Engineering of AAV-Mediated *in Vivo* Targeted DNA Methylation Editing System via *Staphylococcus Aureus* Derived Cas9-SunTag

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Abstract

DNA methylation aberrations have been implicated in the pathogenesis of various psychiatric and developmental disease states. However, basic research and therapeutic *in vivo* applications targeting aberrant DNA methylation are still limited due to the lack of targeted DNA methylation editing systems that work on capacity-limited adeno-associated virus (AAV), a highly versatile *in vivo* gene delivery vector. Here, we report the engineering of AAV-based vector system for *in vivo* targeted DNA methylation/demethylation by integrating small Cas9 orthologues (SaCas9) and a dCas9-SunTag methodology. In this system, dSaCas9-SunTag targeting protein and anti-SunTag-mini-antibody (scFv) fused effector protein were packaged into different vectors for efficient *in vivo* delivery by AAV. We demonstrated that co-introduction of AAV vectors carrying dSaCas9-SunTag and scFv-fused Dnmt3A, Dnmt3L, or Dnmt3A-Dnmt3L fusion proteins induced robust DNA methylation on the *Bdnf* gene locus in a guide RNA dependent manner *in vitro*. The scFv-fused TET1 and TET2 successfully induced robust targeted DNA demethylation in the H19 ICR. We also constructed a doxycycline (Dox)-inducible target DNA methylation system utilizing dSaCas9-SunTag and scFv-Dnmt3AL. Finally, we showed that AAV-mediated delivery of dSaCas9-SunTag system induced DNA methylation on the *Bdnf* gene locus and suppressed activity-dependent but not basal *Bdnf* expression after contextual fear learning in the Hippocampus of adult mouse. Our *in vivo* targeted DNA methylation editing system can be a promising platform for understating the causal relationship between psychiatric disease and aberrant DNA modifications.

Introduction

Aberrant DNA methylation has been widely observed in various psychiatric diseases, such as schizophrenia and major depression [1, 2, 3, 4, 5, 6]. A growing body of research has been aimed at understating the biological function of the local epigenetic status via targeted manipulation of local epigenetic status [7, 8, 9, 10, 11]. Many groups have reported successful attempts of targeted manipulation of local DNA methylation status, called targeted DNA methylation editing, using a fusion protein containing a DNA targeting module and a catalytic domain of DNA methylation modifier [9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. Using targeted DNA methylation editing methodology, the causal relationship between aberrant DNA methylation and abnormal expression disease-related gene was examined *in vitro* [16, 18, 19, 21, 24, 25]. A few studies have reported successful *in vivo* targeted DNA methylation editing by using lentivirus vectors [17, 18], whereas adeno-associated virus (AAV)-based targeted DNA methylation has not yet been reported.

AAV-based targeted DNA methylation editing offers several advantages over a lentivirus-based methodology for *in vivo* application. First, AAV vectors possess higher affinity for neuronal tissue and can induce transgene expression in a broader region [26]. Second, AAV vectors have lower pathogenicity than lentivirus vectors, as AAVs require another helper virus to mediate their replication [27, 28]. Third, lentivirus vectors are randomly integrated into the host cell genome, whereas AAV vectors minimize this possibility, which can in turn reduce the possibility of insertional effects [28, 29]. For these reasons, there are many

on-going clinical trials using AAV targeting Parkinson's disease, Alzheimer's disease, and Epilepsy [30]. Nevertheless, AAV-based targeted DNA methylation editing is hampered by the restrictive cargo size of AAV (4.7 kb of transgene sequence) and bigger size of a fusion protein with catalytically inactive *Streptococcus pyogenes* derived Cas9 (dCas9, 4.2 kb) [31], widely used targeting module, and effector DNA methylation domain (about 1-2 kb) [11, 18, 19, 20, 21, 22, 23].

Aiming to develop AAV-based targeted DNA methylation editing systems, we focused a new targeted methylation editing system using dCas9 fused with a GCN4 peptides repeat sequence (SunTag) [17]. In the dCas9-SunTag system, multiple copies of anti-GCN4 antibody-fused effector domain were recruited to dCas9-SunTag protein on target gene locus [17, 32, 33]. By recruiting multiple copies of effector proteins, dCas9-SunTag system exhibits a much higher induction rate of DNA methylation/demethylation at target sites with greater specificity than other methylation editing systems that recruit only one dCas9-effector fusion protein to the target gene locus [17, 33]. The dCas9-SunTag system offers another advantage for AAV-based targeted methylation editing; unlike a dCas9-effector direct fusion protein, it is easy for the dCas9-SunTag system to be split into smaller units, reducing the size of the transgene payloads within the AAV vectors. In this study, we employed smaller dCas9 orthologues derived from *Staphylococcus aureus* (dSaCas9) as a targeting module [31]. Comparing previously used *Streptococcus pyogenes* derived dCas9 (4.2kb), the smaller dSaCas9 (3.1kb) can maximize the number of fused SunTag units within the loading limit of AAVs (4.7 kb) and amplify the catalytic activity on the target locus by recruiting more copies of the antibody-fused effector protein. Furthermore, given the flexible vector design space of this splitting vector system, we examined the efficacy of various sizes of effector domains mediating DNA methylation or demethylation.

