demethylation and/or activation are desirable.

SUMMARY ~~OF INVENTION~~

Provided herein are methods and compositions for gene specific demethylation and/or activation. As described in detail herein below, oligonucleotides and methods have now been developed for providing targeted demethylation of one or more genes of interest, leading to activation and increased expression thereof. Using targeted oligonucleotide constructs designed for inhibiting DNA methyltransferase 1 (DNMT1) activity, and deactivated (dead) Cas9 (dCas9), it is shown herein that DNA methylation may be decreased in target methylated genomic regions, leading to increased gene expression of target gene(s) of interest. In certain ~~embodiment~~examples, methods described herein may provide a more natural and targeted demethylation effect as compared with traditional non-specific demethylating agents, and results provided herein observed demethylation and activation over extended periods of time. Remarkably, as described herein it is found that targeting the non-template strand of the genomic DNA with the oligonucleotide(s) provided notably better gene demethylation/activation as compared with targeting the template strand of the genomic DNA.

In ~~an embodiment~~one aspect, ~~there is provided herein~~the present disclosure refers to an oligonucleotide comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within a gene, near a gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR.

In another ~~embodiment~~example of the above oligonucleotide, the targeting portion may have sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both.

In still another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the R2 and R5 stem loops of DiR may be from extra-coding CEBPA (ecCEBPA).

In still another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the targeting portion may target a methylated region of the genomic DNA.

In yet another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the targeting portion may target the genomic DNA region within or near a promoter region or within or near a demethylation core region (for example, a region encompassing a proximal promoter-exon 1-beginning of intron 1 region) of the gene, preferably wherein the targeting portion may target a region at or near the 5' end of the first exon (for example, a proximal promoter region) or a region at or near the 3' end of the first exon (for example, a beginning portion of intron 1) of the gene or a middle region (e.g. a region positioned between a proximal promoter on one side and the beginning of intron 1 on the other side) of the first exon of the gene. In certain ~~embodiment~~examples, the middle region may comprise any portion or region within exon 1. In certain ~~embodiment~~examples, the targeting portion may target a region at or near a proximal promoter region associated with the first exon and/or a region at or near the beginning of the first intron and/or a middle region of the first exon of the gene. Preferably, in certain ~~embodiment~~examples, at least two oligonucleotides may be used, one having a targeting portion targeting a region at or near the 5' end of the first exon (for example, a proximal promoter region), and one having a targeting portion targeting a region at or near the 3' end of the first exon (for example, a beginning portion of intron 1) of the gene, so as to simultaneously target both ends of the demethylation core region. In certain ~~embodiment~~examples, an oligonucleotide may be used having a targeting portion targeting a middle region (e.g. a region positioned between a proximal promoter on one side and the beginning of intron 1 on the other side) of the first exon of the gene. In certain ~~embodiment~~examples, at least three oligonucleotides may be used, one having a targeting portion targeting a region at or near the 5' end of the first exon (for example, a proximal promoter region), one having a targeting portion targeting a region at or near the 3' end of the first exon (for example, a beginning portion of intron 1) of the gene, and one having a targeting portion targeting a middle region (e.g. a region positioned between a proximal promoter on one side and the beginning of intron 1 on the other side) of the first exon of the gene, so as to simultaneously target both ends and a middle region of the demethylation core region. It is contemplated that where combinations of oligonucleotides are used, the different oligonucleotides may be for administration simultaneously, sequentially, or in combination. Typically, the oligonucleotides may be for administration such that they act simultaneously; however, it is also contemplated that in certain ~~embodiment~~examples different oligonucleotides or oligonucleotide combinations may be used at different time points or at different stages, for regulating gene activation.

