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Carlo Cosimo Campa, Niels R. Weisbach, António J. Santinha, Danny Incarnato ...+2 more authors

Institutions: ETH Zurich, University of Groningen, University of Basel

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Author(s):
Campa, Carlo C.; Weisbach, Niels R.; Santinha, António J.; Incarnato, Danny; Platt, Randall J.

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Multiplexed genome engineering by Cas12a and CRISPR arrays encoded on single transcripts

Carlo C. Campa[1*], Niels R. Weisbach[1*], António J. Santinha[1], Danny Incarnato[2], Randall J. Platt[1,3†]

[1]Department of Biosystems Science and Engineering
ETH Zurich
4058 Basel, Switzerland

[2]Department of Molecular Genetics
Groningen Biomolecular Sciences and Biotechnology Institute
University of Groningen
Nijenborgh 7, 9747 AG Groningen, Netherlands

[3]Department of Chemistry
University of Basel
4003 Basel, Switzerland

*These authors contributed equally to this work.

†To whom correspondence should be addressed: rplatt@ethz.ch.

Abstract

The ability to modify multiple genetic elements simultaneously would help to elucidate and control the gene interactions and networks underlying complex cellular functions. However, current genome engineering technologies are limited in both the number and the type of perturbations that can be performed simultaneously. Here, we demonstrate that both Cas12a and large CRISPR array can be encoded in a single transcript by adding a stabilizer tertiary RNA structure. By leveraging this system, we illustrate constitutive, conditional, inducible, orthogonal and highly multiplexed genome engineering using a single plasmid as a delivery agent. Our method provides a powerful platform to investigate and orchestrate the sophisticated genetic programs underlying complex cell behaviours.

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) nucleases are versatile RNA-guided enzymes that facilitate a wide-range of genome engineering applications[1,2]. Through the binding of short RNA molecules, known as CRISPR RNAs (crRNAs), members of the Cas nuclease family are programmed to edit specific genomic loci thereby facilitating systematic investigation of gene function[3,4]. In addition, nuclease-inactive Cas enzymes fused to transcriptional effectors enable fine control of gene expression[5-10]. Remarkably, use of either different Cas enzymes or engineered crRNAs enable distinct gene perturbations, including gene knockout, gene activation, and gene repression[11-16]. Despite this versatility, either the heterologous expression of different proteins (e.g. Csy4, scFV) or the presence of long RNA-based regulatory regions (e.g. rybozymes, aptamers) are required to permit regulation of multiple genes using current platforms[17-19]. This inevitably limits the scalability of CRISPR-based multiplexed genome engineering approaches and consequently the possibility to investigate complex cell behaviours.

In order to coordinate a myriad of different processes using a limited number of cellular elements, cells evolved to maximize the efficiency of their molecular components. This principle is adopted by distinct organisms to rapidly match different environmental contexts, thereby supporting the notion that maximizing the performance of each molecular element is a general principle on which biological systems based their own survival[20]. As an example, protection against bacteriophages and other foreign genetic elements is mediated by Cas12a, a class II type V CRISPR-Cas system[21,22]. Functioning as a both RNase and DNase, Cas12a controls both processing and maturation of its own crRNA as well as DNA target cleavage[21-23]. CRISPR arrays associated with Cas12a are transcribed as a long RNA transcript, termed pre-crRNA, that contains a succession of ~30-nucleotide (nt) spacer sequences, separated by 36-nt direct repeat sequences (DRs)[21,22]. While DRs direct Cas12a-mediated pre-crRNA processing into mature crRNAs, recognition and cleavage of target DNA is controlled by Watson-Crick base pairing between the spacer and target sequences[21,22]. Transplantation of this microbial immune system into mammalian cells enables genome engineering applications, including gene editing and transcriptional gene control[24]. Optimization of this genome editing system for mammalian cells was obtained by DR length reduction and adoption of distinct promoters that differentially drive transcription of Cas12a and pre-crRNA[25-27]. Although, the implementation of these strategies enables a wide range of applications, they fail to harness the full potential of Cas12a in genome engineering.

Here, we leverage the dual RNase/DNase function of *Acidaminococcus sp.* Cas12a to develop a robust system for multiplexed genome engineering using a single plasmid as a delivery agent. This system, termed SiT-Cas12a, encodes Cas12a and dozens of crRNAs in a single transcript. Stabilization of SiT-Cas12a transcripts through inclusion of a tertiary structural motif improves both pre-crRNA processing as well as Cas12a production. When coupled with transcriptional effectors and engineered spacer sequences, SiT-Cas12a enables multiplexed orthogonal gene transcriptional regulation and editing thereby providing a scalable way to elucidate and control the gene networks underlying cellular functions.

Results

Compact encoding of Cas12a and CRISPR arrays in a single Pol II-derived transcript

In mammalian cells, distinct promoters control transcription of different RNA molecules. While Pol II promoters are mainly used for transcription of coding genes characterized by long RNA sequences, Pol III promoters are employed for production of small non-coding RNAs including CRISPR RNAs (crRNAs)[28]. To assess whether transcripts derived from Pol II promoters could facilitate Cas12a-based genome engineering applications[26], we expressed a crRNA targeting *DNMT1* from either a U6 (Pol III) or EF1a (Pol II) promoter along with ectopically expressed Cas12a followed by quantification of insertions and deletions (indels) 72 hours after transient transfection. Expression of crRNA from both Pol II and Pol III promoters resulted in comparable gene editing efficiencies (Figure 1A, B, conditions I, II), indicating that Cas12a processes and utilizes crRNAs derived from both promoter types.

To determine whether Pol II promoters facilitate simultaneous protein and crRNA expression, we cloned a CRISPR array containing a spacer targeting *DNMT1* within the 3′UTR of the enhanced green fluorescence protein (*EGFP*) gene and assessed both gene editing efficiency at the endogenous *DNMT1* locus and EGFP expression 72 hours after transient transfection in HEK 293T cells (Figure 1B, condition III). In cells harbouring *EGFP* transcripts containing CRISPR arrays, we observed complete loss of EGFP expression (Figure 1B, condition III), suggesting destabilization of *EGFP* transcripts mediated by the RNase activity of Cas12a. Consistently, expression of an RNase dead Cas12a (rdCas12a), but not DNase dead Cas12a (ddCas12a), rescued EGFP expression (Figure 1B, condition IV, V). Taken together, this suggests that Cas12a-mediated crRNA processing via the RNase domain results in efficient cleavage and destabilization of protein-coding mRNAs, which is likely a result of removal of the polyadenylation (poly(A)) tail.

To overcome mRNA destabilization and enable simultaneous expression of protein and crRNA on the same transcript, we leveraged a 110-nt structure derived from the 3′ end of the mouse non-coding RNA Metastasis-associated lung adenocarcinoma transcript 1 (*Malat1*), previously described to stabilize transcripts lacking poly(A) tails through the formation of a tertiary structure element termed "Triplex"[29]. We cloned the Triplex sequence between the *EGFP* coding sequence and the CRISPR array (Figure 1B, condition VI), which effectively rescued EGFP expression without affecting gene editing efficiency (Figure 1B, condition VI). These results indicate that a Triplex sequence positioned at the 3′ of a protein coding gene stabilizes mRNAs after Cas12a-mediated RNA processing, enabling concomitant protein expression and gene editing.

Constitutive, conditional and inducible gene editing with SiT-Cas12a

To determine whether Cas12a and CRISPR arrays could be compactly encoded on single Pol II-driven mRNA, we developed single-transcript Cas12a (SiT-Cas12a). SiT-Cas12a is composed of a: i) Pol II promoter EF1a; ii) Cas12a derived from *Acidaminococcus sp.*; iii) Triplex sequence; iv) CRISPR array containing spacers targeting a set of mammalian genomic loci; and v) poly(A) signal (Figure 1A). We evaluated the platform using a CRISPR array containing a spacer targeting *DNMT1* and observed consistent and efficient gene editing at the *DNMT1* locus (Figure S1A).

Utilization of a single Pol II promoter offers the unique potential for more sophisticated and simultaneous control of both Cas12a and crRNAs expression. To evaluate this possibility, we generated conditional and inducible SiT-Cas12a platforms, termed SiT-Cas12a-[Cond] (Figure S1B) and SiT-Cas12a-[Ind], respectively (Figure S1C). SiT-Cas12a-[Cond] relies on a Lox-Stop-Lox (LSL) cassette positioned downstream of an EF1a promoter and upstream of the SiT-Cas12a coding region. To demonstrate conditional genome editing, we co-transfected HEK 293T cells with SiT-Cas12a-[Cond] and either a Cre-recombinase encoding plasmid or a control plasmid. At 72 hours post-transfection, we detected genome editing events exclusively in Cre-recombinase expressing cells (Figure S1B). SiT-Cas12a-[Ind] relies on a Tetracycline responsive element (TRE) positioned upstream of a minimal CMV promoter (minCMV) that, differently from the constitutive promoter EF1a, resulted in gene editing efficiencies proportional to inducer concentration (doxycycline) both in a Tet-on and Tet-off configuration (Figure S1C). Taken together, SiT-Cas12a enables either constitutive or conditional and inducible gene editing through fine temporal control of Cas12a and crRNA expression.

Multiplexed gene editing with SiT-Cas12a

We evaluated the potential of the SiT-Cas12a platform for multiplexed gene editing using a CRISPR array containing 5 distinct spacers targeting different genomic loci (*FANCF1, EMX1, GRIN2B, VEGF, DNMT1*) and quantified both mature crRNAs and gene editing efficiency (Figure 2A). In the SiT-Cas12a context, we observed expression and processing of each mature crRNA, albeit with varying efficiencies as observed in previous studies[25] (Figure 2A). In addition, transcripts stabilized by the Triplex sequence (Figure 2A, SiT-Cas12a) increased both Cas12a expression (Figure S2A) and processed crRNA abundance compared to transcripts without the Triplex (Figure 2A, Cas12a). In line with these results, the gene editing efficiency was higher in cells expressing SiT-Cas12a compared to a control lacking the Triplex structure between Cas12a and the CRISPR array (Figure 2B). The increased crRNA production facilitated by the Triplex sequence disappeared upon mutation of the RNase domain of Cas12a (SiT-rdCas12a) (Figure 2A). Consistently, gene editing efficiencies were negligible for the SiT-Cas12a RNase inactive mutant and found to be comparable to the DNase inactive mutant of Cas12a (SiT-ddCas12a) (Figure 2B).

Next, we compared the gene editing efficiency of SiT-Cas12a with previously reported Cas12a platforms based on independent transcription of Cas12a and a CRISPR array from distinct promoters[25,26]. Expression of SiT-Cas12a resulted in gene editing efficiencies equal to or higher than other tested platforms (Figure 2C). Taken together, these data demonstrate that compact encoding of Cas12a and a CRISPR array on Pol II transcripts mediates efficient multiplexed gene editing.

In contrast to Pol III promoters, Pol II promoters express transcripts of seemingly unlimited length[30]. To leverage this property, we cloned a CRISPR array harbouring 10 distinct spacer sequences targeting the *CD47* locus in the SiT-Cas12a context, either singularly or jointly, and performed gene editing quantification (Figure S2B-G). While the gene editing efficiency using single-crRNAs ranged from 2 to 17%, simultaneous expression of all crRNAs increased the gene editing efficiency up to 60% (Figure S2C), indicating that the targeting of multiple crRNAs in the same coding gene introduced more loss of function mutations. We obtained similar gene editing efficiency in cells infected with a SiT-Cas12a-based lentiviral vector enabling stable expression and delivery to cells that are difficult to transfect (Figures S3A-E). In agreement, multiple independent indels at each target site as well as large fragment deletions between target sites were detected using deep sequencing in cells expressing such large CRISPR array (Figure S2D-G), confirming the targeting of all crRNAs used. Consistently, single cell analysis of CD47 protein expression showed

a 4-fold increase in CD47-negative cells, reaching 37% of the total population when using multiple crRNAs (Figure S4A).

Finally, we evaluated whether this strategy could be employed to increase the efficiency of generating multi-gene knockouts after a simple transient transfection experiment. We cloned distinct spacers targeting different coding genes (*CD47*, *CD166* and *CD97*) (Figure S4B) in the SiT-Cas12a context and measured the rate of either single (either CD47 or CD166 or CD97) double (CD47/CD166, CD47/CD97, CD166/CD97) or triple (CD47/CD166/CD97) knockout by single cell flow cytometry analysis. Targeting multiple sites per gene using SiT-Cas12a increased the rate of single, double and triple multiplexed gene knockout generation by 2- to 3-fold compared to single crRNA conditions (Figure S4B). Overall, these results demonstrate that SiT-Cas12a facilitates scalable and multiplexed genome engineering.

Multiplexed transcriptional regulation with SiT-Cas12a

By leveraging on the simultaneous transcription of both Cas12a and a large CRISPR array under a single Pol II promoter, we set out to develop SiT-Cas12a-based platforms to control the expression of endogenous genes. We fused either one, two, or three copies of the Krüppel associated box (KRAB) domain of the transcriptional repressor ZNF10[31] to the C-terminus of ddCas12a, thus generating ddCas12a-$KRAB_1$, ddCas12a-$KRAB_2$, and ddCas12a-[Repr], respectively (Figure S5A). To assess the efficiency of the transcriptional repression conferred by multiple KRAB domains, we co-transfected the SiT-Cas12a repressor variants along with different concentrations of CRISPR arrays containing spacers targeting 4 distinct genomic loci (*RAB5A, RAB7A, EEA1, PIK3C3*). We found that three tandem KRAB domains fused at the C-terminus of ddCas12a (ddCas12a-[Repr]) conferred potent transcriptional repression (Figure 3A, S5B) with differential efficiencies according to the target gene and distance from the TSS (Figure S5C). Next, we cloned a CRISPR array harbouring 20 different spacers targeting 10 distinct genes (*RAB5A*, *RAB7A*, *PIK3C3*, *EEA1*, *RAB9A*, *CHRM3*, *PLCB1*, *PRKC1*, *FZD1*) within SiT-ddCas12a-[Repr] and quantified mature crRNAs and transcriptional repression. Small-RNA-seq analysis confirmed generation of mature crRNAs leading to efficient gene repression ranging from 40 to 80% reduction (Figures 3B, C).
To further strengthen the finding that SiT-Cas12a enables simplified multi-gene transcriptional gene control, we replaced the transcriptional repression domain KRAB with either the VPR activator[32] or a combination of the p65 activation domain (p65) together with the Heat Shock Factor 1 (HSF1)[3] to generate two RNA-guided transcriptional activators: ddCas12a-VPR and ddCas12a-p65-HSF1 (Figure S6A). Both of these Cas12a-based chimeric proteins induced gene activation at different

efficiencies (Figure S6B). Comparative analyses of these gene activators (Figure 4A, B) showed a 10-fold increase in activation efficiency of ddCas12a-[Activ] compared to ddCas12a-VPR (Figure S6B) also in the presence of limiting spacer concentrations (Figure 4A, S6C). We also observed that transcriptional activation efficiency varies according to the target gene and distance from the TSS (Figure S6D). Next, to explore the potential of multiplexed transcriptional activation in the SiT-Cas12a context, we combined SiT-ddCas12a-[Activ] with a CRISPR array harbouring 20 spacers targeting 10 distinct genes (*ASCL1*, *ZFP42*, *CCR4*, *CCR10*, *IL1B*, *IL1R2*, *HBB*, *CHRM4*, *HTR6*, *ADRB2*) and quantified mature crRNAs and transcriptional activation. Small RNA-seq analysis confirmed generation of all 20 mature crRNAs (Figure 4B). In addition, we measured robust gene activation (10-1000-fold) for all target genes (Figure 4C, S6E). Overall, these data indicate that both repressor and activator transcriptional domains when combined with SiT-Cas12a enable multi-gene transcriptional control.

