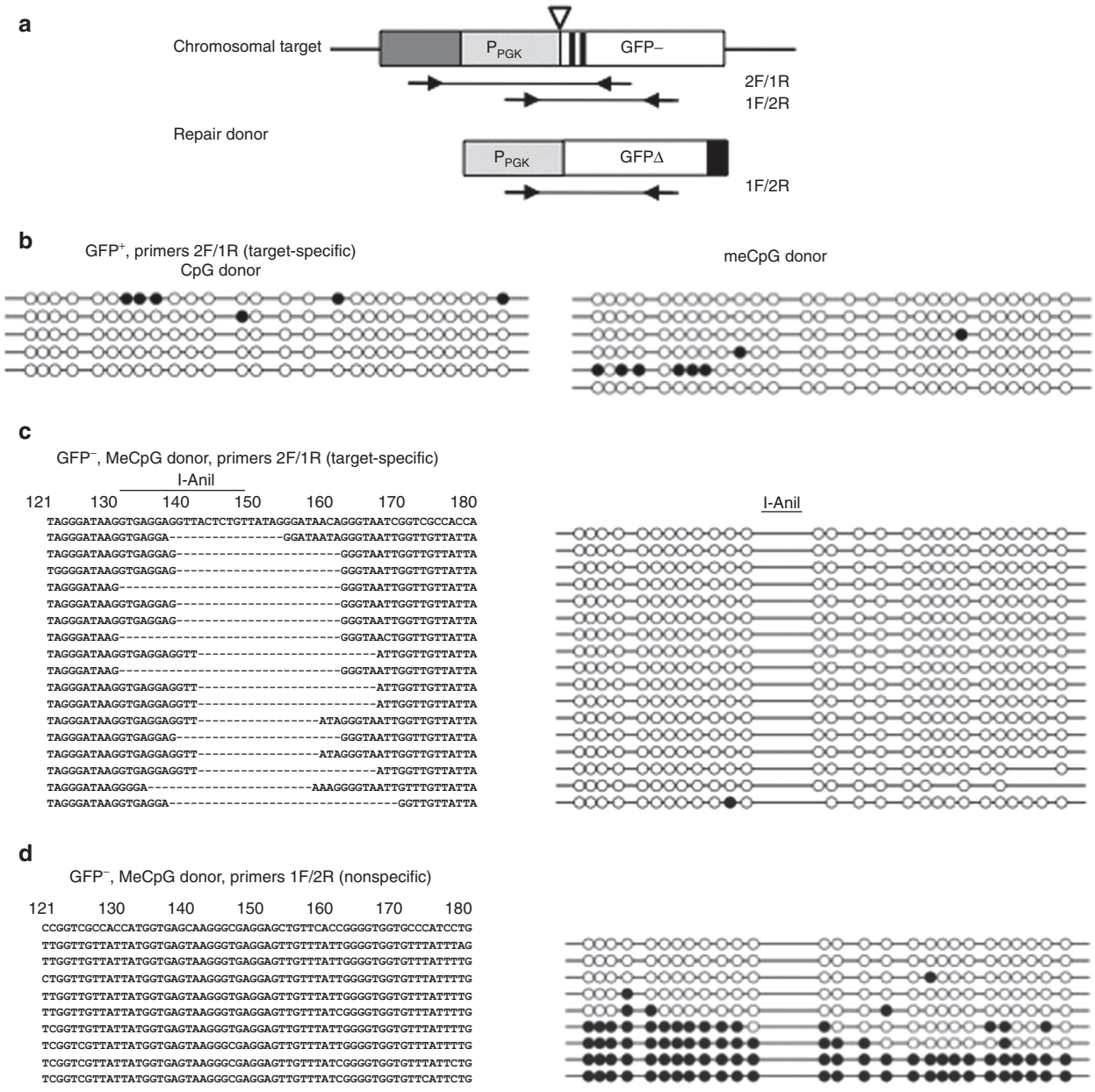

Figure 5 Bisulfite sequencing of the chromosomal target. (**a**) Diagram showing primers used for PCR amplification of bisuflite-treated DNA with respect to the chromosomal target and repair donor. Primers 2F/1R are target-specific whereas primers 1F/2R do not distinguish donor and recipient target. (**b**) CpG methylation sites of five and six clones repaired with unmethylated and methylated donor, respectively. Bisulfite-treated DNA was amplified with target-specific primer pair 2F/1R from GFP+ cells. Circles represent the 27 potential CpG methylation sites in the 250-bp region of the target analyzed by bisulfite sequencing (spanning P_{PGK} promoter, the I-AniI recognition site, and the two stop codons at the 5′ end of the GFP gene). Open and closed circles correspond to unmethylated or methylated CpG dinucleotides, respectively. (**c**) DNA sequence (left) and CpG methylation sites (right) of 17 clones amplified with target-specific primer pair 2F/1R from a GFP– cell population repaired with CpG-methylated donor. Sequence of the region containing the I-AniI site is shown at the top; below, dashes indicate deletion. (**d**) DNA sequence (left) and CpG methylation sites (right) of nine clones amplified with primer pair 1F/2R from the same GFP– population. Sequence of donor DNA is shown (top). GFP, green fluorescent protein; PGK, phosphoglycerol kinase.

derived from the repair donor plasmid, with some methylation marks lost upon DNA replication following transfection.

In summary, we found no evidence for transfer of CpG methylation from the repair donor to the recipient during TGC. Thus CpG methylation of the chromosomal target cannot account for the reduced level of GFP expression seen with a methylated repair template.

HDAC inhibitors partially reverse the inhibitory effect of methylated repair donor on TGC