In still another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the oligonucleotide may comprise the sequence:

(R_a)GUUUR_bAGAGCUA(R_c)UAGCAAGUUR_dAAAUAAGGCUAGUCCGUUAUCAACU U(R_e)AGUGGCACCGAGUCGGUGC(R_f)

(Formula I)

wherein

R_a comprises the targeting portion, and comprises about 20 to about 21 nucleotides in length;

R_b is A, G, or C, and R_d is the complementary base pair of R_b;

R_c comprises the R2 stem loop of DiR, comprising sequence CCCGGGACGCGGGUCCGGGACAG (SEQ ID NO: 7);

R_e comprises the R5 step loop of DiR, comprising sequence CUGAGGCCUUGGCGAGGCUUCU (SEQ ID NO: 8); and

R_f is optionally present, and comprises a poly U transcription termination sequence.

In still another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the oligonucleotide may comprise the sequence:

(R_a)GUUUGAGAGCUACCCGGGACGCGGGUCCGGGACAGUAGCAAGUUCAAAUA AGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCGAGGCUUCUAAGTGGCACC GAGUCGGUGCUUUUUU;

(Formula II)

wherein R_a comprises the targeting portion, and comprises about 20 to about 21 nucleotides in length.

In still another ~~embodiment~~example of any of the above oligonucleotide or oligonucleotides, the gene may be P16, and R_a may comprise:

GCUCCCCCGCCUGCCAGCAA (SEQ ID NO: 9);

GCUAACUGCCAAAUUGAAUCG (SEQ ID NO: 10);

GACCCUCUACCCACCUGGAU (SEQ ID NO: 11); or

GCCCCCAGGGCGUCGCCAGG (SEQ ID NO: 12).

In another ~~embodiment~~example, there is provided herein a plasmid or vector encoding any of the oligonucleotide or oligonucleotides described herein.

In another aspect, the present disclosure refer to a plasmid or vector encoding the oligonucleotide as disclosed herein.

In another ~~embodiment~~example, there is provided herein a composition comprising any of the oligonucleotide or oligonucleotides described herein and a dead Cas9 (dCas9).

In another aspect, the present disclosure refer to a composition comprising an oligonucleotide as disclosed herein and a dead Cas9 (dCas9).

In another ~~embodiment~~example, there is provided herein a composition comprising any one or more of:

an oligonucleotide as described herein;

a plasmid or vector as described herein;

a pharmaceutically acceptable carrier, excipient, diluent, or buffer;

a dead Cas9 (dCas9); or

an oligonucleotide, plasmid, or vector encoding a dead Cas9 (dCas9).

In another aspect, the present disclosure refer to a composition comprising any one or more of:

an oligonucleotide as disclosed herein; and

a plasmid or vector encoding the oligonucleotide as disclosed herein;

wherein the composition further comprises one or more of:

a pharmaceutically acceptable carrier, excipient, diluent, or buffer;

a dead Cas9 (dCas9); or

an oligonucleotide, plasmid, or vector encoding a dead Cas9 (dCas9).

In another ~~embodiment~~example of the above composition, the dCas9 may comprise D10A and H840A mutations.

In still another ~~embodiment~~example, there is provided herein a composition comprising any of the oligonucleotide or oligonucleotides described herein wherein the targeting portion targets a 5’ region of the first exon of a gene; and any of the oligonucleotide or oligonucleotides described herein wherein the targeting portion targets a 3’ region of the first exon of the gene.

In another ~~embodiment~~example, there is provided herein a composition comprising:

any of the oligonucleotide or oligonucleotides as described herein, wherein the targeting portion targets a region at or near the 5’ end of the first exon (for example, a proximal promoter region) of a gene; and

any of the oligonucleotide or oligonucleotides as described herein wherein the targeting portion targets a region at or near the 3’ end of the first exon (for example a beginning region of intron 1) of the gene; and

optionally, further comprising any of the oligonucleotide or oligonucleotides described herein,

wherein the targeting portion targets a middle region of the first exon of the gene;

preferably, wherein the composition comprises an oligonucleotide as described herein wherein the targeting portion targets a region at or near a proximal promoter region associated with the first exon; and an oligonucleotide as described herein wherein the targeting portion targets a region at or near the beginning of the first intron; and optionally further comprises an oligonucleotide as described herein wherein the targeting portion targets a middle region of the first exon of the gene.