We report here the development of AAV vector tool kits for targeted methylation editing via a combination of dSaCas9-SunTag and methylation/demethylation domain [17, 24, 32]. We separately induced vectors expressing dSaCas9 labeled with three SunTag peptides (dSaCas9-SunTag) and a catalytic domain of de novo DNA methyltransferase 3a (Dnmt3ACD) or the C-terminal domain of DNA methyltransferase 3-like (Dnmt3LCD) or a fusion protein with Dnmt3ACD and Dnmt3LCD (Dnmt3AL) fused to a mini-antibody specific for SunTag (scFv). Co-induction of dSaCas9-SunTag and each scFv-Dnmt3ACD or scFv-Dnmt3LCD or scFv-Dnmt3AL could promote DNA methylation on the targeted *Bdnf* locus in a guide RNA dependent manner. Specifically, scFv-Dnmt3AL showed highest methylation efficiency. We also developed a targeted demethylation system using the catalytic domain of TET1 or TET2 (scFv-TET1CD, scFv-TET2CD). Both scFv-TET1CD and scFv-TET2CD induced robust DNA demethylation at the target locus. Furthermore, we showed that Dox-induced temporal expression of dSaCas9-SunTag and scFv-Dnmt3AL could induce DNA methylation at the *Bdnf* gene locus. Finally, we demonstrated that intracranial injection of an AAV cocktail loading a targeted DNA methylation system to the mouse dorsal hippocampus (HPC) suppressed activity-dependent expression of *Bdnf* but not basal expression in adult mouse HPC.

Results

We generated a dSaCas9-SunTag mediated targeted DNA methylation system mediated by multiple AAV vectors (Fig. 1a-d). The first AAV vector contained dSaCas9 fused with SunTag_{3x} which consisted of three tandem arrays of GCN4 peptides (dSaCas9-SunTag_{3x}, SCP3) under the control of the cytomegalovirus (CMV) promoter (Fig. 1a). We employed the optimized length (22 amino acids) for the linker separating each GCN4 peptide unit in the array [17], and optimized locations of the nuclear localization signal (NLS) for improved efficiency (Fig. 1a) [18]. The second AAV vector contained sgRNA under the control of the human U6 promoter, reverse tetracycline-controlled transactivator (rtTA), and red fluorescent tdTomato connected with a self-cleavable peptide (P2A) (Fig. 1b). A third AAV vector contained anti-GCN4 minimum antibody fused with either the catalytic domain of mouse Dnmt3a (scFv-Dnmt3ACD) or the C-terminal domain of Dnmt3l (scFv-Dnmt3LCD) or Dnmt3ACD-Dnmt3LCD fusion proteins (scFv-Dnmt3AL) under the control of the CMV promoter (Fig. 1c). Considering previous reports about the *in vitro* targeted DNA methylation system using Dnmt3ACD [13, 33, 34], we prepared scFv-Dnmt3ACD. The C-terminal domain of Dnmt3l is enzymatically inactive but required for the de novo DNA methylation of imprinted genes via interaction with dimerized Dnmt3l and Dnmt3a [35, 36]. Because enzymatically inactive Dnmt3LCD might reduce potential off-target effects, we also prepared scFv-Dnmt3LCD. Based on a previously reported *in vitro* targeted DNA methylation system using the Dnmt3ACD-Dnmt3LCD fusion proteins [34], we generated an AAV vector expressing scFv-Dnmt3AL. Because the Dnmt3ACD-Dnmt3LCD fusion protein shows about 10-fold higher de novo DNA methylation activity than Dnmt3ACD *in vitro* [15], scFv-Dnmt3AL was expected to have highest DNA methylation efficiency. These systems consisting of dSaCas9-SunTag_{3x} and scFv-Dnmt3ACD or scFv-Dnmt3LCD or scFv-Dnmt3AL, were termed SCP3-A, SCP3-L and SCP3-AL, respectively. We designed three sgRNAs targeting CpGs around the promoter of *Bdnf* exon 4 to determine whether our system can induce DNA methylation on this promoter (Fig. 1e). This region is one of the more well-known key disease-related genomic loci involved in neural activity-dependent transcriptional regulation of *Bdnf* genes via their local DNA methylation profile [18]. Forty-eight hours after transfection of the three sgRNAs and the SCP3-A, SCP3-L or SCP3-AL into N2A cells, tdTomato-expressing cells were selected by fluorescence-activated cell sorting (FACS) and cultured for another 4 days (Fig. 1f). DNA and RNA were extracted for quantitative bisulfite pyrosequencing and quantitative PCR (qPCR) 6 days after transfection, respectively. These cells expressed dSaCas9 and scFv-fused effector genes (Fig. 1g). Around 20% of CpGs at the promoter of *Bdnf* exon 4 were methylated in untreated negative control samples (WT) (Fig. 1h right panel). In contrast, 38.4% and 44.5 % of CpGs were methylated by SCP3-A and SCP3-L in the presence of targeting sgRNA mix but not the sgRNA control, respectively (Fig. 1h, i right panel; SCP3-A: control-sgRNA vs. *Bdnf*-sgRNA, **p < 0.01, SCP3-L: control-sgRNA vs. *Bdnf*-sgRNA, ****p < 0.0001 One-way ANOVA with post-hoc Tukey's multiple comparisons test). SCP3-L showed robust methylation than SCP3-A on almost all examined CpG sites, except for one CpG site located -108 bp upstream from TSS (Fig. 1h, i left panel). Beside a similar amount of the scFv-fused effector protein was expressed in the control and target sgRNA groups, off-target DNA methylation activities derived from over-expressed scFv-Dnmt3ADC or scFv-Dnmt3LDC were not observed (Fig. 1h, i right panel; WT vs. SCP3-A & control-sgRNA or SCP3-L & control-sgRNA, not significant; One-way ANOVA with post-hoc Tukey's multiple comparisons test). Comparing to the DNA methylation levels induced by SCP3-A and SCP3-L, SCP3-AL achieved more efficient methylation on all examined CpG sites (Fig. 1j left