Methylated DNA attracts methyl-CpG binding proteins,[16] which in turn recruit HDACs to repress gene transcription.[17,18] This predicts that treatment with HDAC inhibitors will alleviate, at least in part, the repressive effects of methylation on TGC. Quantification of TGC frequencies by

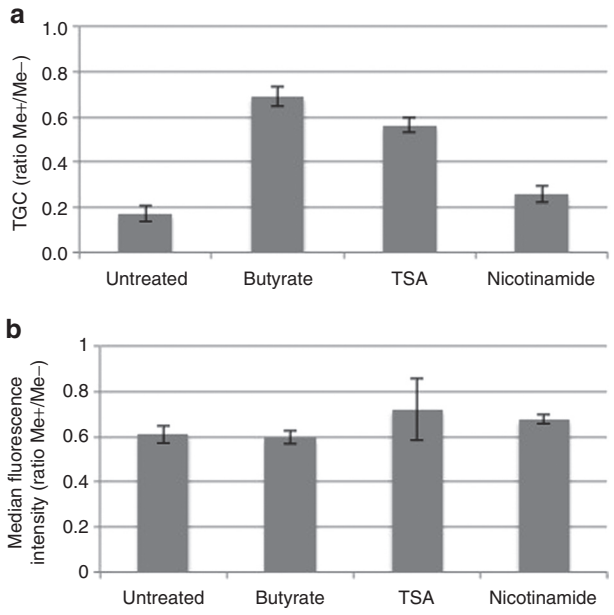

Figure 6 Histone deacetylase inhibitors partially reverse inhibition of TGC by a methylated repair donor. (**a**) Quantification of TGC efficiencies of 293T-GFP15 cells repaired by methylated relative to unmethylated (Me+/Me–) donors. Cells were left untreated or were treated with indicated HDAC inhibitors starting at 24 hours before transfection. Mean TGC was calculated from four independent experiments. (**b**) Quantification of GFP expression by median fluorescence intensity (MFI) of 293T-GFP15 cells repaired by CpG methylated relative to unmethylated (Me+/Me–) donors. Cells were treated with indicated HDAC inhibitors starting at 24 hours before transfection. Mean MFI was calculated from four independent experiments. GFP, green fluorescent protein; HDAC, histone deacetylase; TGC, targeted gene correction; TSA, trichostatin A.

methylated or unmethylated repair donor in cells treated with HDAC inhibitors before transfection showed that treatment of cells with sodium butyrate or trichostatin A (TSA), class I and II HDAC inhibitors, significantly reversed the repressive effect of CpG methylation on TGC. In contrast, treatment with nicotinamide, which inhibits class III HDACs (sirtuins), had no effect (**Figure 6a**). Administration of TSA at 72 hours post-transfection, once most correction events had occurred, did not affect TGC frequency (data not shown). Thus, the repressive effect of donor methylation on TGC frequency can be partially overcome by inhibition of class I or class II HDACs.