In yet another ~~embodiment~~example there is provided herein a combination of any of the oligonucleotide or oligonucleotides described herein wherein the targeting portion targets a region at or near a 5' end of the first exon of a gene; and any of the oligonucleotide or oligonucleotides described herein wherein the targeting portion targets a region at or near a 3' end of the first exon of the gene.

In another ~~embodiment~~aspect, the present disclosure refer to ~~there is provided herein~~ a method for targeted demethylation and/or activation of a gene, said method comprising:

introducing a dead Cas9 (dCas9) and one or more oligonucleotides into a cell, the one or more oligonucleotides each comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR;

thereby demethylating and/or activating the gene by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene.

In another ~~embodiment~~example of the above method, the targeting portion of at least one of the one or more oligonucleotides may have sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both.

In still another ~~embodiment~~example of any of the above method or methods, the step of introducing comprises transfecting, delivering, or expressing the one or more oligonucleotides and the dCas9

in the cell.

In yet another ~~embodiment~~example of any of the above method or methods, the one or more oligonucleotides comprise any one or more of the oligonucleotide or oligonucleotides as described herein.

In still another ~~embodiment~~example of any of the above method or methods, at least two oligonucleotides may be introduced into the cell, wherein the targeting portion of a first oligonucleotide targets a 5' region of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a 3' region of the first exon of the gene.

In still another ~~embodiment~~example of any of the above method or methods, at least two oligonucleotides may be introduced into the cell, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a region at or near the 3' end of the first exon of the gene; preferably wherein the targeting portion of the first oligonucleotide targets a region at or near a proximal promoter region associated with the first exon and the targeting portion of the second oligonucleotide targets a region at or near the beginning of the first intron; optionally wherein a third oligonucleotide may be introduced into the cell, wherein the targeting portion of the third oligonucleotide targets a middle region of the first exon.

In another ~~embodiment~~example of any of the above method or methods, the cell may be exposed to the dCas9 and the one or more oligonucleotides for a period of at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, or at least about 8 days, or about 3 days to about a week.

In another ~~embodiment~~example, there is provided herein a use of any of the oligonucleotide or oligonucleotides, the plasmid or plasmids or vector or vectors, the composition or compositions, or the combination or combinations as described herein, for targeted demethylation and/or activation of a gene.

In another aspect, the present disclosure refer to use of the oligonucleotide as disclosed herein, for targeted demethylation and/or activation of a gene.

In another ~~embodiment~~example, there is provided herein a method for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof, said method comprising:

treating the subject with a dead Cas9 (dCas9) and one or more oligonucleotides, the one or more oligonucleotides each comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR;

thereby demethylating and/or activating the gene by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene, and treating the disease or disorder.

In another aspect, the present disclosure refer to use of a dead Cas9 (dCas9) and one or more oligonucleotides in the manufacture of a medicament for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof,

the one or more oligonucleotides each comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR;

wherein the gene is demethylated and/or activated by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene.

In another ~~embodiment~~example of the above method, the targeting portion of at least one of the one or more oligonucleotides may have sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both.

In still another ~~embodiment~~example of any of the above method or methods, the step of treating may comprise transfecting, delivering, or expressing the one or more oligonucleotides and the dCas9 in at least one cell of the subject.

In still another ~~embodiment~~example of any of the above method or methods, the one or more oligonucleotides may comprise one or more oligonucleotides as described herein.

In still another ~~embodiment~~example of any of the above method or methods, at least two oligonucleotides may be used, wherein the targeting portion of a first oligonucleotide targets a 5' region of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a 3' region of the first exon of the gene.

In still another ~~embodiment~~example of any of the above method or methods, at least two oligonucleotides may be used, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a region at or near a 3' end of the first exon of the gene; preferably wherein the targeting portion of the first oligonucleotide targets a region at or near a proximal promoter region associated with the first exon and the targeting portion of the second oligonucleotide targets a region at or near the beginning of the first intron; optionally wherein a third oligonucleotide is used, wherein the targeting portion of the third oligonucleotide targets a middle region of the first exon.

In yet another ~~embodiment~~example of any of the above method or methods, the subject may be exposed to

the dCas9 and the one or more oligonucleotides for a period of at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, or at least about 8 days, or about 3 days to about a week.

