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Efficient targeted DNA methylation with chimeric dCas9–Dnmt3a–Dnmt3L methyltransferase

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21, 201 [illegible] 23, 201 [illegible] 2 , 201 [illegible] , 201

ABSTRACT

DNA methylation plays a critical role in the regulation and maintenance of cell-type specific transcriptional programs. Targeted epigenome editing is an emerging technology to specifically regulate cellular gene expression in order to modulate cell phenotypes or

rial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (13) system and the nuclease-inactivated CRISPR variant (dCas9) as a programmable genome targeting technology has opened new possibilities to target epigenetic effectors (14–16).

In this work, we show that targeting of an engineered Dnmt3a–Dnmt3L single-chain DNA methyltransferase (Dnmt3a3L) fused to the nuclease-inactivated dCas9 leads to ef cient and widespread DNA methylation of CpG islands located within targeted promoters (up to 1200 bp). Targeted methylation with the Dnmt3a3L fusion protein is □4–5 times stronger than the methylation achieved through targeting of Dnmt3a alone. Peaks of methylation are observed around 25 bp upstream and 40 bp downstream of the PAM site, while 20–30 bp of the dCas9 binding site itself are protected against methylation. In general, targeting with single guide RNAs is suf cient for methylation and multiplexing does not increase its ef ciency. In addition, using this system we illustrate that the multimerization of the Dnmt3a3L protein on DNA contributes to introduction and spreading of DNA methylation within the targeted genomic region.

MATERIALS AND METHODS

Gene a ion of Ca9-Dnm 3a Dnm 3L SC f ion con c

The M-SPn-Cas9-VP64 plasmid (Addgene plasmid #48674) (17) was used as the base for the introduction of the Dnmt3a–Dnmt3L single-chain construct (18). The vector backbone was ampli ed using vector speci c primers listed in Supplementary Table S4. The murine Dnmt3a catalytic domain was ampli ed from ZNF-Dnmt3a CD plasmid and Dnmt3a CD fused to the Dnmt3L C-terminal domain from the ZNF-Dnmt3a3L plasmids (18) with D3a_Cas9_f, D3a_Cas9_r and D3a3L_Cas9_f, D3a3L_Cas9_r primers using Q5® High-Fidelity DNA Polymerase (NEB). The insert and the vector backbone were joined using Gibson assembly (NEB). Proper sequence of the resulting clones was con rmed with Sanger sequencing. The plasmid maps are depicted in Supplementary Figure S2B).

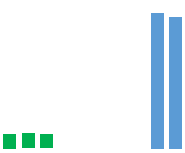
De ign of he g ide RNA fo he EPCAM, CXCR4 and TFRC omo e

The sequences of the promoter regions (containing the CpG islands) of human EpCAM, CXCR4 and TFRC genes were extracted using UCSC genome browser. Potential guide RNA targeting sequences speci c for these promoters were identi ed using the E-CRISP server (19). For EpCAM, a selection of twelve unique guide RNA binding sites was chosen to cover the region with 100–200 bp in between the gRNAs (shown in Supplementary Table S3). For CXCR4 and TFRC, four and ten gRNAs locations were selected, respectively. Each of the guide RNA constructs was generated as a separate gRNA plasmid. The gRNA plasmids were synthesized using overlapping ssDNA oligonucleotides which were cloned into the empty gRNA plasmid (Addgene plasmid # 41824) (20) using Gibson Assembly® Master Mix (NEB) following the manufacturer protocol. All gRNA constructs were validated with Sanger sequencing. Potential off-target binding sites for the gRNAs were predicted using CRISPOR (http://crispor.tefor.net/crispor.py). Four highest scoring off-target sites were selected based on the MIT or CFD scores and primers for bisul te sequencing were designed directly surrounding these regions (primer sequences are provided in Supplementary Table S2).

Cell c l e, an fec ion , MACS elec ion

The human ovary adenocarcinoma cell line, SKOV-3 (a generous gift of Dr Marianne Rots) and HEK293 cells were maintained at 37°C with 5% CO_2 in DMEM media supplemented with 10% FCS (Sigma-Aldrich), 1× penicillin/streptomycin and 8 mM glutamine. For SKOV-3 cells transfection, the cells were trypsinized and seeded in six-well plates or T25 asks at 40% density. The following day, the cells were immersed in the transfection cocktail composed of 5% of a modi ed pVenus-NLS plasmid (derived from Addgene #27794 plasmid) (21), 5% of the pLNGFR plasmid (Miltenyi Biotec), 70–88% of individual or equimolar mixture of pooled gRNA plasmids and 2–20% of SPn-Cas9-Dnmt3a3L-SC plasmid (% of total DNA, 2 or 4 μg for six wells or T25s, respectively) containing 6 or 12 μg PEI MAX MW 40 000 (Polysciences). The cells were washed with Dulbecco's PBS and immersed in normal cell media 14 h post-transfection. Subsequently, the medium was exchanged every two days. Magnetic activated

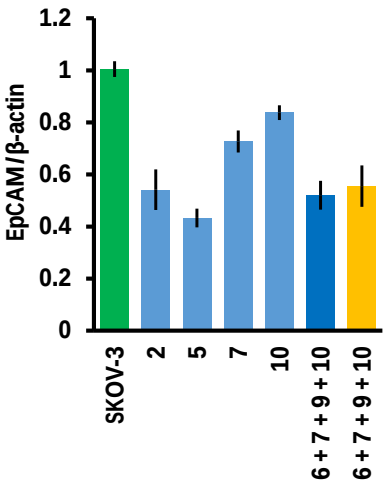
mixed in an equimolar ratio, end repaired and A-tailed using the components of the SureSelect (Agilent Technolo-