panel), and higher average methylation level (65.6 %) without off-target DNA methylation activity derived from over-expressed scFv-Dnmt3AL (Fig. 1j right panel; SCP3-AL & control-sgRNA vs. SCP3-AL & Bdnf-sgRNA, SCP3-A & Bdnf-sgRNA vs. SCP3-AL & Bdnf-sgRNA, SCP3-L & Bdnf-sgRNA vs. SCP3-AL & Bdnf-sgRNA **** $p < 0.0001$; One-way ANOVA with post-hoc Tukey's multiple comparisons test). Because basal expression of *Bdnf* was quite low in N2A cells (Data not shown), we did not examine effects of targeted methylation editing on the *Bdnf* gene. From these results, we concluded that targeted methylation could be achieved using a novel combination of dSaCas9 and SunTag system, and the SCP3-AL system possesses higher DNA methylation efficiency than either SCP3-A and SCP3-L.

We also engineered an AAV-mediated targeted demethylation system. In previous reports, targeted DNA demethylation was achieved by a combination of dCas9-SunTag and the catalytic domain of human TET1 DNA demethylation catalyzing enzyme fused with anti-GCN4 minimum antibody (scFv-TET1CD) [17, 24]. In addition to scFv-TET1CD, we prepared a smaller effector domain, the catalytic domain of the human TET2 enzyme fused with scFv (scFv-TET2CD) (Fig. 2a). Referring to the crystal structure data of human TET2 [37], we cloned the minimum catalytic domains of human TET2 and connected them with a 15 bp linker sequence. The resulting scFv-TET2CD was 774 bp smaller than scFv-TET1CD. These targeted demethylation systems consisting of dSaCas9-SunTag_{3x} and scFv-TET1CD or scFv-TET2CD were termed SCP3-TET1 and SCP3-TET2, respectively. To assess the DNA demethylation efficiencies of SCP3-TET1 and SCP3-TET2 *in vitro*, we designed five sgRNAs targeting the methylation-sensitive CTCF-binding sites harboring the H19 ICR locus (Fig. 2b). DNA and RNA were extracted from FACS-sorted cells 6 days after transfection of AAV constructs expressing the five sgRNAs targeting the H19 ICR locus and either SCP3-TET1 or SCP3-TET2 (Fig. 2c). The dSaCas9 gene and effector genes were expressed in sorted cells (Fig. 2f and g). The CpGs of the targeted CTCF-binding site was highly methylated in untreated cells (81.8%, Fig. 2d, e right panel). Both SCP3-TET1 and SCP3-TET2 induced CpG demethylation on the targeted CTCF-binding site in an sgRNA-dependent manner (Fig. 2d and e right panel; SCP-TET1: control-sgRNA vs. H19-sgRNA, ** $p < 0.01$, SCP3-TET2: control-sgRNA vs. H19-sgRNA, **** $p < 0.0001$; WT vs. control-sgRNA, not significant; One-way ANOVA with post-hoc Tukey's multiple comparisons test). Off-target demethylation on the target site via overexpressing of scFv-TET1 or scFv-TET2 gene was not observed on all examined CpG sites (Fig. 2d, e left panel; WT vs. control-sgRNA, not significant; Two-way ANOVA with post-hoc Tukey's multiple comparisons test). In addition, we observed significant upregulation of the *H19* gene by SCP-TET2 (Fig. 2h and i, SCP-TET2: control-sgRNA vs. H19-sgRNA, * $p < 0.05$, t-test). From these results, we concluded that the targeted DNA demethylation was also achieved by our dSaCas9-SunTag system.

As mentioned above, one of the therapeutic merits of targeted DNA manipulation technology is a long-lasting effect on transcriptional regulation even after expression of the effector genes has ceased [21, 38]. From this point of view, we applied our targeted methylation system to a doxycycline (Dox)-inducible gene expression system. We generated dox-inducible AAV plasmids carrying dSaCas9-SunTag_{3x} or scFv-Dnmt3AL, termed treSCP3-AL (Fig. 3a-c). We examined whether the treSCP3-AL system could induce targeted methylation *in vitro* on the promoter of *Bdnf* exon 4 after Dox-treatments. The same sgRNA set

targeting *Bdnf* exon 4 was used (Fig. 3d). The tdTomato-expressing cells were sorted by FACS forty-eight hours after transfection of the sgRNAs and the components of treSCP3-AL into N2A cells. DNA and RNA were extracted after 4 days following culture in presence of Dox (Fig. 3e). Beside slight off-target methylation observed on a CpG site (Fig. 3f left panel; -65 bp from TSS: WT vs. control-sgRNA * $p < 0.05$; Two-way ANOVA with post-hoc Tukey's multiple comparisons test), on average the treSCP3-AL system induced a significant increase of methylation on the target locus in sgRNA dependent manner (Fig. 3f right panel; WT vs. *Bdnf*-sgRNA, control-sgRNA vs. *Bdnf*-sgRNA, **** $p < 0.0001$; WT vs. control-sgRNA, not significant; One-way ANOVA with post-hoc Tukey's multiple comparisons test). The methylation efficiency of dox-induced treSCP3-AL was comparable to that of SCP3-AL in which dSaCas9-SunTag_{3x} and scFV-Dnmt3AL are controlled by CMV promoter (Fig. 3f right panel, treSCP3-AL & *Bdnf*-sgRNA vs. SCP3-AL & *Bdnf*-sgRNA, not significant; One-way ANOVA with post-hoc Tukey's multiple comparisons test).