In another ~~embodiment~~example there is provided herein a use any of the oligonucleotide or oligonucleotides, the plasmid or plasmids or vector or vectors, the composition or compositions, or the combination or combinations as described herein, for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof.

In another aspect, the present disclosure refer to use of the oligonucleotide as disclosed herein in the manufacture of a medicament for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof.

In another ~~embodiment~~example of any of the above methods or uses, the targeting portion of at least one of the one or more oligonucleotides may target a site within or near a promoter region of the gene or within or near a demethylation core region of the gene, preferably wherein the targeting portion targets a region at or near a 5' end of the first exon or a region at or near a 3' end of the first exon of the gene.

In another ~~embodiment~~example of any of the above methods or uses, at least two oligonucleotides may be used, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a region at or near a 3' end of the first exon of the gene.

In another ~~embodiment~~example of any of the above methods or uses, the promoter region may be a CpG-rich region having at least some methylation.

In still another ~~embodiment~~example of any of the above methods or uses, the disease or disorder may comprise cancer.

In yet another ~~embodiment~~example of any of the above methods or uses, the gene may be a tumor suppressor gene.

In another ~~embodiment~~example of any of the above methods or uses, the targeting portion of at least one of the one or more oligonucleotides may target a site within or near a promoter region of the gene or within or near a demethylation core region of the gene, in particular wherein the targeting portion may target a region at or near a 5' end of the first exon or a region at or near a 3' end of the first exon of the gene, wherein the gene is a tumor suppressor gene.

In another ~~embodiment~~example of any of the above methods or uses, the promoter region may be a CpG-

rich region having at least some methylation.

In still another ~~embodiment~~example of any of the above methods or uses, the targeting portion of at least one of the one or more oligonucleotides may target the D1 or D3 region of the P16 gene.

In another ~~embodiment~~example of any of the above methods or uses, the one or more oligonucleotides may comprise at least one oligonucleotide with a targeting portion targeting the D1 region, and at least one oligonucleotide with a targeting portion targeting the D3 region, and optionally further comprising at least one oligonucleotide with a targeting portion targeting the D2 region.

In another ~~embodiment~~example of any of the above methods or uses, the one or more oligonucleotides may comprise one or more of:

G19sgR2R5 (SEQ ID NO: 1):

GCUCCCCCGCCUGCCAGCAAGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G36sgR2R5 (SEQ ID NO: 2):

GCUAACUGCCAAAUUGAAUCGGUUUGAGAGCUACCCGGGACGCGGGUCCGGG
ACAGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGG
CGAGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G110sgR2R5 (SEQ ID NO: 3):

GACCCUCUACCCACCUGGAUGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G111sgR2R5 (SEQ ID NO: 4):

GCCCCCAGGGCGUCGCCAGGGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G108sgR2R5 (SEQ ID NO: 5):

GUGGCCAGCCAGUCAGCCGAGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU; or

G122sgR2R5 (SEQ ID NO: 6):

GCCGCAGCCGCCGAGCGCACGGUUUGAGAGCUACCCGGGACGCGGGUCCGGGA
CAGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGC
GAGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

or any combinations thereof.

In another ~~embodiment~~example, there is provided herein a method for identifying one or more target sites for demethylation to activate expression of a gene in a cell, said method comprising:

treating the cell with a non-specific demethylation agent;

identifying one or more regions around the transcription start site of the gene which are most demethylated by treatment with the non-specific demethylating agent; and

using the identified one or more regions as target sites for demethylation to activate gene expression.

In another ~~embodiment~~example of the above method, the non-specific demethylation agent may comprise Decitabine (2'-deoxy-5-azacytidine).

In yet another ~~embodiment~~example of any of the above method or methods, the treatment with the non-specific demethylation agent may be for about 3 days.

In another ~~embodiment~~example of any of the above method or methods, the step of identifying the one or more regions around the transcription start site of the gene which are most demethylated by treatment with the non-specific demethylating agent may comprise performing Bisulfite Sanger-sequencing or whole genomic Bisulfite sequencing and, optionally, comparing results with a control untreated cell.