Orthogonal gene editing and transcriptional control with SiT-Cas12a

The Cas12a endonuclease, similarly to Cas9, is characterized by unique DNA binding kinetics, which enables binding while avoiding cleavage in the presence of truncated crRNAs[13-15,33]. To fully harness the potential of Cas12a for genome engineering, we set out to explore these unique properties for generating a SiT-Cas12a-based platform that could facilitate orthogonal gene editing and transcriptional gene control.

Towards developing such a platform, we assessed *Acidaminococcus sp.* Cas12a (*As*Cas12a) processing efficiency using CRISPR arrays containing both long (20 bp) and short (15 bp) spacers (Figure S7A). We measured 3- to 5-fold higher amounts of mature crRNAs in cells expressing arrays containing short spacers compared to those with long spacers (Figure S7B). Furthermore, both short and long spacers generated comparable transcriptional gene control when combining SiT-Cas12a-[Repr], SiT-Cas12a-p65-HSF1, and SiT-Cas12a-[Activ] with 2 distinct CRISPR arrays (Figure S7C, D). This strategy does not extend with a similar efficiency to *Lachnospiraceae bacterium* Cas12a (*Lb*Cas12a), whereby crRNAs containing short spacers did not induce significant gene activation (Figure S7E). Next, we evaluated the SiT-Cas12a platform in an orthogonal transcriptional control and gene editing context (Figure 5A). We combined both active and inactive DNase versions of SiT-Cas12a-based transcriptional repressor and activator (SiT-ddCas12a-[Repr], SiT-Cas12a-[Repr], SiT-ddCas12a-[Activ] and SiT-Cas12a-[Activ]) with 2 sets of CRISPR arrays harbouring spacers targeting 2 distinct promoters using either short or long spacers (Figure 5B, C). Subsequently, we quantified gene expression and genome editing and determined that only DNase

active SiT-Cas12a effectors combined with 20 bp spacers facilitated gene editing. In contrast, SiT-Cas12a effectors combined with 15 bp spacers induced either gene repression (Figure 5B) or gene activation (Figure 5C) with comparable efficiencies and without any detectable gene editing events. Lastly, large CRISPR arrays containing both short and long spacers enable coordinated and highly multiplexed regulation of 10 distinct genes simultaneously with gene editing of another 5 distinct genes (Figure 6A, B). Taken together, SiT-Cas12a effectors, based on *As*Cas12a, facilitate orthogonal transcriptional control and gene editing simply by altering spacer length.

Discussion

In this work we have described SiT-Cas12a, a platform for constitutive, conditional, inducible, orthogonal and highly multiplexed genome engineering. We have demonstrated its potential in numerous multiplexed genome engineering applications, including both multi-gene transcriptional regulation, multi-gene knockout and coordinated multiplexed orthogonal gene transcriptional control and editing. By encoding dozens of crRNAs in a single transcript, SiT-Cas12a provides a powerful and highly flexible tool for highly multiplexed genome engineering thereby exceeding previous published reports [25-27].

In contrast to most CRISPR-Cas gene editing expression strategies, where Cas enzymes and guide RNAs are expressed from distinct promoters, in our platform a single Pol II promoter expresses a single transcript harbouring both Cas12a and a CRISPR array. Consequently, the ratio of Cas12a and CRISPR array is fixed, which introduces distinct disadvantages and advantages. A potential disadvantage is that the fixed ratio of the two components may be suboptimal, especially in expression-limited conditions. Such theoretical disadvantage could be overcome by either increasing SiT-Cas12a transcript abundance or encoding multiple crRNAs per gene. On the other hand, the advantage of the fixed ratio is that it enables tight control of both Cas12a and crRNA expression, facilitating conditional and inducible genome engineering applications. Lastly, as the extent of genome editing reflects the underlying expression of the components, derived from either the intrinsic variability among different cell types or the cellular environment (e.g. Cre recombinase, rtTA, tTA in this work), the SiT-Cas12a platform empowers further applications in DNA-writing, molecular recording and synthetic biology[34-36].

Considering the mean natural length of protein coding transcripts found within mammalian cells (13.5 kb), the potential for expressing multiple crRNAs in the SiT-Cas12a context is profound. In the future this could theoretically be used to enable massively multiplex expression of hundreds to thousands of independent crRNAs, opening up avenues for large-scale genome engineering efforts[30,37]. While SiT-Cas12a offers a seemingly unlimited potential for crRNA expression, longer pre-crRNA transcripts will inevitably be challenging to synthesize and clone. Furthermore, DRs and spacers containing complementary sequences that could generate complex secondary RNA structures affecting the maturation of crRNAs in cells[38,39]. Consequently, complementary regions in pre-crRNA must be considered in order to improve crRNAs maturation. Future work overcoming these limitations will open up numerous applications for highly multiplexed genome engineering.

In the control of cell behaviour, genetic elements act in concert. Deciphering such complexity requires fine modulation of multiple genetic elements. Simultaneous encoding of dozens of crRNAs in a single plasmid simplifies both guide testing and validation of gene function. Inspired by design principles that embody biological efficiency, our genome engineering platform harnesses the full potential of the Cas12a enzyme, providing an easy and customizable way to allow highly multiplexed gene editing and transcriptional control making it possible, in the future, to systematically interrogate complex genetic interactions and cellular behaviours.

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Author Contributions

C.C.C., N.R.W., and R.J.P. conceived of and designed the experiments. C.C.C. and N.R.W. performed the experiments and analysed the data. A.J.S analysed the deep sequencing data. D.I. analysed the RNA-seq data. C.C.C., N.R.W., and R.J.P. wrote the manuscript. All authors reviewed the paper and provided comments.

Competing financial interests statement

The authors declare that they have no conflicts.

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Figure legends

Figure 1: Simultaneous control of protein and crRNA expression from Pol II promoters.

A. Schematic of a single transcript architecture containing both a protein coding sequence and a CRISPR array. The transcript encodes for: Protein/Cas12a (grey/yellow rectangle); Triplex, a tertiary RNA structural motif (small purple rectangle); direct repeat (DR, grey square); spacer (blue square); and a polyadenylation sequence (Poly(A), red rectangle). After transcription and Cas12a-mediated crRNA processing, the Triplex sequence stabilizes the transcript enabling concomitant Protein/Cas12a expression and gene editing.

B. Validation of Triplex-mediated mRNA stabilization for concomitant gene editing and protein expression. Combinations of crRNA expression constructs and Cas12a proteins used (I to VI). Representative EGFP fluorescent images after co-transfection of different plasmid combinations (I to VI). Scale bar = 1.00 mm. Quantification of gene editing efficiencies (combinations I to VI). Values represent mean ± s.e.m, n = 3 independent experiments.

Figure 2: Multiplexed genome editing by SiT-Cas12a.

A. Schematic of SiT-Cas12a, which includes the Pol II promoter EF1a, Cas12a (yellow rectangle), Triplex (purple rectangle), CRISPR array (coloured and grey squares) and polyadenylation sequence (Poly(A), red rectangle). Alignments and quantification of mature crRNAs. n = 2 independent experiments.

B. Schematics of the Cas12a, SiT-Cas12a, rdSiT-Cas12a and ddSiT-Cas12a constructs used. Cas12a (yellow rectangle), Triplex (purple rectangle), CRISPR array (coloured square) are indicated. The Poly(A) signal is present on the constructs but not displayed. Quantification of multiplexed gene editing efficiencies. Values represent mean ± s.e.m., n = 3 independent experiments.

C. Schematics of the plasmid combinations used. Quantification of multiplexed gene editing efficiencies in cells transfected with either EF1a-Cas12a and U6-crRNA on the same plasmid (Cas12a/U6), EF1a-Cas12a and EF1a-crRNA on 2 different plasmids (Cas12a+EF1a) or SiT-Cas12a on a single plasmid. Values represent mean ± s.e.m., n = 3 independent experiments.

Figure 3: Multiplexed transcriptional repression using SiT-Cas12a-[Repr].

A. Quantification of relative mRNA expression for 4 distinct genes (*EEA1*, *RAB5*, *PIK3C3*, *RAB7A*) in cells co-transfected with ddCas12a fused with either 1 (KRAB), 2 ($KRAB_2$) or 3 ([Repr]) KRAB domains in combination with different crRNA array concentrations. Values represent mean ± s.e.m., n = 3 independent experiments.

B. Alignments of mature crRNAs in cells transfected with SiT-ddCas12a-[Repr] containing a CRISPR array with 20 distinct spacers.

C. Quantification of relative RNA expression for 10 distinct genes after multiplexed expression of 20 distinct crRNAs from SiT-ddCas12a-[Repr]. Values represent mean ± s.e.m., n = 3 independent experiments.

Figure 4: Multiplexed transcriptional activation using SiT-Cas12a.

A. Quantification of relative RNA expression for 4 distinct genes (*IL1B*, *ASCL1*, *ZFP42*, *IL1R2*) in cells co-transfected with ddCas12a fusion proteins (VP128, VPR, [Activ]) in combination with different crRNA concentrations. Values represent mean ± s.e.m., n = 3 independent experiments.

B. Alignments of mature crRNAs in cells transfected with SiT-Cas12a-[Activ] containing a CRISPR array with 20 distinct spacers.

C. Quantification of relative RNA expression for 10 distinct genes after multiplexed expression of 20 distinct crRNAs from SiT-Cas12a-[Activ]. Values represent mean ± s.e.m., n = 3 independent experiments.

Figure 5: Orthogonal transcriptional gene regulation and editing.

A. Schematic of the SiT-Cas12a platform for orthogonal gene editing and transcriptional regulation with the Pol II promoter EF1a, Cas12a (yellow rectangle), [ED] (Effector Domain, grey rectangle), Triplex (purple rectangle), CRISPR array (coloured and grey squares) and polyadenylation sequence (Poly(A), red rectangle) indicated. Delivery of SiT-Cas12a-[ED] coupled to a CRISPR array consisting of either long (20 bp) or short (15 bp) spacer sequences enables orthogonal gene editing and transcriptional gene regulation.

B. Quantification of relative *RAB5A* or *PIK3C3* RNA expression and gene editing efficiencies in cells expressing either long (20 bp) or short (15 bp) spacers in combination with either SiT-Cas12a-[Repr] or ddCas12a-[Repr]. Values represent mean ± s.e.m., n = 3 independent experiments.

C. Quantification of relative RNA expression and gene editing efficiencies for 2 distinct genes (*ASCL1* and *IL1B*) in cells expressing long (20 bp) or short (15 bp) spacers in combination with either SiT-Cas12a-[Activ] or ddCas12a-[Activ]. Values represent mean ± s.e.m., n = 3 independent experiments.

Figure 6: Multiplexed orthogonal gene editing and transcriptional activation.

A. Quantification of relative RNA expression and gene editing efficiencies in cells expressing a large CRISPR array (25 crRNAs) harbouring both short (15 bp) and long (20 bp) spacers using SiT-Cas12a-[Repr]. Values represent mean ± s.e.m., n = 3 independent experiments.

B. Quantification of relative RNA expression and gene editing efficiencies in cells expressing a large CRISPR array (25 crRNAs) harbouring both short (15 bp) and long (20 bp) using SiT-Cas12a-[Activ]. Values represent mean ± s.e.m., n = 3 independent experiments.

Methods:

Mammalian cell culture.

Human embryonic kidney 293T (HEK 293T) cell line (SIGMA-Aldrich) was maintained in Dulbecco's modified Eagle's Medium (DMEM) (SIGMA) supplemented with 10% FBS (HyClone) at 37°C with 5% CO_2.

Transient transfection.

HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transient transfections were performed using either 0.6 µg or 1 µg of plasmid DNA per well.

Plasmids.

All plasmids were generated using either restriction enzyme-based cloning, Golden Gate Cloning or Gibson Assembly. SiT-Cas12a constructs for multiplexed genome editing were generated by replacing the U6-sgRNA from pY026 (Addgene: 84741) with the Triplex sequence and 2 DR sequences, separated by 2 BsmBI restriction sites. SiT-Cas12a constructs for transcriptional control were generated by replacing the U6-gRNA and Cas9-Puromycin resistance from lentiCRISPR v2 (Addgene: 52961) with AsCas12a, Triplex sequence and 2 DR sequences, separated by 2 SapI restriction sites. Constructs for production of lentivirus were generated by replacing the U6-gRNA, Cas9-Puromycin, and WPRE element from lentiCRISPR v2 (Addgene: 52961) with a CMV promoter together with either *EGFP* or AsCas12a, Puromycin, Triplex sequence, 2 DR sequences, separated by 2 BsmBI restriction sites and/or a WPRE element and/or a polyadenylation sequence in inverted orientation. Depending on the experiment setup, either a CMV or an EF1a promoter was used. The ddCas12a and rdCas12a mutants were generated by site-directed mutagenesis through the substitution E993A or H800A, respectively. Transcriptional control elements were amplified from gene fragments (IDT) and integrated 3' of AsCas12a using a BamHI restriction site. Assembly of CRISPR arrays was performed by Golden Gate Cloning via BsmBI or SapI using either annealed oligonucleotides (IDT), for single spacers or small arrays (2 to 4 spacers), or gene fragments (IDT) with flanking Type IIS restriction sites for medium (10 spacers) and large (20-25 spacers) arrays. The tetracycline/doxycycline inducible plasmid was constructed by exchanging the constitutive promoter of SiT-Cas12a with tetracycline response element consisting of 5 repeats of bacterial TetO sequence upstream of a minCMV promoter by Gibson assembly. SiT-Cas12a-[Cond] was generated

by adding a Lox-Stop-Lox (LSL) cassette between the CMV promoter and Cas12a by Gibson assembly. DNA and spacer target sequences were listed in Supplemental File. Relevant plasmids used in this study will be deposited to AddGene.

Large CRISPR array (10-20-25 spacers) assembly.

Large CRISPR arrays containing either 10, 20 or 25 spacer sequences were assembled based on previous procedures[40]. Double stranded DNA fragments encoding for 4-6 crRNAs were purchased from IDT Technologies (gBlock or custom gene synthesis services) and amplified by primers containing type IIS restriction site (BsmBI or SapI). Type IIS recognition sites were designed to allow cleavage, at different positions, on the direct repeat (DR) sequence. This procedure enables both generation of non-identical 5′ or 3′ ends and reduction of repetitive elements (in our case DR) which prohibit the chemical synthesis of double-stranded crRNAs fragments by IDT Technologies. Overhangs generated by type IIS restriction enzyme digestion were intended to be complementary, enabling assembly of a large CRISPR array with defined directionality. DNA fragments encoding for Cas12a crRNAs and the vector backbone were assembled by standard Golden Gate Cloning. In brief, simultaneous type II enzymatic digestion using either BsmBI (Thermo Scientific) or SapI (Thermo Scientific) and ligation using T7 DNA ligase (NEB) in 1xT4 Ligase buffer (NEB) reaction was performed. The reaction was incubated for 30 cycles (37°C for 5 min; 16°C for 5 min) followed by 55°C for 10 min. Ligation reaction was transformed into chemical competent E. coli Stbl3 bacteria strain to avoid potential plasmid recombination. Bacteria cells were plated on agar plates supplemented with ampicillin (100 mg/l). Single colonies were cultivated overnight in liquid LB supplemented with ampicillin (100 mg/l) and DNA were isolated. Correct assembly of CRISPR array was verified by SANGER sequencing.

Inducible gene editing.

HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transient transfections were performed using a total of 0.6 µg (0.3 µg of SiT-Cas12a-[Ind] and 0.3 µg of either rtTA or tTA-Advanced (Clontech) plasmid DNA per well and Doxycycline (Sigma) was added (final concentration: 0, 0.01, 0.10, 1.00 µg/ml). Quantification of gene editing was performed 72 hours post-transfection.

Quantification of gene editing.

564 5 x 10^4 HEK 293T cells per well were seeded in 24-well-plates and transfected with 1 µg of DNA
565 plasmid. 72 hours post-transfection, cells were harvested and lysed in QE buffer (1 mM $CaCl_2$, 3
566 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 10 mM Tris pH 7.5, 0.2 mg/ml Proteinase K) using
567 the following temperature conditions: 65°C (15 min), 68°C (15 min) and 98°C (10 min). Genomic
568 DNA was used as a template for PCR-based amplification of targeted genomic regions using
569 Phusion flash polymerase (Thermo Scientific) and primers specific for each target site
570 (Supplementary material file (Indel analyses primer list)). PCR amplicons were purified using the
571 Zymo PCR purification Kit (Zymo Research) and quantified using Nanodrop 3000 (Thermo
572 Scientific).
573 In order to generate heteroduplex DNA fragments, 250 ng purified PCR amplicons were mixed with
574 10x annealing buffer (500 mM $NaCl_2$, 100 mM $MgCl_2$, 100 mM Tris-HCl) and incubated for 10
575 min at 95°C followed by ramping 95°C to 85°C with 1.34°C/s, 85°C to 75°C with 0.2°C/s, 75°C to
576 65°C with 0.2°C/s, 65°C to 55°C with 0.2°C/s, 55°C to 45°C with 0.2°C/s, 45°C to 35°C with
577 0.2°C/s and 35°C to 25°C with 0.2°C/s. Heteroduplex DNA were treated with Surveyor enhancer
578 and Surveyor nuclease from Surveyor Mutation Detection Kit (IDT) in presence of 15 mM $MgCl_2$
579 according to manufacturer's protocol and separated on 2% E-Gel (Thermo Scientific). Separated
580 cleavage products were imaged using Gel DOC EZ imager (Biorad) and quantified using Image Lab
581 software (Biorad). The percentage of heteroduplex DNA formation was quantified using the
582 formula: (1-(1-(b/(a+b))*0.5))*100 with a being equivalent to the integrated intensity of uncut DNA
583 fragments and b being equivalent to the sum of the integrated intensity of all cleavage products.
584
585 ***FACS analyses.***
586 1.2 x 10^5 HEK 293T cells were plated in 24-well-plate and transfected with 600 ng plasmid. 48 hr
587 after transfection, cells were split and 120 hours post-transfection, cells were harvested (using PBS
588 supplemented with 0.5 mM EDTA) and fixed in 1.8% PFA (Electron Microscopy Sciences). Fixed
589 cells were stained using conjugated antibodies CD166 conjugated PE (Miltenyi Biotec 130-106-
590 575, flow cytometry dilution (FC) 1:11); CD47 conjugated FITC (Miltenyi Biotec 130-101-344, FC
591 1:11); CD97 conjugated APC-Vio770 (Miltenyi Biotec 130-105-526, FC 1:11). Cytometric analysis
592 was performed using 5-color Fortessa (BD). Data from 10000 events was collected and analysed by
593 FlowJo (BD). Cell debris was removed by SSC-A/FSC-A gating and fluorescent intensity (FITC,
594 PE or APC-Vio770) was measured. Cells transfected with empty plasmids were used as positive
595 controls. Non-transfected cells were used as a negative control. Percentage of negative cells was
596 calculated by gating on the positive control cell population. Quantification of EGFP-positive cells
597 and EGFP expression of infected cells (72 hours post infection) were performed by harvesting HEK

293T cells (using PBS supplemented with 0.5 mM EDTA) and fixed in 1.8% PFA (Electron Microscopy Sciences). Data from 10000 events was collected and analysed by FlowJo (BD). Cell debris was removed by SSC-A/FSC-A gating and fluorescent intensity (EGFP) was measured. Non-infected cells were used as a negative control. Both percentage of positive cells and EGFP expression (median fluorescence intensity) were calculated.

Quantification of mRNA expression.

Gene expression analyses were conducted 48 hours after transfection according to a previously established protocol[3]. In brief, RNA was isolated using Quick RNA Miniprep Plus kit (Zymo Research), followed by reverse transcription of 500 ng RNA using Qscript cDNA supermix (Quantabio). A qPCR reaction was performed using Fast Plus EvaGreen qPCR master mix (Biotium) according to manufactory protocol (primers used are indicated in the Supplementary material file (qPCR primer list). Quantification of RNA expression was normalized based on expression of GAPDH and calculated using $\Delta\Delta Ct$[41]. For samples with a nearly undetectable amount of mRNA, Ct values exceeding 45, an arbitrary cycle number of 45 was assigned.

Small RNA-seq library preparation.

1.2 x 10^7 HEK 293T cells were plated in a 150 mm dish (Thermo Scientific) and transfected with 30 µg of plasmid. After 48 hours, purification of small RNAs was conducted using Quick RNA Miniprep Plus kit (Zymo Research) according to the manufacturer's instructions. Small RNA library preparation was performed in line with a previous published protocol[42]. In brief, residual genomic DNA was removed by DNase I digestion. 20 µg of small RNAs were dissolved in 39.5 µl water and denatured for 5 minutes at 65°C. After cooling on ice for 5 minutes, 5 µl 10X DNase I buffer including $MgCl_2$ (NEB), 10 units Superase-In RNase Inhibitor (Thermo Scientific) and 5 units DNaseI (NEB) were added and incubated for 45 minutes at 37°C. Purification of this pure small RNA fraction was conducted using Quick RNA Miniprep Plus kit (Zymo Research) and quantified using Nanodrop 3000 (Thermo Scientific). To capture 5' phosphorylated crRNA, an additional 5' phosphorylation step was performed. RNA samples, treated with 10 units DNaseI (NEB), were denatured for 2 minutes at 90°C and stored on ice for 5 minutes. Subsequently, 20 units of T4 PNK (Thermo Scientific) together with 10 units of Superase-In RNase Inhibitor (Thermo Scientific) were added in the presence of T4 PNK buffer (Thermo Scientific) to a final volume of 50 µl. After 6 hours of incubation at 37°C, an additional 10 units of T4 PNK (Thermo Scientific) and 10 mM ATP (2 mM final concentration) (Thermo Scientific) were added to the samples and incubated for 1 hour at 37°C. Purification of 5' phosphorylated RNA fractions were

performed using Quick RNA Miniprep Plus kit (Zymo Research) and quantified. As T4 PNK treatment enriched transcript with 5' pyrophosphates (PP) thereby interfering with the following step of small RNA library preparations, 5' PP were removed by Tobacco Acid Pyrophosphatase (TAP) treatment (Epicentre). In a total volume of 20 µl, 10 units of TAP, 2 µl TAP buffer, 10 units of Superase-In RNase Inhibitor (Thermo Scientific) and 10 µg of PNK-treated small RNA were mixed and incubated for 1 hour at 37°C and purified using Quick RNA Miniprep Plus kit (Zymo Research). These enriched pre-crRNA transcripts were used as template for the preparation of small RNA library. Library preparation was performed using NEBNext multiplex small rna library prep set (set 1) (NEB), separated on a 6% polyacrylamide gel and purified using SpinX columns (Costar). This step ensured enrichment of mature crRNAs (<60nt). 5 Million reads were sequenced for each sample using Illumina NextSeq 500 (Illumina) and analysed as described below.

Mature crRNA quantification.

Adapter sequences (described in NEBNext multiplex small rna library prep set (set 1)) were clipped from sequencing reads using CutAdapt v1.10 [43]. Only reads ≥ 10 nt were retained. Reads were mapped to the reference sequence of the entire crRNA array, using Bowtie v1.2.2 [44], with parameters "--norc -l 28 -n 2 -m 1 --best --strata". For the quantification of mature crRNAs, a BED file containing the coordinates of each crRNA within the array was created and used to extract the number of reads mapping onto each crRNA using the intersectBed utility from BEDTools v2.27.0 [45]. Raw counts were then normalized to the number of non-crRNA reads mapping on human snoRNAs, through the following formula: Ni = (ci/S)*10^6; where Ni and ci are respectively the normalized count and the raw read count for crRNA i, and S is the count of reads mapping on human snoRNAs, a small RNA population largely recognized for performing housekeeping function and used to normalize human microRNA (miRNA) expression [46,47]. Finally, a fold change over control condition was calculated to quantify changes across distinct experiments.

Deep sequencing-based CD47 editing analyses.

Analyses of deep sequencing data derived from HEK 293T cells stably expressing SiT-Cas12a harbouring 10 distinct crRNAs were performed using CRISPResso2 tool[48] *CD47* edited region was amplified, size selected between 200 bp and 500 bp and sequenced. Analysis was performed by CRISPResso2 tool [48] using the following parameters: -w 10 –cleavage offset 1 –S 20; -w 10 –cleavage offset 1 –g.

Statistics.

Unless otherwise noted, experiments in this study were performed using three independent biological replicates. Tests for determination of statistical significance were not implemented.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The datasets generated during the current study are available in the NCBI Sequence Read Archive (BioProject ID PRJNA530879).

Methods-only References:

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Figure 1

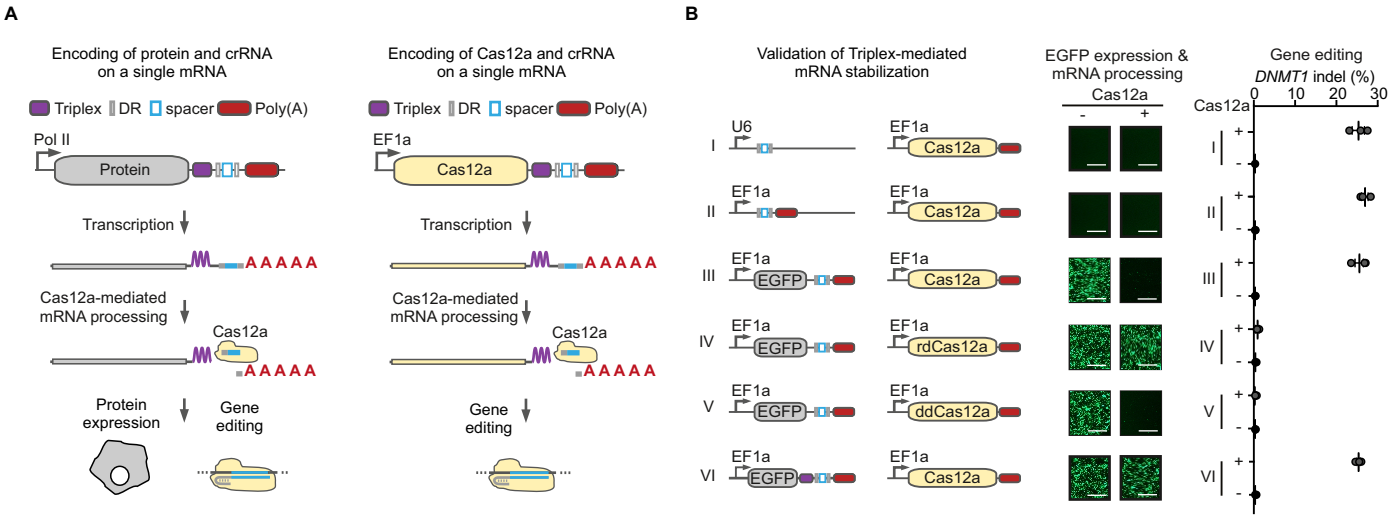

A
Encoding of protein and crRNA on a single mRNA
Triplex
DR
spacer
Poly(A)
Pol II
Protein
Transcription
AAAAA
Cas12a-mediated mRNA processing
Cas12a
Protein expression
Gene editing
Encoding of Cas12a and crRNA on a single mRNA
EF1a
Cas12a
B
Validation of Triplex-mediated mRNA stabilization
U6
EF1a
EGFP
rdCas12a
ddCas12a
EGFP expression & mRNA processing
Cas12a
Gene editing
DNMT1 indel (%)
0
10
20
30

Figure 2

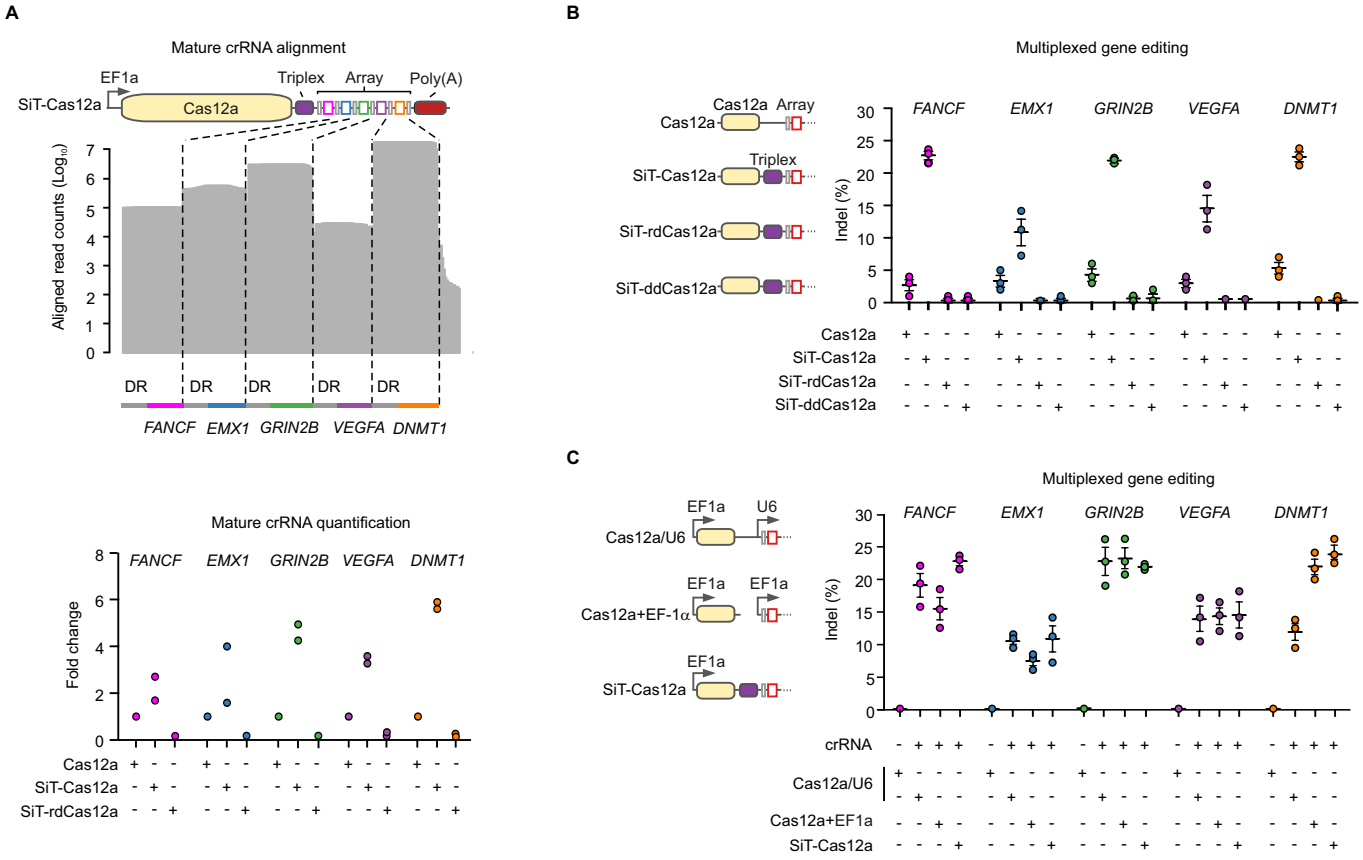

A
Mature crRNA alignment
EF1a
Triplex
Array
Poly(A)
SiT-Cas12a
Cas12a
Aligned read counts (Log10)
DR
FANCF
EMX1
GRIN2B
VEGFA
DNMT1
Mature crRNA quantification
Fold change
Cas12a
SiT-Cas12a
SiT-rdCas12a
B
Multiplexed gene editing
Cas12a Array
SiT-rdCas12a
SiT-ddCas12a
Indel (%)
C
Cas12a/U6
U6
Cas12a+EF-1α
crRNA
Cas12a+EF1a
SiT-ddCas12a