TGC with a methylated donor resulted in reduced expression of the corrected gene to ~60% the level of a gene corrected by an unmethylated donor (**Figure 3e**, right). We asked whether this reduction was alleviated by treatment of cells with the HDAC inhibitors sodium butyrate, TSA or nicotinamide. None of these drug treatments resulted in a significant increase in expression of the corrected target (**Figure 6b**), even though sodium butyrate or TSA treatment stimulated TGC by the methylated donors (**Figure 6a**). This result contrasts with effects of 5-azadC treatment, which stimulated TGC and alleviated the reduced expression caused by a methylated donor (**Figure 4b,c**).

Discussion

We have shown that the epigenetic status of a repair donor determines the efficiency of TGC and influences the level of expression of the corrected gene in our single reporter gene system. These experiments took advantage of cell-based flow assays that permitted both genetic correction and expression levels of the targeted gene to be measured in single cells within a large population. Our results establish a role for chromatin status of the donor for TGC, and provide straightforward guidance in construction of repair donor DNA for TGC in therapeutic contexts.

We found that active transcription of the repair donor stimulated TGC threefold. Comparable stimulation was evident with intact versus truncated promoter carried by a linear DNA donor (**Figure 1**); and activated versus repressed promoter on a circular DNA donor (**Figure 2**). The effect of a donor promoter was previously tested in a seminal report of TGC of a chromosomal reporter gene initiated by zinc finger nucleases.[11] Those experiments described a difference of only 50% in the efficiency of TGC by donors carrying or lacking a promoter, and the statistical significance of that result was not presented. This effect is considerably lower than the threefold stimulation we observe. Those previous experiments employed a circular duplex DNA donor, and read-through transcription may have activated the promoterless donor to cause a high background and diminish the apparent magnitude of stimulation.

The ability of transcription of either the donor (our data) or the target[19] to enhance HDR may reflect activation of chromatin, which will render duplex DNA accessible to factors necessary to promote recombination. In addition, the transient DNA denaturation that accompanies passage of the transcription apparatus may facilitate invasion of the target by donor DNA.

We found that CpG methylation of the repair donor plasmid resulted in a fivefold decrease in TGC efficiency, as well as a subtle but reproducible reduction in expression of the corrected gene (**Figures 3** and **6**). These inhibitory effects are due to CpG methylation, as they are completely overcome by pretreatment of cells with 5-azadC (**Figure 4**). The reduced expression of the target following correction with a methylated donor is distinct from the changes in DNA methylation and chromatin structure described at the site of a DSB in the course of repair,[20] because we strictly observed this effect with methylated donor. A previous report has documented downregulation of two tumor suppressor genes in human mesenchymal stem cells by transfection of methylated denatured linear DNA duplex homologous to the promoters, in the absence of targeted DNA cleavage.[12] That report claimed that downregulation reflected transfer of methylation marks from donor to target but it is not clear whether the primers used in methylation analysis did in fact distinguish between donors and targets. Using primers that made that distinction, we found no evidence of transfer of methylation marks from donor to recipient DNA (**Figure 5**).

Our evidence that epigenetic status of a repair donor provided in *trans* affects the frequency of HDR is consistent with previous examples analyzing the effect of epigenetic status of donors in *cis*. In chicken B cells, diversification of the expressed immunoglobulin genes by gene conversion can be inhibited or stimulated by repressive or activating

modifications of donor chromatin structure.[8,9] In human 293T cells, actively transcribed donors in *cis* preferentially participate in repair of a targeted chromosomal DSB.[7] If results using episomal donor DNA in *trans* can be extended to HDR templated by chromosomal DNA in *trans*, our experiments raise several questions about physiological repair of DSBs. Allele-specific epigenetic regulation has been documented in a significant fraction of human genes.[21] The sister chromatid is thought to serve as the donor for most HDR, which would preserve epigenetic status. However, our results raise the possibility that an active allele on the homolog might compete effectively with a silent allele on the sister to function as a repair donor. If this occurred, activation might accompany repair of the silenced allele. Repair of an active allele directed by a silenced homolog might be predicted to proceed inefficiently, and lead to modest downregulation of gene expression. It should be possible to test these specific predictions by carefully designed experiments.