In another ~~embodiment~~example of any of the above method or methods, selection of the one or more regions around the transcription start site may favour selection of regions at or near the promoter, at or near the first exon of the gene, at or near a first intron of the gene, at or near a region at or

near a 5’ end of the first exon of the gene, at or near a region at or near a 3’ end of the first exon of the gene, at or near a CpG island, at or near another important regulatory region, or any combinations thereof.

In still another ~~embodiment~~example of any of the above method or methods, selection of the one or more regions around the transcription start site may favour selection of at least one region at or near a 5’ region of the first exon of the gene, and at least one region at or near a 3’ region of the first exon of the gene.

In another ~~embodiment~~example of any of the above method or methods, the method may further comprise performing targeted demethylation and gene activation using any of the method or methods described herein, wherein the targeting portions of the one or more oligonucleotides have sequence complementarity with the identified target sites for demethylation.

In another ~~embodiment~~example of any of the above method or methods, the one or more regions may be regions of the non-template strand.

BRIEF DESCRIPTION OF DRAWINGS

These and other features will become further understood with regard to the following description and accompanying drawings, wherein:

FIGURE 1 shows that CRISPR-R2R5 system induced moderate gene activation and demethylation by targeting promoter CpG island. In Figure 1(a), the structure of sgRNA (no DiR) and sgR2R5 (with DiR), the targeting site G2, and the transfection methods, are shown. In Figure 1(b) the p16 mRNA expression in each sample after 72 hours treatment is shown. In Figure 1(c), the MSP data showing the gene demethylation is shown. (Abbreviations - sgOri: Original sgRNA without DiR; sgR2R5: sgRNA fused with R2,R5 loops; G2: guide RNA; MSP: Methylation Specific PCR);

FIGURE 2 shows sequence and structural organization of typical single guide RNA (sgRNA);

FIGURE 3 shows results of CRISPR-DiR targeting P16 Region D1 and D3 simultaneously, with four guides targeting both strands in each region. Figure 3(a) shows the targeting strategy; Figure 3(b) shows the P16 mRNA expression profile; Figure 3(c) shows the P16 protein restoration profile; Figure 3(d) shows the methylation in Region D1 and D3 measured by COBRA; and Figure 3(e) shows the cell cycle analysis of the Day 53 treated samples;

WHAT IS CLAIMED IS:

1. An oligonucleotide comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within a gene, near a gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR.

2. The oligonucleotide of claim 1, wherein the targeting portion has sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both~~.~~;

~~3. The oligonucleotide of claim 1,~~ wherein optionally the R2 and R5 stem loops of DiR are from extra-coding CEBPA (ecCEBPA)~~.~~;

~~4. The oligonucleotide of claim 1,~~ wherein optionally the targeting portion targets a methylated region of the genomic DNA~~.~~;

~~5. The oligonucleotide of claim 1, wherein the targeting portion targets the genomic DNA region within or near a promoter region or within or near a demethylation core region of the gene; preferably wherein the targeting portion targets a region at or near the 5' end of the first exon and/or a region at or near the 3' end of the first exon and/or a middle region of the first exon of the gene; even more preferably wherein the targeting portion targets a region at or near a proximal promoter region associated with the first exon and/or a region at or near the beginning of the first intron and/or a middle region of the first exon of the gene.~~

~~6. The oligonucleotide of claim 1,~~ wherein optionally the oligonucleotide comprises the sequence:

$(R_a)GUUUR_bAGAGCUA(R_c)UAGCAAGUUR_dAAAUAAGGCUAGUCCGUUAUCAACU U(R_e)AGUGGCACCGAGUCGGUGC(R_f)$

(Formula I)

wherein

R_a comprises the targeting portion, and comprises about 20 to about 21 nucleotides in length;

R_b is A, G, or C, and R_d is the complementary base pair of R_b;

R_c comprises the R2 stem loop of DiR, comprising sequence CCCGGGACGCGGGUCCGGGACAG (SEQ ID NO: 7);