Lastly, we investigated whether our targeted methylation system can be applied *in vivo*. For this purpose, we generated AAVs carrying the treSCP3-AL system showed in Fig. 3 and injected the AAV cocktails into the dorsal HPC of adult mice. We used the same sgRNA set targeting *Bdnf* exon 4 that was used for the *in vitro* studies (Fig. 3d). Expression of dSaCas9 and scFv-Dnmt3AL was induced by administration of Dox in drinking water for 4 days, followed by 7 days of rest (Fig. 4a). These animals were sacrificed for subsequent immuno-histochemical (IHC) and quantitative DNA methylation analysis (Fig. 4a). Expression of myc-tagged scFv-Dnmt3AL and tdTomato signal derived from sgRNA-expressing AAV was observed via IHC analyses of dox-administered dorsal HPC (Fig. 4b). We also confirmed strong expression of dSaCas9 and scFV by qPCR analysis (Fig. 4c). These data clearly indicated that the components of treSCP3-AL were properly delivered into the adult mouse HPC via AAV. As a result, treSCP3-AL system successfully induced a significant increase of DNA methylation on the target locus *in vivo* (Fig. 4d right panel; WT vs. *Bdnf*-sgRNA, **** $p < 0.0001$, control-sgRNA vs. *Bdnf*-sgRNA, *** $p < 0.001$; One-way ANOVA with post-hoc Tukey's multiple comparisons test). However, mild but significant off-target methylation by overexpression of scFV-Dnmt3AL was also observed (Fig. 4f right panel; WT vs. control-sgRNA ** $p < 0.01$; Two-way ANOVA with post-hoc Tukey's multiple comparisons test). Consistent with the previous reports that methylation level of *Bdnf* exon 4 was correlated with the activity-dependent expression of *Bdnf* [39, 40, 41], targeted methylation by treSCP3-AL did not affect basal expression of *Bdnf* in adult HPC (Fig. 4e). Previously, it was shown that contextual fear conditioning induced DNA hypomethylation within the *Bdnf* exon 4 promoter in the mouse and rat hippocampal CA1, and that this is correlated with the upregulation of *Bdnf* exon 4 mRNA expression [42, 43]. Based on these reports, we next examined whether the AAV-mediated targeted DNA methylation on the adult mouse CA1 inhibited the activity-dependent transcriptional up-regulation of *Bdnf* gene after fear learning (Fig. 4f). After AAV injection, expression of dSaCas9 and scFv-Dnmt3AL was induced by administration of Dox in drinking water for following 7 days (Fig. 4f). 1 week after injection, animals explored a novel environment and were exposed to foot shock (Contextual fear conditioning, CFC). We repeated CFC to acquire robust activity-dependent *Bdnf* expression, and sacrificed animals 1 hr after 2nd CFC (Fig. 4f). All animals showed robust freezing behavior after foot shocks (Fig. 4g). We also prepared AAV-injected controls that were never given a foot shock and taken from the home cage. The expression of dSaCas9-SunTag_{3x} and scFV-Dnmt3AL were

properly induced into the adult mouse CA1 via AAV (Fig. 4h). We observed up-regulation of *Bdnf* from animals after contextual fear learning compared to the inexperienced animals (Fig. 4i; con no CFC vs. con 1hr after CFC, $*p < 0.05$; One-way ANOVA with post-hoc Tukey's multiple comparisons test). The activity dependent up-regulation of *Bdnf* contextual fear learning was inhibited only in the presence of the sgRNA mix targeting the promoter of *Bdnf* exon 4 but not control sgRNA (Fig. 4i; mix no CFC vs. mix 1hr after CFC, not significant; con 1hr after CFC vs. mix 1hr after CFC, $*p < 0.05$; One-way ANOVA with post-hoc Tukey's multiple comparisons test). Our *in vivo* and *in vitro* results strongly supported the conclusion that AAV-mediated induction of SCP3-AL and targeting sgRNAs into the adult mouse HPC inhibited the learning induced transcriptional up-regulation of the *Bdnf* gene via targeted DNA methylation and the successful development of Dox-inducible AAV-mediated *in vivo* targeted DNA methylation editing system.

Discussion

We here describe the engineering of an AAV-mediated *in vivo* targeted DNA methylation editing system with a combination of dSaCas9 and SunTag system. In this system, we reduced the size of the transgene to the load capacity-limit of AAVs by splitting into two smaller vectors carrying the targeting domain and effector domain independently. Several groups reported new effector domain proteins using dCas9-SunTag to accomplish more efficient and specific target DNA methylation editing including the functional domains of TET1, Dnmt3a and Dnmt3l [17, 22, 33, 44]. Given the flexible vector design space of this split vector system, we could examine the efficacy of various sizes of effector domains, such as scFV-Dnmt3LCD and scFV-Dnmt3AL, a fusion protein with Dnmt3ACD and Dnmt3LCD. We found scFV-Dnmt3AL showed significantly higher methylation efficiency than cFV-Dnmt3LCD or previously reported scFV-Dnmt3ACD (Fig. 1j). In addition, we also designed a novel effector domain, scFv-TET2, whose size is two-thirds of the previously reported scFv-TET1 and induced robust sgRNA-dependent DNA demethylation (Fig. 2e). The extensibility of our system could be a useful for assessing other effector domains *in vivo* with more robust and less off-target activity, such as scFV-Dnmt3AL variants reported to reduced their endogenous DNA binding [44].