Figure 3

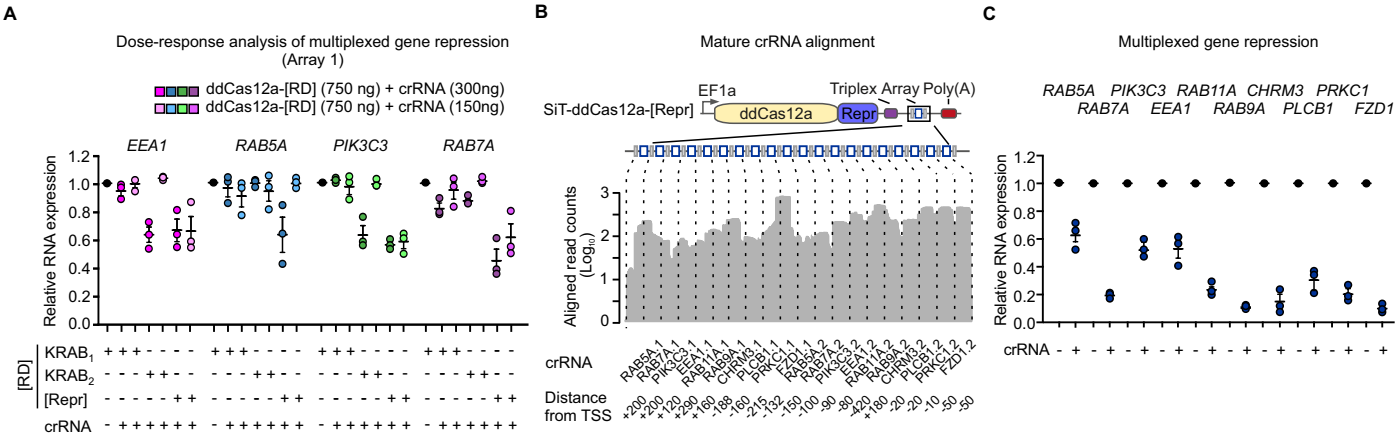

A
Dose-response analysis of multiplexed gene repression
(Array 1)
ddCas12a-[RD] (750 ng) + crRNA (300ng)
ddCas12a-[RD] (750 ng) + crRNA (150ng)
EEA1
RAB5A
PIK3C3
RAB7A
Relative RNA expression
[RD]
KRAB1
KRAB2
[Repr]
crRNA
B
Mature crRNA alignment
EF1a
Triplex
Array
Poly(A)
SiT-ddCas12a-[Repr]
ddCas12a
Repr
Aligned read counts
(Log10)
crRNA
Distance
from TSS
C
Multiplexed gene repression
RAB5A PIK3C3 RAB11A CHRM3 PRKC1
RAB7A EEA1 RAB9A PLCB1 FZD1
Relative RNA expression
crRNA

Figure 4

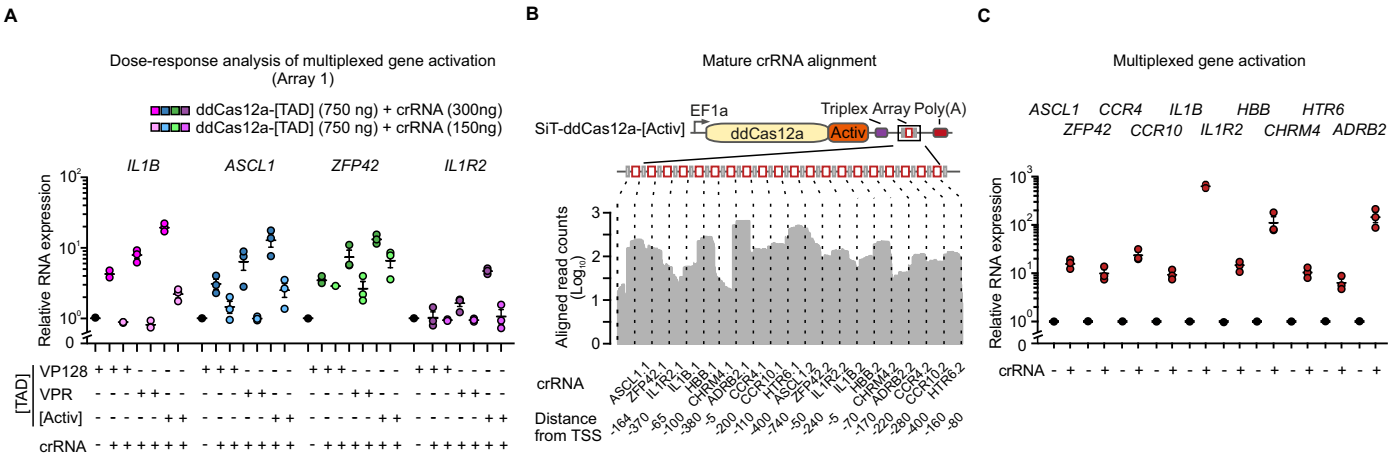

A
Dose-response analysis of multiplexed gene activation
(Array 1)
ddCas12a-[TAD] (750 ng) + crRNA (300ng)
ddCas12a-[TAD] (750 ng) + crRNA (150ng)
IL1B
ASCL1
ZFP42
IL1R2
Relative RNA expression
[TAD]
VP128
VPR
[Activ]
crRNA
B
Mature crRNA alignment
EF1a
SiT-ddCas12a-[Activ]
ddCas12a
Activ
Triplex Array
Poly(A)
Aligned read counts
(Log10)
crRNA
Distance
from TSS
C
Multiplexed gene activation
ASCL1 CCR4 IL1B HBB HTR6
ZFP42 CCR10 IL1R2 CHRM4 ADRB2
Relative RNA expression
crRNA

Figure 5

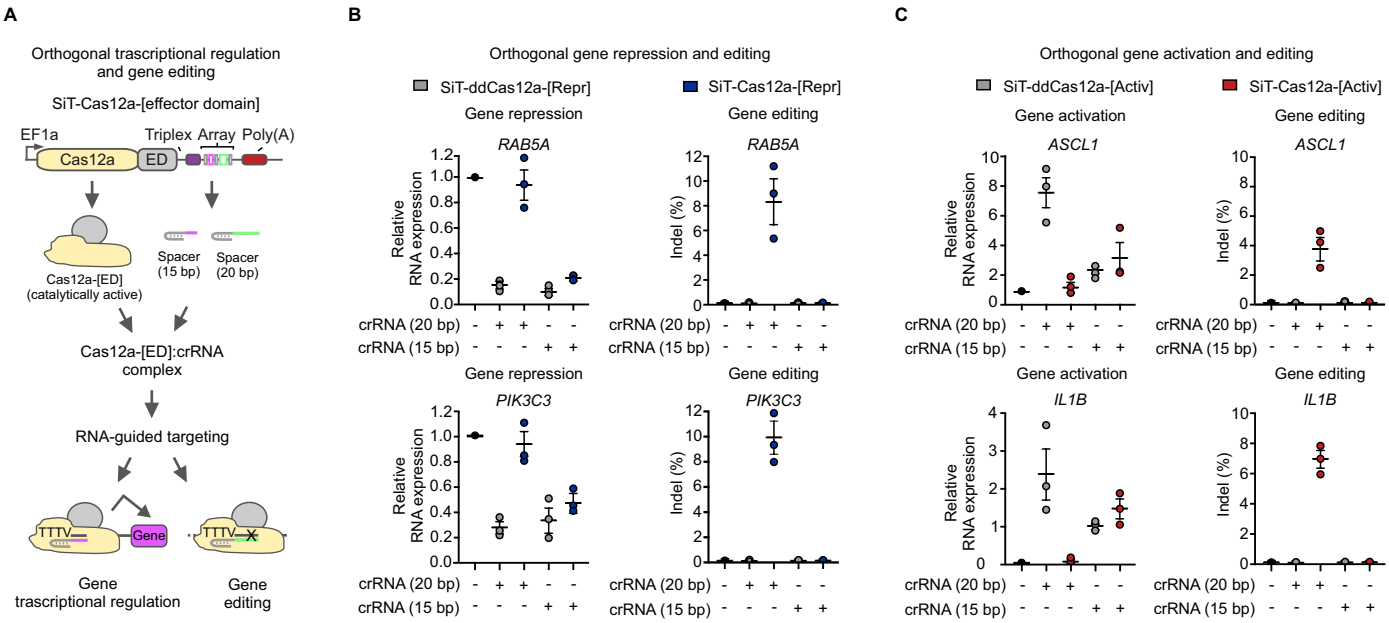
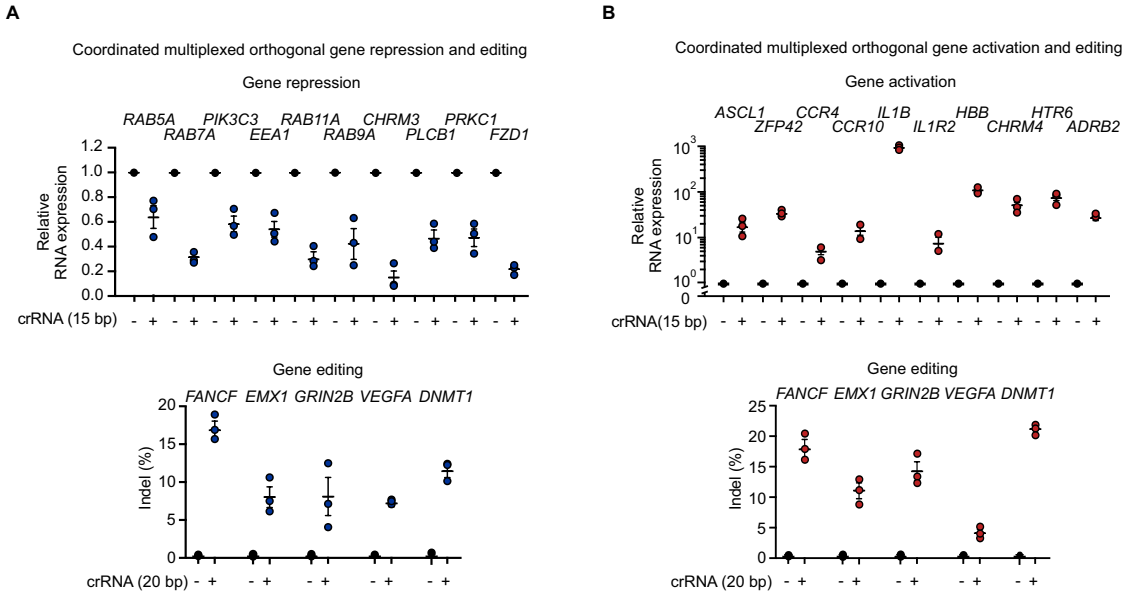
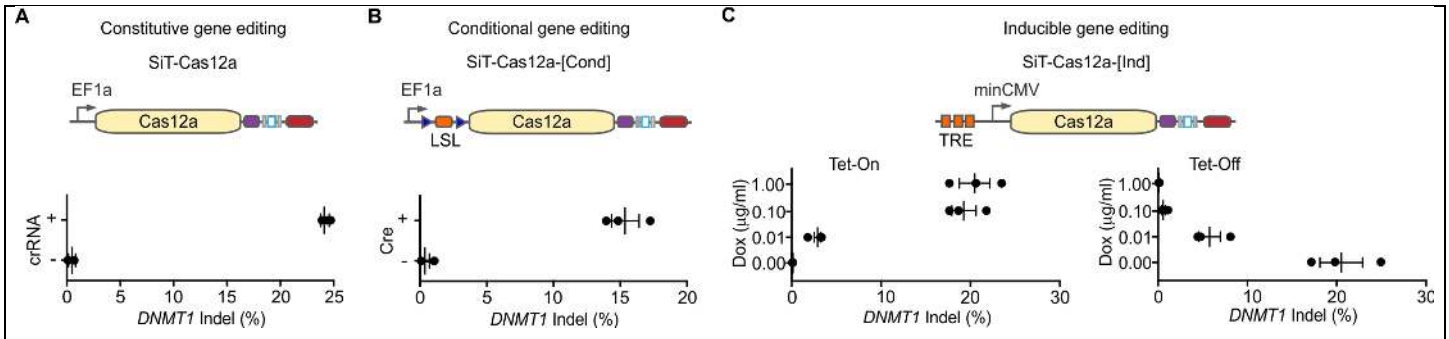

A
Orthogonal trascriptional regulation and gene editing
SiT-Cas12a-[effector domain]
EF1a
Triplex Array
Poly(A)
Cas12a
ED
Cas12a-[ED] (catalytically active)
Spacer (15 bp)
Spacer (20 bp)
Cas12a-[ED]:crRNA complex
RNA-guided targeting
TTTV
Gene
Gene trascriptional regulation
Gene editing
B
Orthogonal gene repression and editing
SiT-ddCas12a-[Repr]
SiT-Cas12a-[Repr]
Gene repression
Gene editing
RAB5A
PIK3C3
Relative RNA expression
Indel (%)
crRNA (20 bp)
crRNA (15 bp)
C
Orthogonal gene activation and editing
SiT-ddCas12a-[Activ]
SiT-Cas12a-[Activ]
Gene activation
Gene editing
ASCL1
IL1B
Relative RNA expression
Indel (%)
crRNA (20 bp)
crRNA (15 bp)

Figure 6


A
Coordinated multiplexed orthogonal gene repression and editing
Gene repression
RAB5A
RAB7A
PIK3C3
EEA1
RAB11A
RAB9A
CHRM3
PLCB1
PRKC1
FZD1
Relative RNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
crRNA (15 bp) - + - + - + - + - + - + - + - + - + - +
Gene editing
FANCF EMX1 GRIN2B VEGFA DNMT1
Indel (%)
20
15
10
5
0
crRNA (20 bp) - + - + - + - + - +
B
Coordinated multiplexed orthogonal gene activation and editing
Gene activation
ASCL1
ZFP42
CCR4
CCR10
IL1B
IL1R2
HBB
CHRM4
HTR6
ADRB2
Relative RNA expression
10^3
10^2
10^1
10^0
0
crRNA(15 bp) - + - + - + - + - + - + - + - + - + - +
Gene editing
FANCF EMX1 GRIN2B VEGFA DNMT1
Indel (%)
25
20
15
10
5
0
crRNA (20 bp) - + - + - + - + - +