Methylated DNA recruits class I and II HDACs to establish repressive histone modifications and chromatin structure.[22] Consistent with this, the decrease in TGC efficiencies by methylated donor was largely but not completely overcome by pretreatment of cells with sodium butyrate or TSA, while nicotinamide had no effect (**Figure 6a**). One likely source of reduced gene expression following correction with a methylated donor is redistribution of repressive histones from the donor to the target during recombination, analogous to the redistribution of histones from the parental duplex to daughters upon replication.[23] Pretreatment of cells with sodium butyrate or TSA did not alleviate the reduced expression characteristic of genes repaired by methylated donors (**Figure 6b**). These drugs clearly had an effect on TGC frequency, but they were unable to counteract all nucleosome acetylation at dose and time frame that are well tolerated by cultured cells, or compatible with efficient TGC.

HDAC inhibitors have many effects on cells. Microarray analyses have documented downregulation of RAD51 and of other genes involved in DNA repair and DNA damage response following HDAC inhibition, with a corresponding decrease in HDR and in the cellular response to DNA damage.[24,25] The ability of HDAC inhibitors to downregulate HDR is now being exploited therapeutically, as this will sensitize cells to radiation and other kinds of DNA damage.

The results we report greatly strengthen the previous rationale for including a functional promoter in donor gene design, and for developing repair donors with CpG-free promoters to circumvent transcriptional silencing by *de novo* methylation.[26] Our results also caution against attempts to overcome marginal correction frequencies ascribable to donor silencing by treatment with HDAC inhibitors. Drugs that inhibit HDACs have multiple effects, among them downregulation of HDR, and they are therefore likely to be incompatible with TGC.

Materials and methods

Cell culture, transfection, and flow cytometry. All cell lines were derivatives of HEK293T, an SV40-transformed human embryonic kidney cell line. Cells were cultured at 37 °C, 5% CO_2 in Dulbecco-modified Eagle's medium (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA) and 200 units/ml penicillin, 200 µg/ml streptomycin (Hyclone), and 2 mmol/l L-glutamine (Hyclone). Trichostatin A (Sigma-Aldrich, St Louis, MO) was added at a final concentration of 80 nmol/l, sodium butyrate (Sigma-Aldrich) at 1.25 µmol/l, nicotinamide (Sigma-Aldrich) at 20 µmol/l, and 5-azadC (Sigma-Aldrich) at 1 µmol/l.

Transfections were performed using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 24 hours before transfection, cells were seeded in 0.5–1.0 ml of serum-free medium at 2.5×10^5 cells/ml, then transfected with 1 µg DNA and 2.5 µl of lipofectamine per ml. Transfection efficiency for each experiment was controlled with pEGFP-N1 vector (Clontech, Mountain View, CA) and was typically 80–100%. GFP expression assays used 300 ng of linear or circular DNA, and expression was assayed at 48 hours post-transfection.

Cells were fixed in 2% formaldehyde and analyzed on a LSRII flow cytometer (Becton Dickinson, San Jose, CA). GFP fluorescence was detected with a 488 nm laser (FITC channel); and mTagBFP fluorescence was detected with a 405 nm laser (Pacific Blue channel). Data was compensated and analyzed with FlowJo software (TreeStar, Ashland, OR).

TGC reporter assay and repair donor constructs. The GFP reporter assay for measuring TGC has been previously described.[27–29] The cell line carrying the TGC reporter was generated by transduction of HEK293T cells with self-inactivating lentivirus made with the pRSCSMPG'ISce_AniCS_w2reporter construct[28] at low multiplicity of infection. Integrants were selected with 50 µmol/l O^6-benzylguanine and 200 µmol/l 1,3-Bis(2-chloroethyl)-1-nitrosourea.[29] TGC was analyzed in the clonal population 293T-GFP15. TGC was typically induced by transfection of 250–500 ng repair donor DNA and 150 ng of I-AniI expressing construct per 1.25×10^5 cells, and measured at 72 hours post-transfection. The I-AniI gene is under control of the EF1, a promoter and coupled to mTagBFP by a T2A translational linker, to enable identification of transfectants as BFP+cells.