R_e comprises the R5 step loop of DiR, comprising sequence CUGAGGCCUUGGCGAGGCUUCU (SEQ ID NO: 8); and

R_f is optionally present, and comprises a poly U transcription termination sequence~~.~~;

~~7. The oligonucleotide of claim 6,~~ wherein optionally the oligonucleotide ~~comprising~~ comprises the sequence:

(R_a)GUUUGAGAGCUACCCGGGACGCGGGUCCGGGACAGUAGCAAGUUCAAAUAAG GCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCGAGGCUUCUAAGTGGCACCGAGU CGGUGCUUUUUU;

(Formula II)

wherein R_a comprises the targeting portion, and comprises about 20 to about 21 nucleotides in length~~.~~; and

~~8. The oligonucleotide of claim 6,~~ wherein optionally the gene is P16, and R_a comprises:

GCUCCCCCGCCUGCCAGCAA (SEQ ID NO: 9);

GCUAACUGCCAAAUUGAAUCG (SEQ ID NO: 10);

GACCCUCUACCCACCUGGAU (SEQ ID NO: 11); or

GCCCCCAGGGCGUCGCCAGG (SEQ ID NO: 12).

~~9.~~ 3. A plasmid or vector encoding the oligonucleotide of ~~any one of claims 1-8~~claim 1 or claim 2.

~~10.~~ 4. A composition comprising an oligonucleotide of claim 1 or claim 2~~any one of claims 1-8~~ and a dead Cas9 (dCas9).

~~11.~~ 5. A composition comprising any one or more of:

an oligonucleotide of claim 1 or claim 2~~any one of claims 1-8~~; and

a plasmid or vector encoding the oligonucleotide of claim 1 or claim 2~~any one of claims 1-8~~;

wherein the composition further comprises one or more of:

a pharmaceutically acceptable carrier, excipient, diluent, or buffer;

a dead Cas9 (dCas9); or

an oligonucleotide, plasmid, or vector encoding a dead Cas9 (dCas9).

~~12.~~ 6. The composition of claim ~~11~~5, wherein the dCas9 comprises D10A and H840A mutations.

~~13. A composition comprising:~~

~~an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near the 5' end of the first exon of a gene; and~~

~~an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near the 3' end of the first exon of the gene; and~~

~~optionally, further comprising an oligonucleotide of any one of claims 1-7, wherein the targeting portion targets a middle region of the first exon of the gene;~~

~~preferably, wherein the composition comprises an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near a proximal promoter region associated with the first exon; and an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near the beginning of the first intron; and optionally further comprises an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a middle region of the first exon of the gene.~~

~~14. A combination of an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near the 5' end of the first exon of a gene; and an oligonucleotide of any one of claims 1-7 wherein the targeting portion targets a region at or near a 3' end of the first exon of~~

~~the gene.~~

~~15.~~ 7. A method for targeted demethylation and/or activation of a gene, said method comprising:

introducing a dead Cas9 (dCas9) and one or more oligonucleotides into a cell, the one or more oligonucleotides each comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR;

thereby demethylating and/or activating the gene by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene.

~~16.~~ 8. The method of claim ~~15~~7, wherein the targeting portion of at least one of the one or more oligonucleotides has sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both~~.~~;

~~17. The method of claim 15,~~ wherein optionally the step of introducing comprises transfecting, delivering, or expressing the one or more oligonucleotides and the dCas9 in the cell~~.~~;

~~18. The method of claim 15,~~ wherein optionally the one or more oligonucleotides comprise

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within a gene, near a gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR~~.~~; and

~~19. The method of claim 15, wherein at least two oligonucleotides are introduced into the cell, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a~~

~~region at or near the 3' end of the first exon of the gene; preferably wherein the targeting portion of the first oligonucleotide targets a region at or near a proximal promoter region associated with the first exon and the targeting portion of the second oligonucleotide targets a region at or near the beginning of the first intron; optionally wherein a third oligonucleotide is introduced into the cell, wherein the targeting portion of the third oligonucleotide targets a middle region of the first exon.~~

~~20. The method of claim 15,~~ wherein optionally the cell is exposed to the dCas9 and the one or more oligonucleotides for a period of at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, or at least about 8 days, or about 3 days to about a week.