BDNF is widely produced in the cortex throughout life and is essential for normal physiological processes in the adult mammalian brain. For example, postmortem studies showed the downregulation of *BDNF* expression in patients with schizophrenia and mood disorders, which are often associated with increased DNA methylation at the *BDNF* promoter [3, 4, 5]. In animal experiments, infusions of a DNA methyltransferase inhibitor significantly altered DNA methylation around *Bdnf* exon 4 and triggered changes in exon specific *Bdnf* mRNA levels, indicating that altered DNA methylation is sufficient to drive expression of *Bdnf* both *in vivo* and *in vitro* [42, 45]. The therapeutic effects of *Bdnf* were assessed in animal models. Viral vector-mediated gene delivery of *Bdnf* to the hippocampal region exhibits potent therapeutic effects including restoring disease-associated aberrant gene expression, prevention of cell death and improvements in learning and memory abilities in animal models of Alzheimer's disease [46, 47]. Compared to AAV-mediated gene delivery, our AAV-mediated epigenetic manipulation has several potential advantages towards the development of precision medicine. Firstly, targeted DNA manipulation technology has a long-lasting effect on the transcriptional regulation of target genes even after

expression of the effector genes has ceased [21, 38]. Because the effector genes and dSaCas9 were under control of a dox-inducible promoter in our *in vivo* targeted methylation system, the off-target effects derived from dSaCas9 and effector proteins can be minimized. Secondly, targeted epigenetic therapy can recover gene function by restoring the transcriptional regulation of the target gene in a diseased cell by restoring normal epigenetic patterning. On the other hand, AAV-mediated gene therapy aims to recover gene function with forced expression of the target gene under the control of an artificial promoter, perhaps a constitutively active promoter. Many biological processes require rigorous transcriptional timing of genes for their proper function. For example, precisely regulated oscillatory expression of key transcription factors for proper neuronal development [48, 49] and activity-dependent transcriptional regulation is important for spike-timing-dependent plasticity of neurons in our brain [50, 51]. We showed that AAV-mediated delivery of the dSaCas9-SunTag targeted DNA methylation system induced DNA methylation on *Bdnf* gene locus and impaired activity-dependent expression of *Bdnf* but not basal expression in hippocampal HPC of adult mouse (Fig. 4). These results suggest that *in vivo* DNA methylation editing technology could be useful to normalize transcriptional regulation of target genes. Although, *in vivo* DNA methylation efficiency was still not particularly high (Fig. 4d), it could be improved by using the optimal promoter for *in vivo* expression of *rtTA*, such as the human Synapsin 1 (hSyn) promoter.

In summary, we successfully developed an AAV-mediated *in vivo* targeted DNA methylation system. We applied small Cas9 orthologues to the previously reported dCas9-SunTag epigenome editing system to address the difficulty in packaging a large dCas9 fusion protein required for targeted DNA methylation editing into an AAV delivery vehicle. Our dSaCas9-SunTag system induced both DNA methylation and demethylation on the target gene locus in an sgRNA-dependent manner *in vitro*. Furthermore, we showed that AAV-mediated delivery of the dox-inducible dSaCas9-SunTag and sgRNAs complex induced methylation on the *Bdnf* gene locus and specifically suppressed activity-dependent expression but not basal expression of *Bdnf* in the hippocampus of adult mouse. Based on our results, our dSaCas9-SunTag system appears promising for future applications of *in vivo* targeted DNA methylation editing-based therapy.

Material And Methods

Cloning

The CMV-dSaCas9-SunTag_{x3} vector was cloned based on the Addgene plasmid #61594 [52], in which 3x GCN4 sequence inserted after 3xHA tag by In-Fusion system (Takara). The 3x GCN4 sequence was cloned from Plasmid #82560 [17]. The CMV-scFv-Dnmt3ACD, -Dnmt3LCD, -Dnmt3AL, -TET1CD and -TET2CD vectors were cloned into a AAV vector backbone Addgene plasmid #61594 [52], in which the dSaCas9 sequence was replaced by each effector domain. The scFv sequence was cloned from Plasmid #82559 [17]. The catalytic domain of mouse Dnmt3a (598–908 aa), C-terminal domain of mouse Dnmt3l (194–415 aa) and Dnmt3AL were cloned based on previous *in vitro* study [13, 34]. Catalytic domains of human TET2 (1129–1936 and 1481–1843 aa) were cloned based on the reported structure [37] and