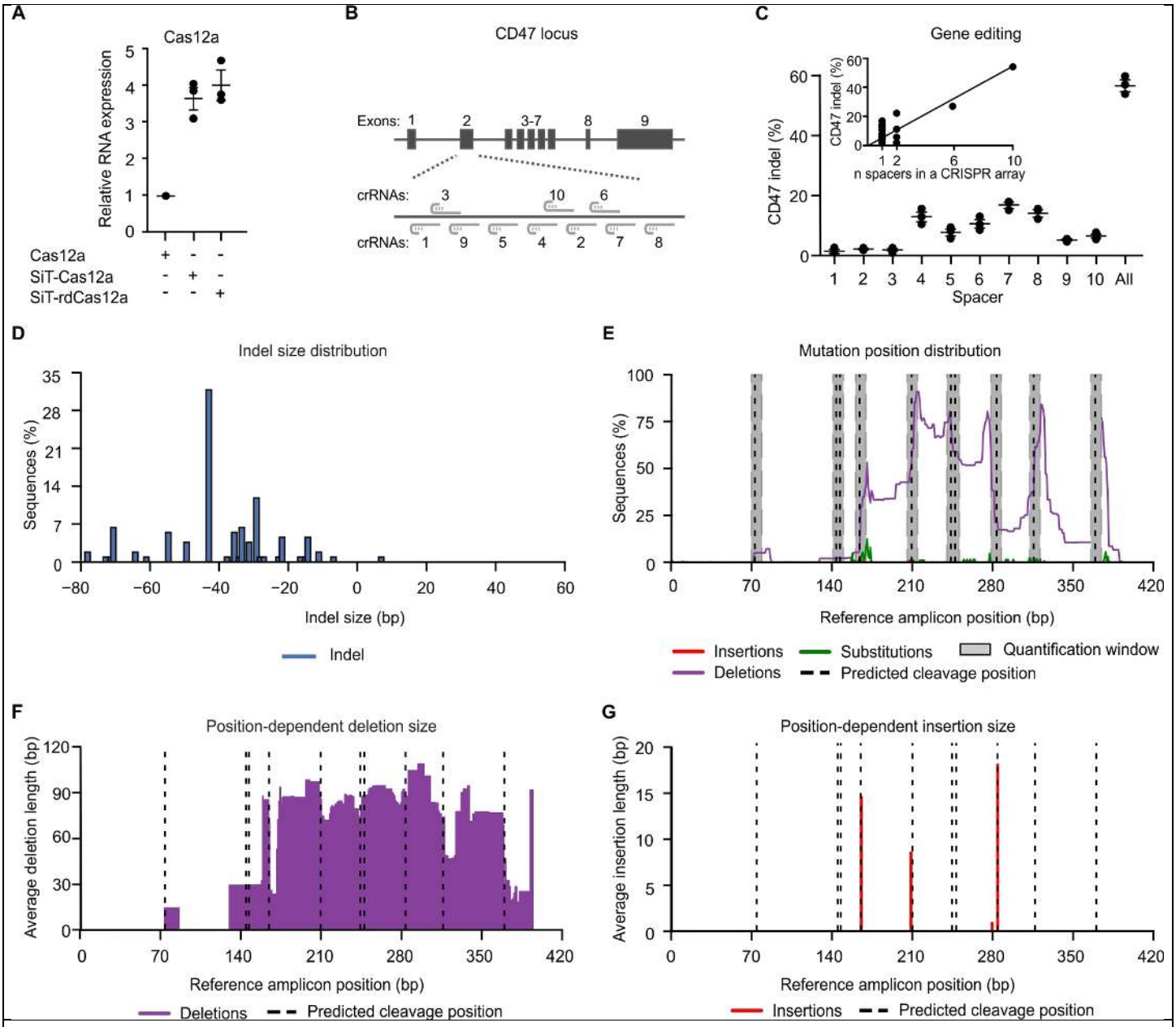
Supplementary Figure 1

Constitutive, conditional and inducible gene editing with SiT-Cas12a.

A. Quantification of gene editing efficiencies by constitutive SiT-Cas12a construct. Values represent mean ± s.e.m., n = 3 independent experiments.

B. Quantification of gene editing efficiencies by conditional SiT-Cas12a (SiT-Cas12a-[Cond]) construct. Values represent mean ± s.e.m., n = 3 independent experiments.

C. Quantification of gene editing efficiencies by inducible SiT-Cas12a (SiT-Cas12a-[Ind]) construct in either Tet-Off or Tet-On configuration. Values represent mean ± s.e.m., n = 3 independent experiments.



Supplementary Figure 2

Quantification of SiT-Cas12a-induced gene mutations

A. Quantification of relative Cas12a mRNA expression in either Cas12a, SiT-Cas12a or SiT-rdCas12a expressing cells. Values represent mean ± s.e.m., n = 3 independent experiments.

B. Schematic of the *CD47* locus and the target position of crRNA used.

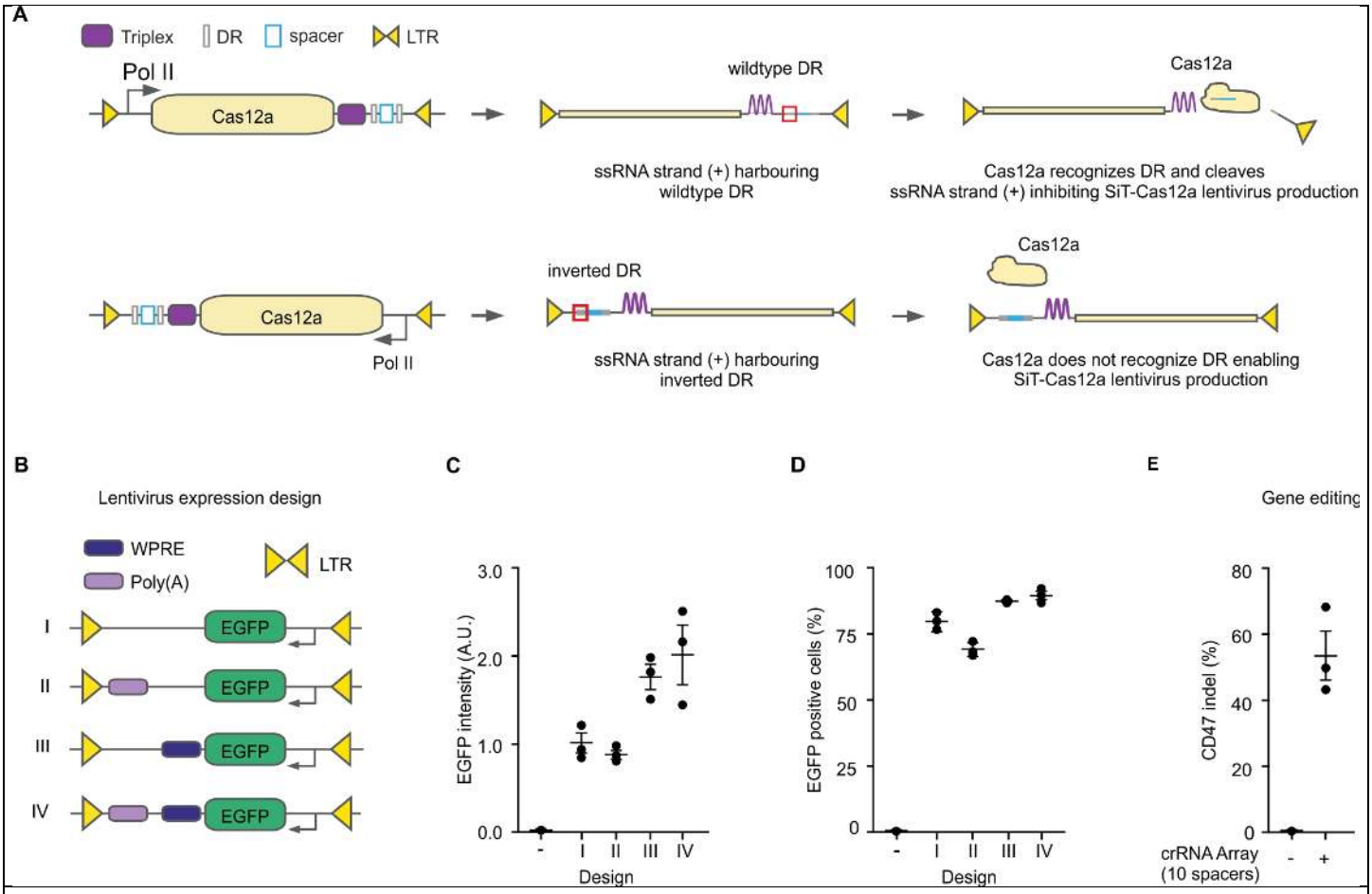
C. Quantification of SiT-Cas12a gene editing efficiencies using either single (crRNA 1-10), double (crRNAs 1-2, 3-4, 5-6, 7-8, 9-10), or multiple (comprising crRNA from 4 to 9 or from 1 to 10) spacer sequences targeting the *CD47* gene. Values represent mean ± s.e.m., n = 3 independent experiments.

D. Quantification of indel size distribution generated in *CD47* locus using a CRISPR array containing 10 distinct spacer sequences. Similar results for n = 3 independent experiments.

E. Quantification of mutation position distribution generated in *CD47* locus using a CRISPR array containing 10 distinct spacer sequences. Similar results for n = 3 independent experiments.

F. Quantification of position-dependent deletion size generated in *CD47* locus using a CRISPR array containing 10 distinct spacer sequences. Similar results for n = 3 independent experiments.

G. Quantification of position-dependent insertion size generated in *CD47* locus using a CRISPR array containing 10 distinct spacer sequences. Similar results for n = 3 independent experiments.



Supplementary Figure 3

Generation of SiT-Cas12a-based lentiviral vector

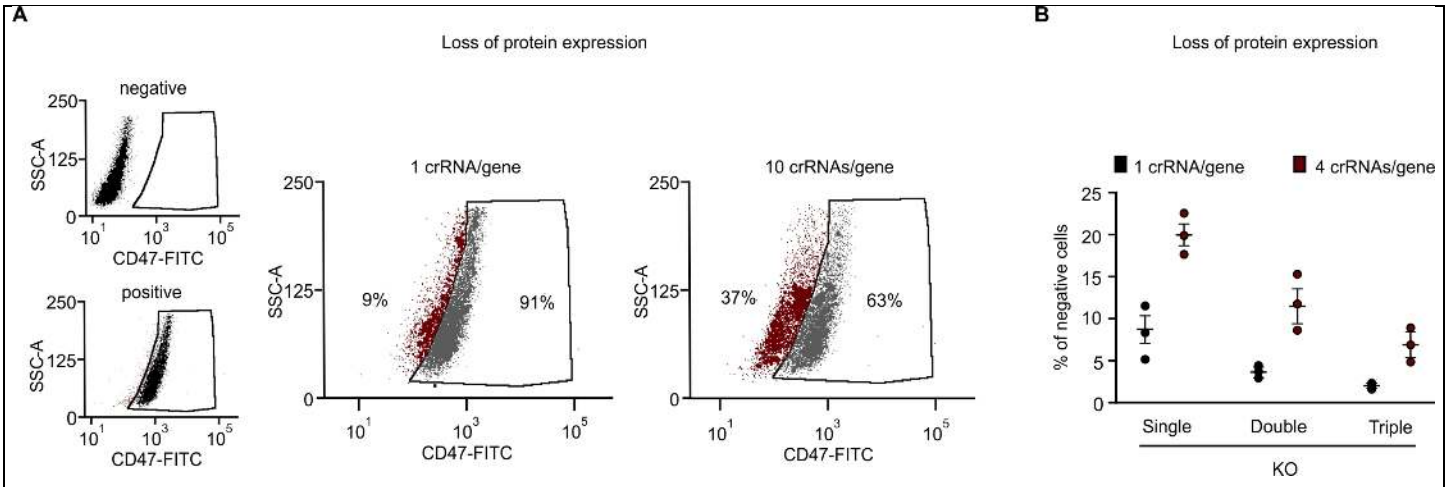
A. Schematic of a single transcript lentivirus architecture containing both Cas12a and a CRISPR array in different orientations and its effect on lentivirus production. The transcript encodes for: Cas12a (yellow rectangle); Triplex, a tertiary RNA structural motif (small purple rectangle); direct repeat (DR, grey square); spacer (blue square). The (+)-strand RNA contains a recognizable direct repeat prone to Cas12a-mediated cleavage, preventing functional assembly of virions. On the contrary, direct repeats positioned in inverted orientation are not recognized by Cas12a enabling lentivirus production.

B. Schematic of a single transcript lentivirus architecture containing both EGFP and mRNA stabilizer sequences in inverted orientation. The transcript encodes for: EGFP (green rectangle); Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) (small blue rectangle); and a polyadenylation sequence (Poly(A), lilac rectangle).

C. Validation of single transcript lentivirus architecture in the inverted orientation. Values represent mean ± s.e.m., n = 3 independent experiments.

D. Quantification of percentage of EGFP-positive cells by single transcript lentivirus architectures in the inverted orientation. Values represent mean ± s.e.m., n = 3 independent experiments.

E. Quantification of gene editing efficiencies by SiT-Cas12a-based lentivirus harbouring 10 distinct spacer sequences targeting the *CD47* gene. Values represent mean ± s.e.m., n = 3 independent experiments.

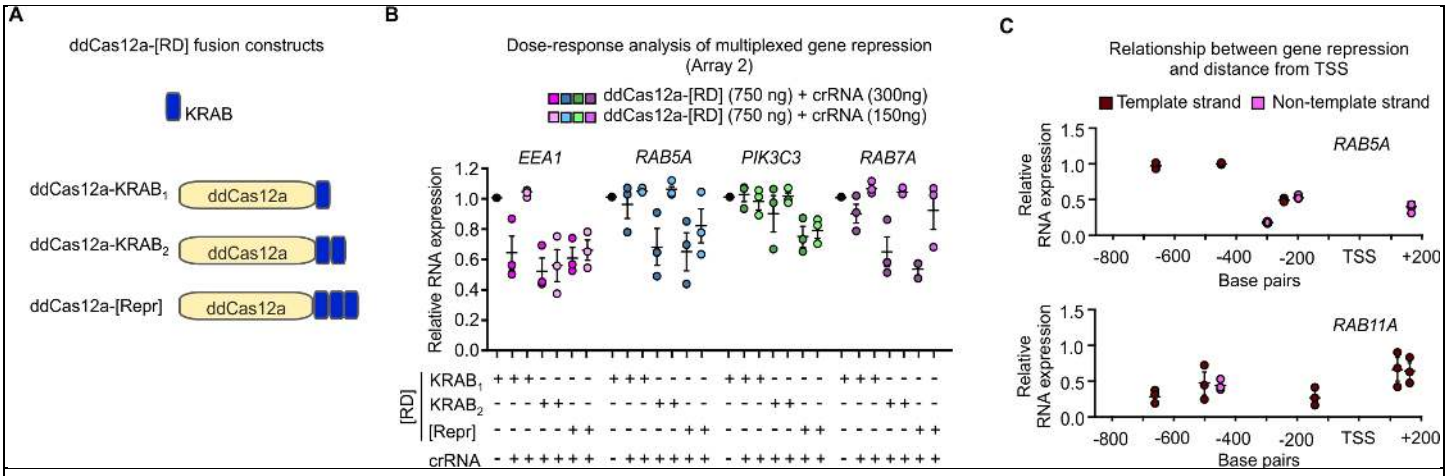


Supplementary Figure 4

Multiplexed gene editing with SiT-Cas12a.

A. Quantification of residual CD47-positive cells after SiT-Cas12a-mediated gene editing using single or multiple (10) crRNAs targeting the *CD47* gene. Similar results for n = 3 independent experiments.

B. Quantification of single, double and triple-negative cells after SiT-Cas12a-mediated gene editing using single or multiple (4) crRNAs targeting the *CD47, CD97,* and *CD166* genes. Values represent mean ± s.e.m., n = 3 independent experiments.