Repair donor plasmid pP_{PGK}-GFPΔ, previously described as pRSCSIPΔ14Gw2,[28] contains an SV40 origin of replication to allow replication in 293T cells. Linear donors bearing an intact (516 bp) or truncated (89 bp) P_{PGK} promoter were generated by PCR amplification of plasmid pP_{PGK}-GFPΔ using primers WPRE-R (5′-GCAACCAGGATTTATACAAGGAGG), and PGK-F1 (5′-GGTGTTCCGCATTCTGCAAGC) or PGK-F2 (5′-GGATGACGGTGGCAAATGGGA), respectively. Plasmids carrying the TetO2 promoter were generated by cloning functional or mutant GFP genes into the BamHI/NotI sites of pcDNA4/TO (T-Rex system; Invitrogen), which carries two tetracycline operator sequences (TetO2) just downstream of the TATA box of a cytomegalovirus promoter. TetR was expressed by transient transfection with pcDNA6/TR (Invitrogen).

In each TGC experiment, cells were transfected with the donor construct alone or with the I-AniI construct alone as controls (one example is shown in **Figure 1c**). Each TGC experiment was repeated multiple times as indicated in the figure legends. Statistical significance was determined by a two-tailed *t*-test. Error bars represent SEM.

Plasmid recovery from transfectants. Plasmid DNA pP_{PGK}-GFP was extracted and isolated from transfected 293T cells at 72 hours post-transfection and purified by Qiagen chromatography.[30] Briefly, cells were collected and lysed using buffers P1 and P2 following the manufacturer's handbook (Qiaprep; Qiagen, Valencia, CA), and digested with 800 µg/µl proteinase K (Fermentas, Glen Burnie, MD) at 55 °C for 2 hours. Plasmid DNA was transformed in competent XL1-Blue MRF' *Escherichia coli* (Agilent Technologies, Santa Clara, CA), deficient in all known restriction modification systems, grown on selective media, and colonies were enumerated.

In vitro methylation and bisulfite sequencing. Plasmids were methylated *in vitro* with M. SssI or M. MspI methyltransferases (New England Biolab, Ipswich, MA) as recommended by the manufacturer, purified using DNA clean and concentrator-5 kit (Zymo Research, Irvine, CA) and OD_{260} quantified. Methylation was verified by assaying resistance to HpaII endonuclease (New England Biolab), which cleaves the sequence 5′-CCGG-3′ in unmethylated but not methylated DNA.

For bisulfite sequencing, 1 µg genomic DNA, isolated by phenol/chloroform extraction, was treated with sodium bisulfite to convert C to U, using the Epitect Bisulfite Kit (Qiagen) as recommended by the manufacturer. To enrich for clones that had undergone I-AniI cleavage and correction in the GFP– population, DNAs were digested by nuclease targeting the I-AniI region before bisulfite treatment. Primers for amplification of bisulfite-treated DNA were designed to lack CpG dinucleotides using Epidesigner (Sequenom, San Diego, CA). Primer sequences were: 1F, 5′-TAGTGTGGGTTTTGTTTTTGTT; 1R, 5′-ATAAACTTCAAAATCAACTTACC; 2F, 5′-GGAGAAGTGATTTTTTATTAGTAATTGG; 2R, 5′-AATTATACTCCAACTTATACCCCAA. First round amplification was with 1F/2R or 2F/1R and second round amplification was with 1F/1R, using 2 µl of the first reaction. DNA was amplified with Taq polymerase (New England Biolab) and PCR fragments were purified from gels using the DNA extraction kit (Qiagen) and cloned into pCR2.1-TOPO TA vector (Invitrogen). Inserts were amplified with primers M13R/F (Invitrogen), purified and sequenced (Eurofins MWG operon, Huntsville, AL). Sequences were analyzed with QUMA software.[31]

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