connected with 15bp of GS linker sequence (GGGGSGGGGSGGGGS). The AAV vector co-expressing sgRNA rtTA and tdTomato was generated based on the Addgene plasmid #61593 [52], in which the TBG promoter and dSaCas9 sequence were replaced by the CMV promoter and rtTA and tdTomato genes. The rtTA and tdTomato gene were connected with P2A peptide sequence. For Dox-inducible AAV vectors, CMV promoter were replaced by TRE promoter cloned from Addgene plasmid #50916 [53]. The sequences of the sgRNAs were GTAATTCGTGCACTAGAGTGTC (Bdnf-1), CGAGGAGAGGACTGCTCTCGC (Bdnf-2), ACGCCTTCAGCGAGAAGCTCC (Bdnf-3), ATCAGTTGTGGGGTTTATACGC (H19-1), GACCCACAGCATTGCCATTT (H19-2), GTCACCCAAGTTCAGTACCTCAG (H19-3), GTGACCCCCCTGAGGTACTGAAC (H19-4), GCCGACCTTGTTGATTTGGGAGT (H19-5) and TGAGACCTGCCGTGGTCTCC (non-targeting control). The sequences of the sgRNAs were designed with the E-CRISP algorithm [54]. Non-targeting control sequence does not target any genome loci in mouse. All reagents described in this manuscript have been deposited with Addgene (plasmid IDs 177342 – 177365, including CMV, hSyn or Dox-inducible promoter).

Cell Culture Co-Transfections and FACS

Neuro2A cells and AAVpro 293T cells (Takara) were cultivated at 37 °C in a cell culture incubator with 95% relative humidity and 5% (v/v) CO₂. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WAKO) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) Penicillin/Streptomycin (Sigma-Aldrich). Transfections of N2A cells were carried out at 80% confluency 24 h post-seeding in a 6 well dish using using polyethyleneimine MAX (PEI-MAX; 1 mg/mL in milliQ water, pH 7.2; Polysciences Inc., Warrington, PA, USA) as a transfection reagent following the manufacturer's instructions. For transfections, 2 µg of dSaCas9-SunTag_{3x} construct, 2 µg of the effector construct, 2 µg of the control sgRNA coding vectors construct or 500 ng of three the sgRNA targeting Bdnf exon 4 or five the sgRNA targeting H19 ICR coding vectors were mixed. The medium was changed 12 hr after transfection. Cells were harvested by trypsinization 36 h later and filtered through a 35 µm pre-separation filter (Stellar Scientific) followed by preparative cell sorting by flow cytometry. The tdTomato-positive cells were collected using a FACSaria II (BD Bioscience). Dead cells and debris were discriminated with propidium iodide. The collected cells (>5 x 10⁴ cells) were cultured with 6 well dish for 48hr and harvested for further analysis. To examine the effect of the treSCP3-AL system, FACS sorted tdTomato-positive cells were cultured with Dox (10 ug/ml) for 3 days.

Quantitative DNA Methylation Analysis

The pyrosequencing technique was used for quantitative DNA methylation analysis. Total DNA was prepared from isolated tissues using the Allprep DNA/RNA mini kit (QIAGEN). 1 µg of the purified DNA was used for bisulfite treatment with MethylEasy Xceed kit (Takara). The target DNA sequence was amplified using biotin-labeled one-sided PCR primers with 2x Gotaq2 G2 Hot Start Polumerase MasterMix (Promega). PCR conditions were as follows: 2 min at 98 °C, 45 cycles of 30 s at 98 °C, 30 s at 50 °C, 1 min at 72 °C, and finally 10 min at 72 °C. Small aliquots from PCR products were subjected to 2% agarose gel electrophoresis for validation of specificity. The PCR products were used for pyrosequencing

with PyroMarkGoldQ24 (QIAGEN) and analyzed PyroMarkQ24 software (QIAGEN) according to the manufacturer's instructions. The PCR primers: ATGGGAAAGTGGGTGGGAGTTTA and biotin-ACATCTACAAAACATACAATACCCTAAAA (Bdnf exon 4 #1), AGGA^tTGGAAGTGAAA^tAT^tTAtAAAGT and biotin-aCTaTCATATaATACCTCCTCTaCCT (Bdnf exon 4 #2), biotin-GGAGGtATAAGAATTCTGtAAGGAG and ACATTTATAACCCCCCTAAAATACTA (H19-ICR). The sequence primers: GGGAGTTTAAGAGAGGGTTTTA (Bdnf exon 4 #1 sequence), tTatAAAGtATGtAATGtttTGGA (Bdnf exon 4 #2 sequence), CCAATACCAAAAATAAAAAAAC (H19-ICR sequence).

Quantitative RT-PCR analysis.

Total RNA was using the Allprep DNA/RNA mini kit (QIAGEN) with following DNase (Wako) treatment (15 min at 37 °C, 10 min at 70 °C). 1 ng of purified RNA was subjected to following reverse transcription (15 min at 37 °C, 5 sec at 85 °C) with PrimeScript RT Master Mix (Takara). Gene expression levels were measured with StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq II (Takara) according to the manufacturer's instructions. *Gapdh* was used as an inner control. The sequences of PCR primer were following. The PCR primers: TGTGTCCGTCGTGGATCTGA and CCTGCTTCACCACCTTCTTGA (*Gapdh*), GCTTCAGCCTGACCGACTAC and TACACGGTGTTCTTGCCGTT (scFv), AGCTGATCAACAAGAGCCCC and ATGGGCGTTCAGTTTGTTC (dSaCas9), CAGGAGTACATATCGGCCACCA and GTAGGCCAAGTTGCCTTGTCCGT (Bdnf exon 4) and CACTGTATGCCCTAACCGCT and CCAACCTCCCTCCCTAGAA (H19).

Virus production and titration.