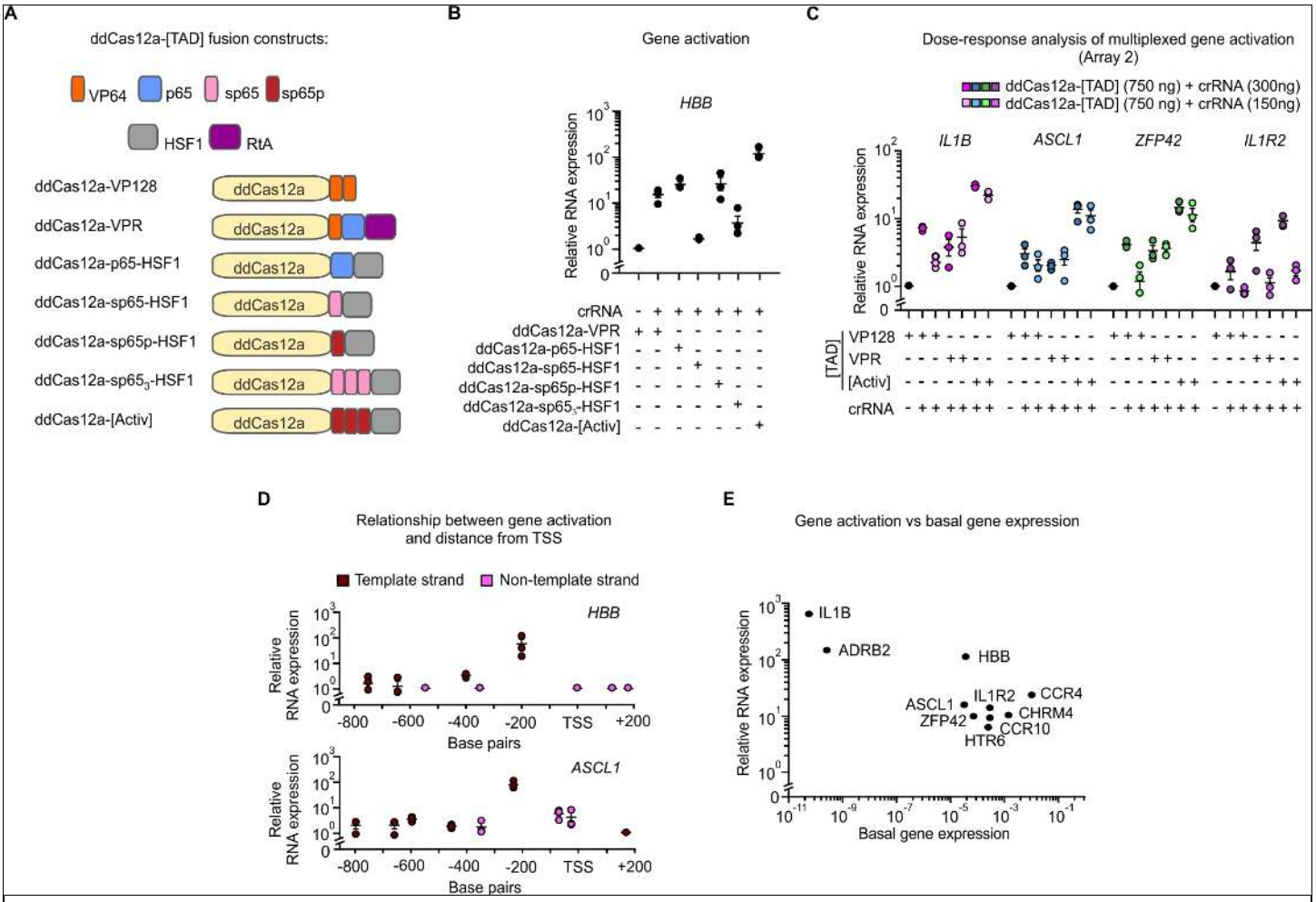
Supplementary Figure 5

Optimization and characterization of a SiT-Cas12a transcriptional repressor.

A. Schematic of Cas12a transcriptional repressors. Cas12a (yellow rectangle) and repression domain fused (RD, blue rectangle) are indicated.

B. Quantification of relative RNA expression for 4 distinct genes (*EEA1*, *RAB5*, *PIK3C3*, *RAB7A*) using ddCas12a fused with either 1 ($KRAB_1$), 2 ($KRAB_2$) or 3 ([Repr]) KRAB domains and in combination with different crRNA array concentrations. Values represent mean ± s.e.m., n = 3 independent experiments.

C. Quantification of relative RNA expression for 2 distinct genes (*RAB5A*, *RAB11A*) as a function of the distance between the target and the transcription start site (TSS). Values represent mean ± s.e.m., n = 3 independent experiments.



Supplementary Figure 6

Optimization and characterization of a SiT-Cas12a transcriptional activator.

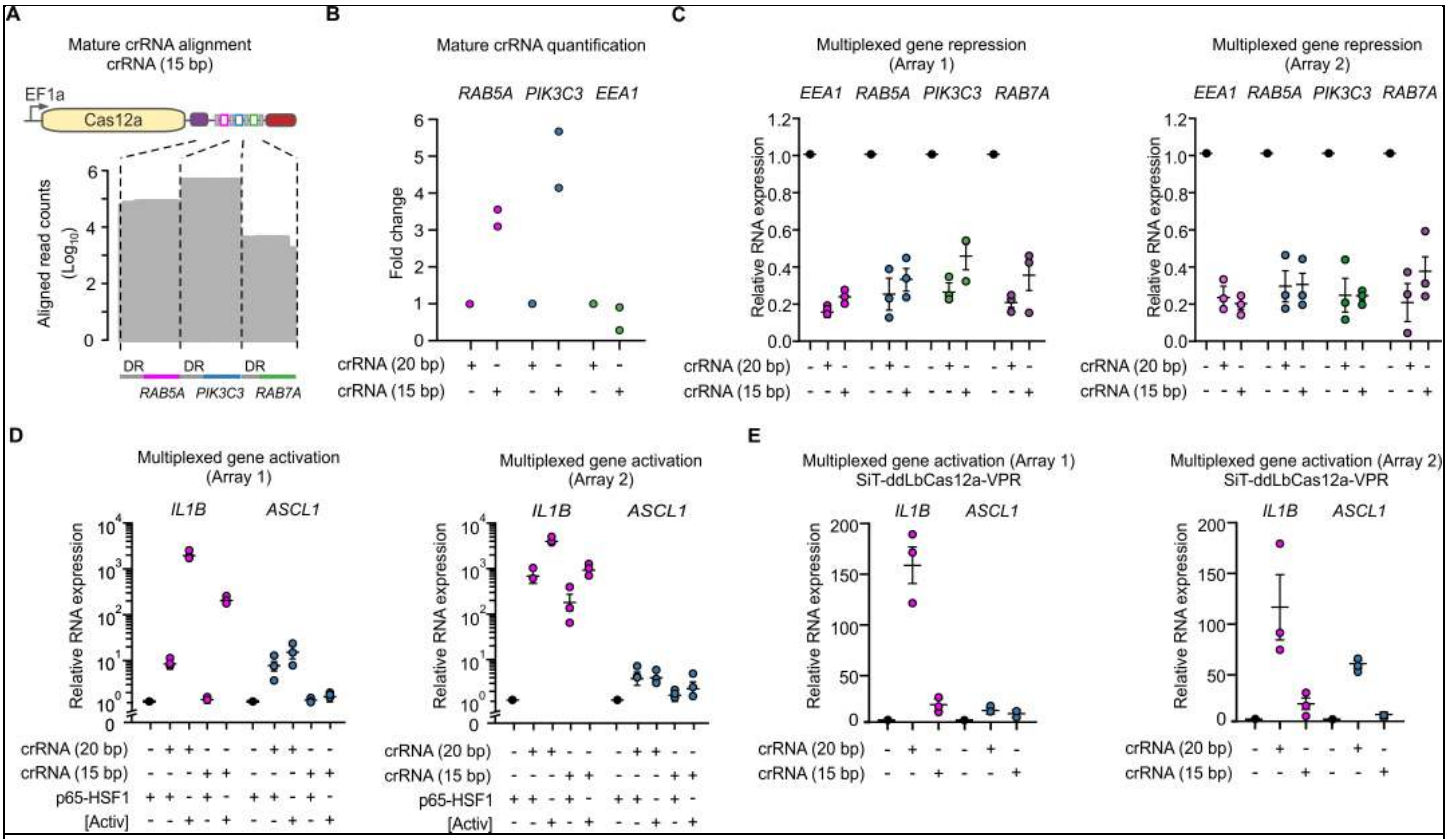
A. Schematic of Cas12a transcriptional activators. Cas12a (yellow rectangle) and transactivation domains fused (TAD) are indicated.

B. Quantification of relative *HBB* RNA expression using different Cas12a transcriptional activation domain fusion protein. Values represent mean ± s.e.m., n = 3 independent experiments.

C. Quantification of relative RNA expression for 4 distinct genes (*IL1B*, *ASCL1*, *ZFP42*, *IL1R2*) using ddCas12a fusion proteins (VP128, VPR, [Activ]) in combination with different crRNA array concentrations. Values represent mean ± s.e.m., n = 3 independent experiments.

D. Quantification of relative RNA expression of 2 distinct genes (*HBB*, *ASCL1*) as a function of the distance between the target and the transcription start site (TSS). Values represent mean ± s.e.m., n = 3 independent experiments.

E. Quantification of relative RNA expression as a function of basal gene expression for 10 distinct genes (*IL1B*, *ADRB2*, *HBB*, *IL1R2*, *CCR4*, *ASCL1*, *ZFP42*, *CHRM4*, *CCR10*, *HTR6*) induced by SiT-Cas12a-[Activ] and 20 distinct spacers targeting those 10 genes. Similar results for n = 3 independent experiments.



Supplementary Figure 7

Orthogonal genome engineering in the SiT-Cas12a context.

A. Small RNA-seq alignment showing mature short crRNAs (15 bp).

B. Quantification of mature crRNAs. n = 2 independent experiments.

C. Quantification of relative RNA expression of 4 distinct genes (*EEA1*, *RAB5*, *PIK3C3*, *RAB7A*) using SiT-ddCas12a-[Repr]. Results from 2 distinct CRISPR arrays are represented. Values represent mean ± s.e.m., n = 3 independent experiments.

D. Quantification of relative RNA expression of 2 distinct genes (*IL1B*, *ASCL1*) using either SiT-ddCas12a-p65-HSF1 or SiT-ddCas12a-[Activ]. Results from 2 distinct CRISPR arrays are represented. Values represent mean ± s.e.m., n = 3 independent experiments.

E. Quantification of relative RNA expression of 2 distinct genes (*IL1B*, *ASCL1*) using SiT-ddLbCas12a-VPR. Results from 2 distinct CRISPR arrays are represented. Values represent mean ± s.e.m., n = 3 independent experiments.

Supplementary Note 1

Single-transcript Cas12a construct:

AsCas12a-NLS-HAx3-Triplex-DR-BsmbI-BsmBI-DR

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Single-transcript Cas12a construct for Lentivirus production:

[CMV-AsCas12a-NLS-P2A-Puro-HAx3-Triplex-DR-BsmbI-BsmBI-DR-WPRE-Poly(A)] inverted

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Cas12a variants:

ddCas12a (E993A)

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ddLbCas12a (D832A)

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Single-transcript Cas12a – [TAD] construct:

AsCas12a-NLS-[Aktiv]-HAx3-Triplex-DR-SapI-SapI-DR

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Single-transcript Cas12a – [Repr] construct:

AsCas12a-NLS-[Repr]-HAx3-Triplex-DR-SapI-SapI-DR

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Transcriptional control elements:

KRAB

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KRAB$_2$

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[Repr]

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VP64$_2$

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p65

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sp65

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sp65$_3$

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sp65p$_3$

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HSF1

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RtA

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[Activ]

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CTTTTCAGCTCTCCTCAGCCAGATAAGCTCTGGAAGTAGTGGGCTTCCAAATGGGCTTGACGGTGACGAAGATTT
CTCTGATATAGCGGACATGGACTTCTCAGCTCTGCTCTCCCAGATTTCTTCAGGGTCCAGCGGCTTGCCCAACGG
CCTCGATGGCGACGAGGATTTTTCTGACATAGCAGACATGGACTTTTCCGCACTCCTCAGTCAGATTTCTTCTGG
AGGCGGTGGAAGCGGCTTCAGCGTGGACACCAGTGCCCTGCTGGACCTGTTCAGCCCCTCGGTGACCGTGCCCGA
CATGAGCCTGCCTGACCTTGACAGCAGCCTGGCCAGTATCCAAGAGCTCCTGTCTCCCCAGGAGCCCCCCAGGCCT
CCCGAGGCAGAGAACAGCAGCCCGGATTCAGGGAAGCAGCTGGTGCACTACACAGCGCAGCCGCTGTTCCTGCTG
GACCCCGGCTCCGTGGACACCGGGAGCAACGACCTGCCGGTGCTGTTTGAGCTGGGAGAGGGCTCCTACTTCTCC
GAAGGGGACGGCTTCGCCGAGGACCCCACCATCTCCCTGCTGACAGGCTCGGAGCCTCCCAAAGCCAAGGACCCC
ACTGTCTCC

Direct Repeat (DR)

AATTTCTACTCTTGTAGAT

Gene editing

Gene	Target Sequence
CD47.1	ATTAAATAGTAGCTGAGCTGATC
CD47.2	GCACTACTAAAGTCAGTGGGGAC
CD47.3	GTAATGACACTGTCGTCATTCCA
CD47.4	GAGCTCCATCAAAGGTGTAAATA
CD47.5	CGTATACTTCAGTAGTGTTTTGT
CD47.6	GTAGTGCAAAAATTGAAGTCTCA
CD47.7	CTGTGTGTGAGACAGCATCACTC
CD47.8	GCTCGATGATCGTTTCACCTTCT
CD47.9	GTGCCTCCATATTAGTAACAAAG
CD47.10	ATGGAGCTCTAAACAAGTCCACT
CD97.1	AGCAGGGCTTCCTCTGGAGCTTC
CD97.2	TTGTGGTGCGCGTGTTCCAAGGC
CD97.3	CTGGCCGCCTTCTGCTGGATGAG
CD97.4	CGTGACTACCGTCTGGAAGCTCA
CD166.1	TAGAGGATCTGAAGGCAATAAAT
CD166.2	AGGCACCTACAATAGTCAAGGTG
CD166.3	TGTGCATGCTAGTAACTGAGGAC
CD166.4	ATCACTGATCCTTGCATTACTGA
DNMT1	CTGATGGTCCATGTCTGTTACTC
FANCF	GGCGGGGTCCAGTTCCGGGATTA
EMX1	TGGTTGCCCACCCTAGTCATTGG
GRIN2B	GTGCTCAATGAAAGGAGATAAGG
VEGFA	CTAGGAATATTGAAGGGGGCAGG

Transcriptional activator

Gene	Target Sequence
ZFP42.1	TTGAGCGCTCACCAC
ZFP42.2	CGTGCGGGCCGGGTG
ASCL1.1	GGGAGTGGGTGGGAG
ASCL1.2	CAATGGGACACCCAG
IL1B.1	CATGGTGATACATTT
IL1B.2	CTACTCCTTGCCCTT
IL1R2.1	CTTGGCCACTTCCCC
IL1R2.2	CCTATTTTTCTGTGA
ADRB2.1	GCAGTAAAGTCACAT
ADRB2.2	GTTACACTTCATGAA
CCR4.1	GCACATCTTCTTGGC
CCR4.2	ATTTTTGGGGAGATA
CCR10.1	CCCTACTCCACTTTG
CCR10.2	GTAAAATCCAGATCC
CHRM4.1	TCTCCCCTTCCTCCC
CHRM4.2	CATGTCTCCCCCCAT
HBB.1	TCTACCATAATTCAG
HBB.2	AAGTCCAACTCCTAA
HTR6.1	CCAACTCCTGGCTCC
HTR6.2	CCAGGGGCGGCTTTG