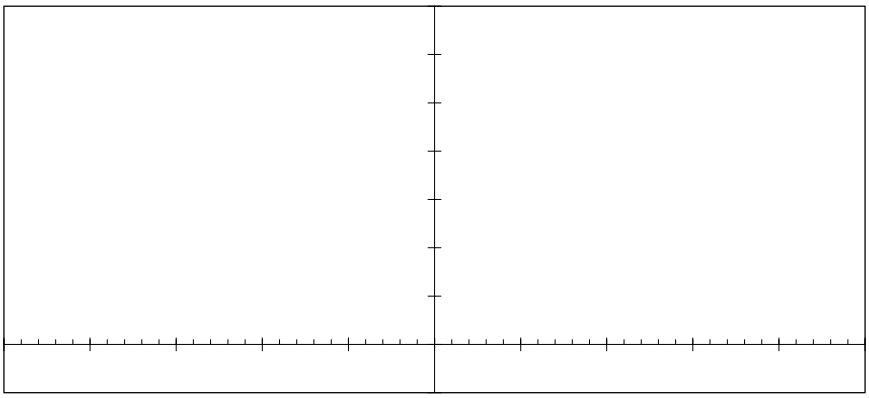


respectively. gRNA^{CXCR4} 4, which binds to a site □370 bp upstream of the bisul te amplicons, caused introduction of 13.3 ± 1.5% methylation within the analyzed amplicons. In the EpCAM promoter, targeting the dCas9–Dnmt3a3L construct with the individual gRNAs^{EpCAM} 1–3 or 5–10 triggered methylation between 25% (for gRNA^{EpCAM} 9) to 33.7% (for gRNA^{EpCAM} 2). gRNA^{EpCAM} 11 and 12, which are targeting the boundary of the CpG island, caused only weak methylation, comparable to the methylation increase observed with untargeted dCas9–Dnmt3a3L. Interestingly, targeted methylation with gRNA^{EpCAM} 4 in the EpCAM promoter was less ef cient than with other neighboring gRNAs, which might be explained by the fact that the gRNA binding site overlaps with the annotated TSS, where the transcriptional machinery could prevent stable binding of the dCas9 fusion.

In summary, we conclude that single gRNAs co-transfected with dCas9–Dnmt3a3L can cause robust DNA methylation. Strikingly, although the deposited methylation was most prominent on the 3□ side next to the dCas9 targeted sites, where based on modeling the fusion partner is presented (reaching up to 60.5 ± 1% for gRNA^{TFRC} 3 or 49.1 ± 2.7% for gRNA^{CXCR4} 2), the DNA methylation signal was propagated over the entire promoter in most cases (Figures 1B, C, 2A and Supplementary Figures S3, S5 and S9).

Our data show that the ef ciency of dCas9 mediated targeted methylation is comparable to previous targeting systems. For example, targeting the EpCAM promoter in SKOV-3 cells Nunna 2*2.7%Tf -0.0459r32*8*.7% .3147343.5 (gR303%-1,13.6656g)nor13.6654000-g..6634.4 (om615.3nE*


1.2
1
0.8
0.6
0.4
0.2
0
EpCAM/β-actin
SKOV-3
2
5
7
10
6 + 7 + 9 + 10
6 + 7 + 9 + 10



which raises the probability that a transient DNA encounter leads to the formation of a stable Dnmt3a3L–DNA complex followed by DNA methylation. Notably, formation of a long Dnmt3a3L ber that would cover the whole CpG island (□1000 bp) is rather unlikely, as this would cause a larger distortion of the local chromatin structure, including displacement of nucleosomes. However, we envision a dynamic formation of shorter multimers that would cause local methylation of the bound DNA. Methylation of remote regions within the locus could be achieved through DNA looping and subsequent multimer formation and methylation at the distant site. As the propensity of a diffusional encounter of the tethered enzyme and DNA declines with the distance of the DNA site to the targeted region, ef cient binding of the tethered MTase to remote DNA sites is even more dependent on the ability of Dnmt3a to multimerize on DNA.

We show here the targeted methylation of larger DNA regions by dCas–Dnmt3a3L. This approach mimics establishment of natural DNA methylation patterns and it increases the probability of causing a strong biological response (as for example changes in gene expression as shown here). Additionally, we observed that the CpG sites that are covered by the bound dCas9 protein are almost completely protected from DNA methylation, illustrating that inactivated Cas9 stays rmly bound to its target site. This explains why dCas9 alone can be used to out-compete and displace DNA binding proteins (such as transcription factors) to directly affect transcription, as observed by others (24). In a more general view, it suggests that targeting of dCas9 to sites bound by other protein factors can be applied in order to investigate their functions at a speci c genomic site. The pattern of the targeted DNA methylation suggests that Dnmt3a preferentially methylates linker DNA, which is in agreement with earlier *in vitro* data and cellular methylation studies (35–39).

Targeting of our epigenetic editing tools based on DNA methyltransferase domains caused down-regulation of gene expression at three different promoters tested, indicating that it could be used as a universal tool for gene repression. Alternative tools, like CRISPRi or based on the targeted recruitment of transcriptional repressors (like for example KRAB domain) have been developed and successfully used to down-regulate gene expression as well (14,24,29). The possible advantage of targeted methylation is that it has the potential of causing durable effects. Future work will show which of these techniques nally will prove most useful for targeted gene regulation.

During preparation of this manuscript, dCas9 targeted DNA methylation was described by two other groups using

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