For in-house viral production, AAVpro 293T cells (Takara) were maintained as described above in 150 mm plates. For each transfection, 8 µg of pAAVdj serotype packaging plasmid, 8 µg of helper plasmid, and 6 µg of reconstructed AAV plasmid carrying the construct of interest mixed with 1000 mL of OPTI-MEM (Thermo) containing 48 µl of PEI-MAX (1 mg ml⁻¹, pH 7.2) was prepared. After 10 min incubation, the mixture was applied to each dish. Medium was changed 12 hr after transfection. Cells were harvested between 120 h post transfection by scraping and pelleting by centrifugation. The AAV2/dj viral particles were then purified with AAVpro Purification Kit (Takara). Purified viruses were titred by real-time qPCR using primer against AAV-ITR. AAVs with a titer of more than 3 x10¹¹ genome copy/ml were used in following *in vivo* experiments.

Animals

Male C57BL/6J mice (8 weeks old, SLC) were used in this study. Animals were housed in the animal facilities at the School of Pharmacy and Pharmaceutical Sciences Hoshi University under specific pathogen-free conditions, maintained at 24°C on a 12 h light/dark cycle. Food and water were provided *ad libitum*. The animals were acclimatized for 1 week prior to the start of the experiments. All procedures were approved by the animal experiment ethics committee of Hoshi University and performed in accordance with the Hoshi University guidelines for the care and use of laboratory animals.

Mouse stereotaxic surgery

Mice were anesthetized with 0.8%–1.5% isoflurane on a stereotaxic device. A small amount of 2 % lidocaine was placed on and under the skin as a topical analgesic and a small hole was drilled above each injection site. Viruses were injected using a glass micropipette attached to a 1 ml Hamilton microsyringe through a microelectrode holder filled with mineral oil. A microsyringe pump and its controller were used to control the speed of the injection. The needle was slowly lowered to the target site and remained for 5 min after the injection. Viral injections were performed at a rate of 2 nL/second. Bilateral viral delivery into HPC of the WT male mice was targeted at AP: -2.0 mm, ML, ± 1.5 mm, DV, -1.5 mm from Bregma. WT mice were injected with 250 nl of AAV cocktail (AAV2/dj-TRE-dSaCas9-SunTag_{x3} : AAV2/dj-TRE-scFv-Dnmt3AL : AAV2/dj-U6-sgRNA;CMV-rtTA-tdTomato mix=2:2:1) with 1 % fast green dye (Sigma-Aldrich). An equal amount of three AAVs expressing sgRNA targeting the Bdnf exon 4 were mixed. At the end of surgery, mice were given intraperitoneal injections of 0.9 % sterile saline and meloxicam (2 mg/kg) as an analgesic, placed on a heating pad until fully recovered from the anesthesia. After finishing all behavioral procedures, we performed histology to verify the target sites and expression of AAV injection. For DNA/RNA extraction, AAV-injected site on dorsal HPC labelled with fast green dye was manually isolated.

Immunohistochemistry

AAV injected mice were anesthetized using intraperitoneal injection of urethane (1.25 g/kg) and transcardially perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in PBS. Doxycycline (1mg/ml) was administered with drinking water 4 days before perfusion. The brains were post-fixed overnight at 4 °C in the same fixative solution, transferred to 30% sucrose in PBS for 48 h, and then cut into 30 μ m coronal sections using a CM1510-11 cryostat (Leica). The free-floating sections were blocked with 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 1 h at room temperature and incubated overnight at 4 °C with the following antibodies: mouse monoclonal anti-myc (1:200; 9B11, Cell Signaling). Alexa 488-conjugated donkey anti-mouse IgG (1:1,000; A21202, Thermo Fisher Scientific) was used for the following secondary antibody. Labeled sections were mounted onto microscope slides with Prolong Diamond Antifade Reagent containing DAPI (Thermo Fisher Scientific).

Contextual fear conditioning

Contextual fear conditioning was performed on WT male mice aged between 7 and 12 weeks during the light cycle. All animals were handled 5 minutes a day for 5 consecutive days prior to experimentation. A white plastic conditioning chamber (17 $\times$ 10 $\times$ 10 cm) was cleaned with water prior to the introduction of each individual mouse. Subject mice were kept in the conditioning chamber for total 270 s. A foot-shock (1 s, 0.3mA) was delivered at 120 s, 150 s, 180 s, 210 s and 240 s. Mice remained in the conditioning chamber for a total of 270 s. All sessions were video-recorded for automatic scoring of freezing (TimeFZ software, O'HARA & Co., LTD.) which was defined as a period of immobility, lasting for at least 2 s. 2nd

time CFC was performed after within 1hr after 1st CFC. Half of AAV-injected animals were subjected to CFC and sacrificed 1 hr later. The remaining half was sacrificed without CFC (no CFC).

Statistical analysis

All data excluding Fig. 4g are expressed as the mean $\pm$ standard deviation. Fig. 4g is expressed as the mean $\pm$ standard error of the mean. Statistical analyses were performed using the Graphpad Prism 9 software. P values of less than 0.05 were considered statistically significant. For all figures, ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

Abbreviations

AAV: Adeno-associated virus

Bdnf: Brain derived neurotrophic factor

Cas9: CRISPR associated protein

CFC: Contextual fear conditioning

CRISPR: Clustered regularly interspaced short palindromic repeat

dCas9: dead Cas9

Dox: Doxycycline

Dnmt: DNA methyltransferase

HPC: Hippocampus

hSyn: human Synapsin 1

ICR: Imprinting control region

ITR: Inverted terminal repeat

NLS: Nuclear localization signal

N2A: Neuro 2A

rtTA: Reverse tetracycline-controlled transactivator

sgRNA: Single guide RNA

SaCas9: *Staphylococcus aureus* derived smaller dCas9

scFv: Single-chain variable fragment

Tet: Tet Methylcytosine Dioxygenase

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the animal experiment ethics committee of Hoshi University and performed in accordance with the Hoshi University guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The authors plan to make the plasmids widely available to the academic community through Addgene.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