Transcriptional activator

Gene	Target Sequence
ASCL1.3 (15 bp)	GGGAGTGGGTGGGAG
ASCL1.3(20 bp)	GGGAGTGGGTGGGAGGAAGA
ASCL1.4(15 bp)	CAATGGGACACCCAG
ASCL1.4 (20 bp)	CAATGGGACACCCAGCCCCA
IL1B.3 (15 bp)	CATGGTGATACATTT
IL1B.3 (20 bp)	CATGGTGATACATTTGCAAA
IL1B.4 (15 bp)	CTACTCCTTGCCCTT
IL1B.4 (20 bp)	CTACTCCTTGCCCTTCCATG
IL1R2.3 (15 bp)	CTTGGCCACTTCCCC
IL1R2.3 (20 bp)	CTTGGCCACTTCCCCATCTG
IL1R2.4 (15 bp)	CCTATTTTTCTGTGA
IL1R2.4 (20 bp)	CCTATTTTTCTGTGACTCGC
ZFP42.3 (15 bp)	TTGAGCGCTCACCAC
ZFP42.3 (20 bp)	TTGAGCGCTCACCACGTGCC
ZFP42.4 (15 bp)	CGTGCGGGCCGGGTG
ZFP42.4 (20 bp)	CGTGCGGGCCGGGTGCCTGG

Transcriptional repression

Gene	Target Sequence
CHRM3.1	AGCCAACCCACCCCA
CHRM3.2	GTCTCAAGCACGCAG
FZD1.1	TGCCTGAGTAGTGCC
FZD1.2	CGCCGGGAAGCCGGA
PLCB1.1	CCAGCCAGTTGGGAT
PLCB1.2	CACGCTGGGTCAGGC
PRKC1.1	ACAGCTCGCGTGAAA
PRKC1.2	TCTGCCCCCGTAAGG
RAB11A.1	AAGAGGTAGTCGTAC
RAB11A.2	AAGGTGAGGCCATGG
RAB9A.1	GTACTCGCTGTCGCC
RAB9A.2	CCTGGCCCCGCCCCG
RAB7A.1	GTCTCCTCCTCGGCG
RAB7A.2	TAGCACGAGATCCAG
EEA1.1	ACAGAGGGTAAGAGA
EEA1.2	CAGCAGAAACTAGCA
PIK3C3.1	ACTACATCTATAGTT
PIK3C3.2	GGCGGGGAGTTCCGC
RAB5A.1	TCCTCCTCCGCCGCC
RAB5A.2	TTCGCGGGGCGGGGC

Transcriptional repression

Gene	Target Sequence
EEA1.3 (15 bp)	ACAGAGGGTAAGAGA
EEA1.3 (20 bp)	ACAGAGGGTAAGAGAGTGAA
EEA1.4 (15 bp)	ACCACCACCCGGCGC
EEA1.4 (20 bp)	ACCACCACCCGGCGCCGCCG
PIK3C3.3 (15 bp)	ACTACATCTATAGTT
PIK3C3.3 (20 bp)	ACTACATCTATAGTTGTGAC
PIK3C3.4 (15 bp)	CAGACGGTGCGATGG
PIK3C3.4 (20 bp)	CAGACGGTGCGATGGGGGAA
RAB5A.3 (15 bp)	TCCTCCTCCGCCGCC
RAB5A.3 (20 bp)	TCCTCCTCCGCCGCCGCCGC
RAB5A.4 (15 bp)	TTCGCGGGGCGGGGC
RAB5A.4 (20 bp)	TTCGCGGGGCGGGGCGAGGC
RAB5A.5 (15 bp)	GGTTCGTGAAGGAGC
RAB5A.5 (20 bp)	GGTTCGTGAAGGAGCCGGCG
RAB7A.3 (15 bp)	GTCTCCTCCTCGGCG
RAB7A.3 (20 bp)	GTCTCCTCCTCGGCGGGAGC
RAB7A.4 (15 bp)	TGGCCAAGACTCCAG
RAB7A.4 (20 bp)	TGGCCAAGACTCCAGGCCCG

Distance from promoter TSS

Gene	Target Sequence
HBB.1	CCAAAACCTAATAAGTAACT
HBB.2	TTAGCATGCATGAGCAAATT
HBB.3	GATTAAAACCTTCTGGTAAG
HBB.4	GTGCATCAACTTCTTATTTG
HBB.5	GAATCACAGCTTGGTAAGCA
HBB.6	AAGTCCAACTCCTAAGCCAG
HBB.7	CTTCTGACACAACTGTGTTC
HBB.8	TGATAGGCACTGACTCTCTC
HBB.9	TTGCCATGAGCCTTCACCTT
ASCL1.3	GAGCTGAATGGGACATTAGA
ASCL1.4	ACATAGTCCAGCACTTTTTT
ASCL1.5	CTCCAATTTCTAGGGTCACC
ASCL1.6	CTTCAAGTTCTTAGTAGAAT
ASCL1.7	GGAAGGGGGTGGGGGGCGTC
ASCL1.8	CAAGGAGCGGGAGAAAGGAA
ASCL1.9	AATGGGACACCCAGCCCCAC
ASCL1.10	ACTCGCCCTCCCTGGCCGGA
ASCL1.11	CTGCTGCTTCTGCTTTTTTT
RAB5A.1	TCCTCCTCCGCCGCCGCCGCCGC
RAB5A.2	TTCGCGGGGCGGGGCGAGGCAGG
RAB5A.4	GTACAGTAAAGAGCGAAGGGAAA
RAB5A.5	GGCTGGGGGGTCTCTGGGCTCCT
RAB5A.6	GTTCGTGAAGGAGCCGGCGGCTG
RAB5A.7	GGGACTGACTGAGGGAGCGACGG
RAB11A.1	AAGAGGTAGTCGTACTCGTCGTC
RAB11A.2	AAGGTGAGGCCATGGGCTCTCGC
RAB11A.4	GTCACTAAGTAATTGAACAACTA
RAB11A.5	CCCTTTGAGCCTCCTTTAGCGAC
RAB11A.6	GCGACTAAAGCTTGAAGCCCCAC
RAB11A.7	GCTCCTCGGCCGCGCAATGGGCA

qPCR Primer

Gene	sequence
ADRB2_forward	TTGCTGGCACCCAATAGAAGC
ADRB2_reverse	CAGACGCTCGAACTTGGCA
ASCL1_forward	CCCAAGCAAGTCAAGCGACA
ASCL1_reverse	AAGCCGCTGAAGTTGAGCC
CCR4_forward	TCTCGCCAAGACACTGAACAG
CCR4_reverse	GGCCCTGCATTCCTCAAGAAG
CHRM3_forward	GGCCTGTGCCGATCTGATTAT
CHRM3_reverse	CGGCCTCGTGATGGAAAAG
CHRM4_forward	CAGCTCGGGCAATCAGTCC
CHRM4_reverse	GCCTATGATGAGATCAGCACAC
EEA1_forward	AGCAACTCCTATAAACACAGTGG
EEA1_reverse	AGCAAGATTAGACTCTCCTCCAT
FZD1_forward	AGCCATCCAGTTGCACGAG
FZD1_reverse	GAGTCGGGCCACTTGAAGTT
HBB_forward	AGGAGAAGTCTGCCGTTACTG
HBB_reverse	CCGAGCACTTTCTTGCCATGA
HTR6_forward	GCAACACGTCCAACTTCTTCC
HTR6_reverse	TGCAGCACATCACGTCGAA
IL1B_forward	AAACAGATGAAGTGCTCCTTCC
IL1B_reverse	AAGATGAAGGGAAAGAAGGTGC
IL1R2_forward	ATGTTGCGCTTGTACGTGTTG
IL1R2_reverse	CCCGCTTGTAATGCCTCCC
PIK3C3_forward	CCTGGAAGACCCAATGTTGAAG
PIK3C3_reverse	CGGGACCATACACATCCCAT
PLCB1_forward	GGAAGCGGCAAAAAGAAGCTC
PLCB1_reverse	CGTCGTCGTCACTTTCCGT
PRKC1_forward	GACAACGAACAGCTCTTCACC
PRKC1_reverse	CCAGGACGTTCTGGTACACA
RAB5A_forward	AGACCCAACGGGCCAAATAC
RAB5A_reverse	GCCCCAATGGTACTCTCTTGAA
RAB7A_forward	GTGTTGCTGAAGGTTATCATCCT
RAB7A_reverse	GCTCCTATTGTGGCTTTGTACTG
RAB9A_forward	AGGGACAACGGCGACTATC
RAB9A_reverse	TCTGACCTATCCTCGGTAGCA
RAB11A_forward	CAACAAGAAGCATCCAGGTTGA
RAB11A_reverse	GCACCTACAGCTCCACGATAAT
ZFP42_forward	AGAAACGGGCAAAGACAAGAC
ZFP42_reverse	GCTGACAGGTTCTATTTCCGC

NGS primer

CD47_forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNAAGATCTTACAGTACAGACTTC
CD47_reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC NNCAGGACAAATAAAAAAAGAAGC

Indel analysis primer

ASCL1_forward	CGAGGAACCCGAAGAGAATAACAGTGAG
ASCL1_reverse	CCGCTGGCGCCTTCTTGTTTCTA
CD47_forward	TATCAGTTCAGCAAGTTCTATTTAGCAGTGTGTT
CD47_reverse	AGTACATTACCCCAGGACCAATCAGCCAAA
DNMT1_forward	CTGGGACTCAGGCGGGTCAC
DNMT1_reverse	CCTCACACAACAGCTTCATGTCAGC
EMX1_forward	TGGATGCCCGTGTCATTAAGAGAGAGACTTT
EMX1_reverse	CCCTTCTGTGAATGTTAGACCCATGGGAGC
FANCF_forward	GTATTAGGGCTTTTAAGTTGCCCAGAGTCAAGGA
FANCF_reverse	GTCTGTTAGCAGACCCAGATAGACAGGAGAC
GRIN2B_forward	GAATGCAGGGCTTGTGTACTTATAGCCCC
GRIN2B_reverse	ACAAATGCATGGTTTAGTCCTCAGCACAAAC
IL1B_forward	CCATGAGATTGGCTAGGGTAACAGCA
IL1B_reverse	AGAGACAGAGAGACTCCCTTAGCACC
PIK3C3_forward	GTGGGCGCCTTGTGCACATGC
PIK3C3_reverse	CACCTCCCGTGCTAATACACCATGTGCTC
RAB5A_forward	CGCCCCGCGAACAAACCTAGGC
RAB5A_reverse	CCAGGACGGAGACCAGGCGGAACC
VEGFA_forward	AAACTCCCCCCACCCCCTTTCC
VEGFA_reverse	ATTCCAGCACCGAGCGCCC

Supplementary Note 1

Single-transcript Cas12a construct:

AsCas12a-NLS-HAx3-Triplex-DR-BsmbI-BsmBI-DR

ATGACACAGTTCGAGGGCTTTACCAACCTGTATCAGGTGAGCAAGACACTGCGGTTTGAGCT
GATCCCACAGGGCAAGACCCTGAAGCACATCCAGGAGCAGGGCTTCATCGAGGAGGACAAGG
CCCGCAATGATCACTACAAGGAGCTGAAGCCCATCATCGATCGGATCTACAAGACCTATGCCG
ACCAGTGCCTGCAGCTGGTGCAGCTGGATTGGGAGAACCTGAGCGCCGCCATCGACTCCTATA
GAAAGGAGAAAACCGAGGAGACAAGGAACGCCCTGATCGAGGAGCAGGCCACATATCGCAAT
GCCATCCACGACTACTTCATCGGCCGGACAGACAACCTGACCGATGCCATCAATAAGAGACAC
GCCGAGATCTACAAGGGCCTGTTCAAGGCCGAGCTGTTTAATGGCAAGGTGCTGAAGCAGCT
GGGCACCGTGACCACAACCGAGCACGAGAACGCCCTGCTGCGGAGCTTCGACAAGTTTACAAC
CTACTTCTCCGGCTTTTATGAGAACAGGAAGAACGTGTTCAGCGCCGAGGATATCAGCACAG
CCATCCCACACCGCATCGTGCAGGACAACTTCCCCAAGTTTAAGGAGAATTGTCACATCTTCA
CACGCCTGATCACCGCCGTGCCCAGCCTGCGGGAGCACTTTGAGAACGTGAAGAAGGCCATCG
GCATCTTCGTGAGCACCTCCATCGAGGAGGTGTTTTCCTTCCCTTTTTATAACCAGCTGCTGA
CACAGACCCAGATCGACCTGTATAACCAGCTGCTGGGAGGAATCTCTCGGGAGGCAGGCACC
GAGAAGATCAAGGGCCTGAACGAGGTGCTGAATCTGGCCATCCAGAAGAATGATGAGACAGC
CCACATCATCGCCTCCCTGCCACACAGATTCATCCCCCTGTTTAAGCAGATCCTGTCCGATAG
GAACACCCTGTCTTTCATCCTGGAGGAGTTTAAGAGCGACGAGGAAGTGATCCAGTCCTTCT
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GAAAACCAGCGAGATCCTGTCCCACGCACACGCCGCCCTGGATCAGCCACTGCCTACAACCCT
GAAGAAGCAGGAGGAGAAGGAGATCCTGAAGTCTCAGCTGGACAGCCTGCTGGGCCTGTACC
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GCCACCAAGAAGCCCTACTCCGTGGAGAAGTTCAAGCTGAACTTTCAGATGCCTACACTGGCC
TCTGGCTGGGACGTGAATAAGGAGAAGAACAATGGCGCCATCCTGTTTGTGAAGAACGGCCT
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CGGCATCTCCAATCAGGACTGGCTGGCCTACATCCAGGAGCTGCGCAACAAAAGGCCGGCGGC
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AGACGCAAATTCTAATCCAGCTTCAACAGCTATTTCACAAGCTTTCATGATTTCTCGTCTCTA
ATTTCTACTCTTGTAGAT