~~21.~~ 9. Use of the oligonucleotide of ~~any one of claims 1-8~~claim 1 or claim 2, for targeted demethylation and/or activation of a gene.

~~22.~~ 10. Use of a dead Cas9 (dCas9) and one or more oligonucleotides ~~A method~~ in the manufacture of a medicament for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof, ~~said method comprising:~~

~~treating the subject with a dead Cas9 (dCas9) and one or more oligonucleotides,~~ the one or more oligonucleotides each comprising:

a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR;

~~thereby~~ wherein the gene is ~~demethylating~~ demethylated and/or ~~activating~~ activated~~the gene~~ by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene~~, and treating the disease or disorder~~.

~~23.~~ 11. The ~~method~~ use of claim ~~22~~10, wherein the targeting portion of at least one of the one or more oligonucleotides has sequence complementarity and binding affinity with a non-template strand of the genomic DNA within the gene, near the gene, or both~~.~~;

~~24. The method of claim 22,~~ wherein optionally ~~the step of treating comprises transfecting, delivering, or expressing~~ the one or more oligonucleotides and the dCas9 are to be transfected, delivered, or expressed in at least one cell of the subject~~.~~;

~~25. The method of claim 22,~~ wherein optionally the one or more oligonucleotides comprise: a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within a gene, near a gene, or both; and

a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR~~.~~;

~~26. The method of claim 22, wherein at least two oligonucleotides are used, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a region at or near a 3' end of the first exon of the gene; preferably wherein the targeting portion of the first oligonucleotide targets a region at or near a proximal promoter region associated with the first exon and the targeting portion of the second oligonucleotide targets a region at or near the beginning of the first intron; optionally wherein a third oligonucleotide is used, wherein the targeting portion of the third oligonucleotide targets a middle region of the first exon.~~

~~27. The method of claim 22,~~ wherein optionally the subject is to be exposed to the dCas9 and the one or more oligonucleotides for a period of at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, or at least about 8 days, or about 3 days to about a week~~.~~;

~~28. Use of the oligonucleotide of any one of claims 1-8, for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof.~~

~~29. The method of claim 22, wherein the targeting portion of at least one of the one or more oligonucleotides targets a site within or near a promoter region of the gene or within or near a demethylation core region of the gene, preferably wherein the targeting portion targets a region at or near a 5' end of the first exon or a region at or near a 3' end of the first exon of the gene, or a middle region of the first exon of the gene.~~

~~30. The method of claim 29, wherein at least two oligonucleotides are used, wherein the targeting portion of a first oligonucleotide targets a region at or near a 5' end of the first exon of the gene; and wherein the targeting portion of a second oligonucleotide targets a region at or near a 3' end of the first exon of the gene.~~

~~31. The method of claim 29,~~ wherein optionally the promoter region is a CpG-rich region having at least some methylation~~.~~;

~~32. The method of claim 22,~~ wherein optionally the disease or disorder comprises cancer~~.~~;

~~33. The method of claim 32,~~ wherein optionally the gene is a tumor suppressor gene~~.~~;

~~34. The method of claim 32, wherein the targeting portion of at least one of the one or more oligonucleotides targets a site within or near a promoter region of the gene or within or near a demethylation core region of the gene, preferably wherein the targeting portion targets a region at or near a 5' end of the first exon or a region at or near a 3' end of the first exon of the gene, wherein the gene is a tumor suppressor gene.~~

~~35. The method of claim 34, wherein the promoter region is a CpG-rich region having at least some methylation.~~

~~36. The method of claim 32,~~ wherein optionally the targeting portion of at least one of the one or more oligonucleotides targets the promoter-exon1-intron1 ~~D1 or D3~~ region of the P16 gene~~.~~; and

~~37. The method of claim 36, wherein the one or more oligonucleotides comprise at least one oligonucleotide with a targeting portion targeting the D1 region, and at least one oligonucleotide with a targeting portion targeting the D3 region, and optionally further comprising at least one oligonucleotide with a targeting portion targeting the D2 region.~~