NY and KI wrote the manuscript. NY performed the experiments. NY and KI conceived the experiments and analyzed the data. HT advised about *in vivo* experiments. SM and IH prepared samples. MIO, HT and MN correct the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Targeted DNA methylation with the dSaCas9-SunTag and antibody-conjugated-Dnmts in vitro (a-c) Structure of AAV vector components. Size of transgene on each vector was shown. (d) Schematic representation of targeted DNA methylation with dSaCas9-SunTag system. (e) The mouse *Gfap* exon 4 locus. Positions of CpG sites are numbered relative to TSS of the *Bdnf* exon 4 and indicated by vertical black bars. The locations of the sgRNA targeting sequence are indicated by black arrows. (f) The

experimental scheme in this experiment. (g) RT-qPCR analysis of dSaCas9 and scFv. (h-k) Left panel: The percent of DNA methylation across individual CpGs on the Bdnf exon 4 as determined by bisulfite pyrosequencing analysis Right panel: The averaged DNA methylation level shown in left panel. (g-j) Values are mean $\pm$ SD from (wt : n =3, SCP3-A and SCL3-L : n = 3, SCP3-Dnmt3AL : n=6, independent transfections). Statistical analysis were performed using two-way repeated measure ANOVA with Tukey's multiple comparison in (h, i left), mixed-effects analysis with Tukey's multiple comparison in (j left) and one-way repeated measure ANOVA with Tukey's multiple comparison in (h, i and j right). ns: not significant; * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001 .

Figure 2

Targeted DNA demethylation with the dSaCas9-SunTag and antibody-conjugated-TET1 or TET2 in vitro (a) Structure of AAV vector expressing scFv-TET1CD or scFv-TET2CD. (b) The mouse H19 ICR locus. CTCF-binding sites indicated by grey box. Positions of CpG sites are numbered relative to edge of H19 ICR and indicated by vertical black bars. The locations of the s-sgRNA targeting sequence are indicated by black arrows. (c) The experimental scheme in this experiment. (d, e) Left panel: The percent of DNA

methylation across individual CpGs on H19 ICR as determined by bisulfite pyrosequencing analysis. Right panel: The averaged DNA methylation level shown in left panel. (f, g) RT-qPCR analysis of dSaCas9 and scFv. (h, i) Fold change in the expression levels of the H19 genes. Mean expression level in control-sgRNA transfected sample was set to 1. (d-g) n = 3 for wt, n = 3 independent transfections for SCP3-TET1, n = 4 independent transfections with SCP3-TET2. Statistical analysis were performed using two-way repeated measure ANOVA with Tukey's multiple comparison in (d and e left), one-way repeated measure ANOVA with Tukey's multiple comparison in (d and e right) and Student's two-tailed t-tests in (h, i).

Figure 3

Dox-inducible in vitro targeted DNA methylation with the dSaCas9-SunTag and antibody-conjugated-Dnmt3AL in vitro (a-c) Structure of AAV vectors for the Dox-inducible SCP3-AL system. Size of transgene on each vector was shown. (d) The mouse Gfap exon 4 locus. (e) The experimental scheme in this experiment. (f) Left panel: The percent of DNA methylation across individual CpGs on the Bdnf exon 4 as determined by bisulfite pyrosequencing analysis. Right panel: The averaged DNA methylation level shown in left panel. (f) n =3 for wt, n = 3 independent transfections for treSCP3-AL. Statistical analysis were performed using two-way repeated measure ANOVA with Tukey's multiple comparison in (f left) and one-way repeated measure ANOVA with Tukey's multiple comparison in (f right).

Figure 4

AAV-mediated in vivo targeted DNA methylation with Dox-inducible the SCP3-AL (a) The experimental scheme in this experiment. After bilateral AAV injection to HPC, Dox was administered in drinking water. (b) The tdTomato-labelled cell bodies (red) on dorsal HPC were immunostained with anti-myc (green). Scale bar: 30 μ m. (c and h) RT-qPCR analysis of dSaCas9 and scFv. (d) Left panel: The percent of DNA methylation across individual CpGs on the Bdnf exon 4 as determined by bisulfite pyrosequencing

analysis. Right panel: The averaged DNA methylation level shown in left panel. (e) Fold change in the expression levels of the Bdnf exon 4. Mean expression level in WT sample was set to 1. (f) The experimental scheme in this experiment. Half of AAV-injected animals were subjected to 2 times of CFC and sacrificed 1 hr later. The remaining half was sacrificed without CFC (no CFC). (g) Freezing rate of animal during CFCs. Red vertical line indicate the timing delivered a foot shock. Mean + SEM. (i) Fold change in the expression levels of the Bdnf exon 4. Mean expression level in control-sgRNA transfected and no-CFC sample was set to 1. (c, e) wt: n=3, control-sgRNA:n=4, Bdnf-mix: n=5. (d and g-i) n = 3. Statistical analyses were performed using Two-way repeated measure ANOVA with Tukey's multiple comparison in (e left) or one-way repeated measure ANOVA with Tukey's multiple comparison in (e right).