~~38. The method of claim 22,~~ wherein optionally the one or more oligonucleotides comprise one or more of:

G19sgR2R5 (SEQ ID NO: 1):

GCUCCCCCGCCUGCCAGCAAGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G36sgR2R5 (SEQ ID NO: 2):

GCUAACUGCCAAAUUGAAUCGGUUUGAGAGCUACCCGGGACGCGGGUCCGGG
ACAGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGG
CGAGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G110sgR2R5 (SEQ ID NO: 3):

GACCCUCUACCCACCUGGAUGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G111sgR2R5 (SEQ ID NO: 4):

GCCCCCAGGGCGUCGCCAGGGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

G108sgR2R5 (SEQ ID NO: 5):

GUGGCCAGCCAGUCAGCCGAGUUUGAGAGCUACCCGGGACGCGGGUCCGGGAC
AGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGCG
AGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU; or

G122sgR2R5 (SEQ ID NO: 6):

GCCGCAGCCGCCGAGCGCACGGUUUGAGAGCUACCCGGGACGCGGGUCCGGGA
CAGUAGCAAGUUCAAAUAAGGCUAGUCCGUUAUCAACUUCUGAGGCCUUGGC
GAGGCUUCUAAGUGGCACCGAGUCGGUGCUUUUUU;

or any combinations thereof.

12. Use of the oligonucleotide of claim 1 or claim 2 in the manufacture of a medicament for treating a disease or disorder associated with decreased expression of at least one gene due to aberrant DNA methylation in a subject in need thereof.

~~39. A method for identifying one or more target sites for demethylation to activate expression of a gene in a cell, said method comprising:~~

~~treating the cell with a non-specific demethylation agent;~~

~~identifying one or more regions around the transcription start site of the gene which are most demethylated by treatment with the non-specific demethylating agent; and~~

~~using the identified one or more regions as target sites for demethylation to activate gene expression.~~

~~40. The method of claim 39, wherein the non-specific demethylation agent comprises Decitabine (2'-deoxy-5-azacytidine).~~

~~41. The method of claim 39, wherein the treatment with the non-specific demethylation agent is for about 3 days.~~

~~42. The method of claim 39, wherein the step of identifying the one or more regions around the transcription start site of the gene which are most demethylated by treatment with the non-specific demethylating agent comprises performing Bisulfite Sanger-sequencing or whole genomic Bisulfite sequencing and, optionally, comparing results with a control untreated cell.~~

~~43. The method of claim 39, wherein selection of the one or more regions around the transcription start site favours selection of regions at or near the promoter, at or near the first exon of the gene, at or near a first intron of the gene, at or near a 5' region of the first exon of the gene, at or near a 3' region of the first exon of the gene, at or near a CpG island, at or near another important regulatory region, or any combinations thereof.~~

~~44. The method of claim 43, wherein selection of the one or more regions around the transcription start site favours selection of at least one region at or near a region at or near a 5' end of the first exon of the gene, and at least one region at or near a region at or near a 3' end of the first exon of the gene.~~

~~45. The method of claim 39, wherein the method further comprises: introducing a dead Cas9 (dCas9) and one or more oligonucleotides into the cell, the one or more oligonucleotides each comprising: a targeting portion having sequence complementarity and binding affinity with a region of genomic DNA within the gene, near the gene, or both; and a single guide RNA (sgRNA) scaffold portion, wherein a tetra-loop portion of the sgRNA is modified and comprises an R2 stem loop of DNMT1-interacting RNA (DiR), and wherein a stem loop 2 portion of the sgRNA is modified and comprises an R5 step loop of DiR; thereby demethylating and/or~~

~~activating the gene by inhibiting DNA methyltransferase 1 (DNMT1) activity on the gene; wherein the targeting portions of the one or more oligonucleotides have sequence complementarity with the identified target sites for demethylation.~~

~~46. The method of claim 39, wherein the one or more regions are regions of the non-template strand.~~