LSL-AsCas12a-NLS-HAx3-Triplex-DR-BsmbI-BsmBI-DR

ATAACTTCGTATAATGTATGCTATACGAAGTTATTCGCGATGAATAAATGAAAGCTTGCAGA
TCTGCGACTCTAGAGGATCTGCGACTCTAGAGGATCATAATCAGCCNTACCACATTTTGTAG
AGGTTTTACTNGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAAT
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TCCAAACTCATCAATGTATCTTATCATGTCTGGATCCCCATCAAGCTGATCCGGAACCCTTAA
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CCACCATGACACAGTTCGAGGGCTTTACCAACCTGTATCAGGTGAGCAAGACACTGCGGTTT
GAGCTGATCCCACAGGGCAAGACCCTGAAGCACATCCAGGAGCAGGGCTTCATCGAGGAGGA
CAAGGCCCGCAATGATCACTACAAGGAGCTGAAGCCCATCATCGATCGGATCTACAAGACCT
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GACAGCCCACATCATCGCCTCCCTGCCACACAGATTCATCCCCCTGTTTAAGCAGATCCTGTC
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TCTCCGAGCTGACAGGCAAGATCACCAAGTCTGCCAAGGAGAAGGTGCAGCGCAGCCTGAAG
CACGAGGATATCAACCTGCAGGAGATCATCTCTGCCGCAGGCAAGGAGCTGAGCGAGGCCTT
CAAGCAGAAAACCAGCGAGATCCTGTCCCACGCACACGCCGCCCTGGATCAGCCACTGCCTAC
AACCCTGAAGAAGCAGGAGGAGAAGGAGATCCTGAAGTCTCAGCTGGACAGCCTGCTGGGCC
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TGCCTACCTGAAGGAGCACCCCGAGACACCTATCATCGGCATCGATCGGGGCGAGAGAAACCT
GATCTATATCACAGTGATCGACTCCACCGGCAAGATCCTGGAGCAGCGGAGCCTGAACACCA
TCCAGCAGTTTGATTACCAGAAGAAGCTGGACAACAGGGAGAAGGAGAGGGTGGCAGCAAGG
CAGGCCTGGTCTGTGGTGGGCACAATCAAGGATCTGAAGCAGGGCTATCTGAGCCAGGTCAT
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CGTGCTGAACCCATACCAGCTGACAGACCAGTTCACCTCCTTTGCCAAGATGGGCACCCAGTC
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TCGACTTTCTGCACTACGACGTGAAAACCGGCGACTTCATCCTGCACTTTAAGATGAACAGA
AATCTGTCCTTCCAGAGGGGCCTGCCCGGCTTTATGCCTGCATGGGATATCGTGTTCGAGAAG
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TRE-miniCMV-AsCas12a-NLS-HAx3-Triplex-DR-BsmbI-BsmBI-DR

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Single-transcript Cas12a construct for Lentivirus production:

[CMV-AsCas12a-NLS-P2A-Puro-HAx3-Triplex-DR-BsmbI-BsmBI-DR-WPRE-Poly(A)] inverted

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Cas12a variants:

ddCas12a (E993A)

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rdCas12a (H800A)

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ddLbCas12a (D832A)

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Single-transcript Cas12a – [TAD] construct:

AsCas12a-NLS-[Aktiv]-HAx3-Triplex-DR-SapI-SapI-DR

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Single-transcript Cas12a – [Repr] construct:

AsCas12a-NLS-[Repr]-HAx3-Triplex-DR-SapI-SapI-DR

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TCTAATTTCTACTCTTGTAGAT

Transcriptional control elements:

KRAB

CGGACACTGGTGACCTTCAAGGATGTATTTGTGGACTTCACCAGGGAGGAGTGGAAGCTGCT
GGACACTGCTCAGCAGATCGTGTACAGAAATGTGATGCTGGAGAACTATAAGAACCTGGTTT
CCTTGGGTTATCAGCTTACTAAGCCAGATGTGATCCTCCGGTTGGAGAAGGGAGAAGAGCCC

KRAB$_2$

CGGACACTGGTGACCTTCAAGGATGTATTTGTGGACTTCACCAGGGAGGAGTGGAAGCTGCT
GGACACTGCTCAGCAGATCGTGTACAGAAATGTGATGCTGGAGAACTATAAGAACCTGGTTT
CCTTGGGTTATCAGCTTACTAAGCCAGATGTGATCCTCCGGTTGGAGAAGGGAGAAGAGCCC
GGATCCGGGGGTCGGACACTGGTGACCTTCAAGGATGTATTTGTGGACTTCACCAGGGAGGA
GTGGAAGCTGCTGGACACTGCTCAGCAGATCGTGTACAGAAATGTGATGCTGGAGAACTATA

AGAACCTGGTTTCCTTGGGTTATCAGCTTACTAAGCCAGATGTGATCCTCCGGTTGGAGAAG
GGAGAAGAGCCC

[Repr]

GGTGGGCGGACCCTTGTTACTTTCAAGGATGTATTCGTGGATTTCACACGCGAGGAGTGGAA
ATTGTTGGATACTGCCCAGCAGATTGTATACCGCAATGTAATGCTCGAAAACTACAAAAATC
TCGTGAGTCTCGGATATCAACTGACCAAGCCTGACGTTATCCTCCGATTGGAGAAAGGCGAG
GAACCTGGGAGCGGAGGAAGGACGCTTGTCACGTTTAAGGATGTTTTTGTTGACTTCACTCG
AGAGGAGTGGAAGCTGTTGGATACTGCCCAACAAATTGTGTATCGCAACGTGATGCTCGAGA
ACTACAAGAACCTTGTGTCTTTGGGCTATCAGCTGACTAAACCTGATGTAATACTCCGCCTCG
AGAAAGGAGAGGAGCCTGGTAGTGGGGGACGGACCCTGGTAACATTCAAGGATGTATTCGTG
GATTTCACGCGGGAAGAATGGAAACTCCTCGATACTGCCCAACAGATAGTGTATCGAAACGT
AATGCTCGAAAACTACAAAAACTTGGTCAGCCTCGGATACCAACTTACAAAACCTGATGTTA
TCCTCAGACTTGAGAAGGGCGAGGAACCC

VP64$_2$

GACGCATTGGACGATTTTGATCTGGATATGCTGGGAAGTGACGCCCTCGATGATTTTGACCT
TGACATGCTTGGTTCGGATGCCCTTGATGACTTTGACCTCGACATGCTCGGCAGTGACGCCCT
TGATGATTTCGACCTGGACATGCTGGGATCCGGTGGGGACGCATTGGACGATTTTGATCTGG
ATATGCTGGGAAGTGACGCCCTCGATGATTTTGACCTTGACATGCTTGGTTCGGATGCCCTTG
ATGACTTTGACCTCGACATGCTCGGCAGTGACGCCCTTGATGATTTCGACCTGGACATGCTG

p65

CCTTCAGGGCAGATCAGCAACCAGGCCCTGGCTCTGGCCCCTAGCTCCGCTCCAGTGCTGGCC
CAGACTATGGTGCCCTCTAGTGCTATGGTGCCTCTGGCCCAGCCACCTGCTCCAGCCCCTGTG
CTGACCCCAGGACCACCCCAGTCACTGAGCGCTCCAGTGCCCAAGTCTACACAGGCCGGCGAG
GGGACTCTGAGTGAAGCTCTGCTGCACCTGCAGTTCGACGCTGATGAGGACCTGGGAGCTCT
GCTGGGGAACAGCACCGATCCCGGAGTGTTCACAGATCTGGCCTCCGTGGACAACTCTGAGTT
TCAGCAGCTGCTGAATCAGGGCGTGTCCATGTCTCATAGTACAGCCGAACCAATGCTGATGG
AGTACCCCGAAGCCATTACCCGGCTGGTGACCGGCAGCCAGCGGCCCCCCGACCCCGCTCCAA
CTCCCCTGGGAACCAGCGGCCTGCCTAATGGGCTGTCCGGAGATGAAGACTTCTCAAGCATCG
CTGATATGGACTTTAGTGCCCTGCTGTCACAGATTTCCTCTAGTGGGCAG

sp65

AGCGGCCTGCCTAATGGGCTGTCCGGAGATGAAGACTTCTCAAGCATCGCTGATATGGACTT
TAGTGCCCTGCTGTCACAGATTTCCTCT

sp65$_3$

AGCGGGTTGCCTAATGGGTTGAGTGGTGACGAGGATTTTAGCAGTATCGCCGATATGGACTT
TTCAGCTCTCCTCAGCCAGATAAGCTCTGGAAGTAGTGGGCTTCCAAATGGGCTTAGCGGTG
ACGAAGATTTCTCTAGTATAGCGGACATGGACTTCTCAGCTCTGCTCTCCCAGATTTCTTCAG
GGTCCAGCGGCTTGCCCAACGGCCTCAGTGGCGACGAGGATTTTTCTAGCATAGCAGACATG
GACTTTTCCGCACTCCTCAGTCAGATTTCTTCT

sp65p$_3$

AGCGGGTTGCCTAATGGGTTGGATGGTGACGAGGATTTTAGCGATATCGCCGATATGGACTT
TTCAGCTCTCCTCAGCCAGATAAGCTCTGGAAGTAGTGGGCTTCCAAATGGGCTTGACGGTG
ACGAAGATTTCTCTGATATAGCGGACATGGACTTCTCAGCTCTGCTCTCCCAGATTTCTTCAG
GGTCCAGCGGCTTGCCCAACGGCCTCGATGGCGACGAGGATTTTTCTGACATAGCAGACATG
GACTTTTCCGCACTCCTCAGTCAGATTTCTTCT

HSF1

GGCTTCAGCGTGGACACCAGTGCCCTGCTGGACCTGTTCAGCCCCTCGGTGACCGTGCCCGAC
ATGAGCCTGCCTGACCTTGACAGCAGCCTGGCCAGTATCCAAGAGCTCCTGTCTCCCCAGGAG
CCCCCCAGGCCTCCCGAGGCAGAGAACAGCAGCCCGGATTCAGGGAAGCAGCTGGTGCACTAC
ACAGCGCAGCCGCTGTTCCTGCTGGACCCCGGCTCCGTGGACACCGGGAGCAACGACCTGCCG
GTGCTGTTTGAGCTGGGAGAGGGCTCCTACTTCTCCGAAGGGGACGGCTTCGCCGAGGACCCC
ACCATCTCCCTGCTGACAGGCTCGGAGCCTCCCAAAGCCAAGGACCCCACTGTCTCC

RtA

CGGGATTCCAGGGAAGGGATGTTTTTGCCGAAGCCTGAGGCCGGCTCCGCTATTAGTGACGT
GTTTGAGGGCCGCGAGGTGTGCCAGCCAAAACGAATCCGGCCATTTCATCCTCCAGGAAGTCC
ATGGGCCAACCGCCCACTCCCCGCCAGCCTCGCACCAACACCAACCGGTCCAGTACATGAGCC
AGTCGGGTCACTGACCCCGGCACCAGTCCCTCAGCCACTGGATCCAGCGCCCGCAGTGACTCC
CGAGGCCAGTCACCTGTTGGAGGATCCCGATGAAGAGACGAGCCAGGCTGTCAAAGCCCTTC
GGGAGATGGCCGATACTGTGATTCCCCAGAAGGAAGAGGCTGCAATCTGTGGCCAAATGGAC
CTTTCCCATCCGCCCCCAAGGGGCCATCTGGATGAGCTGACAACCACACTTGAGTCCATGACC
GAGGATCTGAACCTGGACTCACCCCTGACCCCGGAATTGAACGAGATTCTGGATACCTTCCTG
AACGACGAGTGCCTCTTGCATGCCATGCATATCAGCACAGGACTGTCCATCTTCGACACATCT
CTGT

[Activ]

GGTGGGGGTGGGTCCAGCGGGTTGCCTAATGGGTTGGATGGTGACGAGGATTTTAGCGATAT
CGCCGATATGGACTTTTCAGCTCTCCTCAGCCAGATAAGCTCTGGAAGTAGTGGGCTTCCAAA
TGGGCTTGACGGTGACGAAGATTTCTCTGATATAGCGGACATGGACTTCTCAGCTCTGCTCTC
CCAGATTTCTTCAGGGTCCAGCGGCTTGCCCAACGGCCTCGATGGCGACGAGGATTTTTCTGA
CATAGCAGACATGGACTTTTCCGCACTCCTCAGTCAGATTTCTTCTGGAGGCGGTGGAAGCGG
CTTCAGCGTGGACACCAGTGCCCTGCTGGACCTGTTCAGCCCCTCGGTGACCGTGCCCGACAT
GAGCCTGCCTGACCTTGACAGCAGCCTGGCCAGTATCCAAGAGCTCCTGTCTCCCCAGGAGCC
CCCCAGGCCTCCCGAGGCAGAGAACAGCAGCCCGGATTCAGGGAAGCAGCTGGTGCACTACAC
AGCGCAGCCGCTGTTCCTGCTGGACCCCGGCTCCGTGGACACCGGGAGCAACGACCTGCCGGT
GCTGTTTGAGCTGGGAGAGGGCTCCTACTTCTCCGAAGGGGACGGCTTCGCCGAGGACCCCAC
CATCTCCCTGCTGACAGGCTCGGAGCCTCCCAAAGCCAAGGACCCCACTGTCTCC

Direct Repeat (DR)

AATTTCTACTCTTGTAGAT

Gene editing

Gene	Target Sequence
CD47.1	ATTAAATAGTAGCTGAGCTGATC
CD47.2	GCACTACTAAAGTCAGTGGGGAC
CD47.3	GTAATGACACTGTCGTCATTCCA
CD47.4	GAGCTCCATCAAAGGTGTAAATA
CD47.5	CGTATACTTCAGTAGTGTTTTGT
CD47.6	GTAGTGCAAAAATTGAAGTCTCA
CD47.7	CTGTGTGTGAGACAGCATCACTC
CD47.8	GCTCGATGATCGTTTCACCTTCT
CD47.9	GTGCCTCCATATTAGTAACAAAG
CD47.10	ATGGAGCTCTAAACAAGTCCACT
CD97.1	AGCAGGGCTTCCTCTGGAGCTTC
CD97.2	TTGTGGTGCGCGTGTTCCAAGGC
CD97.3	CTGGCCGCCTTCTGCTGGATGAG
CD97.4	CGTGACTACCGTCTGGAAGCTCA
CD166.1	TAGAGGATCTGAAGGCAATAAAT
CD166.2	AGGCACCTACAATAGTCAAGGTG
CD166.3	TGTGCATGCTAGTAACTGAGGAC
CD166.4	ATCACTGATCCTTGCATTACTGA
DNMT1	CTGATGGTCCATGTCTGTTACTC
FANCF	GGCGGGGTCCAGTTCCGGGATTA
EMX1	TGGTTGCCCACCCTAGTCATTGG
GRIN2B	GTGCTCAATGAAAGGAGATAAGG
VEGFA	CTAGGAATATTGAAGGGGGCAGG

Transcriptional activator

Gene	Target Sequence
ZFP42.1	TTGAGCGCTCACCAC
ZFP42.2	CGTGCGGGCCGGGTG
ASCL1.1	GGGAGTGGGTGGGAG
ASCL1.2	CAATGGGACACCCAG
IL1B.1	CATGGTGATACATTT
IL1B.2	CTACTCCTTGCCCTT
IL1R2.1	CTTGGCCACTTCCCC
IL1R2.2	CCTATTTTTCTGTGA
ADRB2.1	GCAGTAAAGTCACAT
ADRB2.2	GTTACACTTCATGAA
CCR4.1	GCACATCTTCTTGGC
CCR4.2	ATTTTTGGGGAGATA
CCR10.1	CCCTACTCCACTTTG
CCR10.2	GTAAAATCCAGATCC
CHRM4.1	TCTCCCCTTCCTCCC
CHRM4.2	CATGTCTCCCCCCAT
HBB.1	TCTACCATAATTCAG
HBB.2	AAGTCCAACTCCTAA
HTR6.1	CCAACTCCTGGCTCC
HTR6.2	CCAGGGGCGGCTTTG

Transcriptional activator

Gene	Target Sequence
ASCL1.3 (15 bp)	GGGAGTGGGTGGGAG
ASCL1.3(20 bp)	GGGAGTGGGTGGGAGGAAGA
ASCL1.4(15 bp)	CAATGGGACACCCAG
ASCL1.4 (20 bp)	CAATGGGACACCCAGCCCCA
IL1B.3 (15 bp)	CATGGTGATACATTT
IL1B.3 (20 bp)	CATGGTGATACATTTGCAAA
IL1B.4 (15 bp)	CTACTCCTTGCCCTT
IL1B.4 (20 bp)	CTACTCCTTGCCCTTCCATG
IL1R2.3 (15 bp)	CTTGGCCACTTCCCC
IL1R2.3 (20 bp)	CTTGGCCACTTCCCCATCTG
IL1R2.4 (15 bp)	CCTATTTTTCTGTGA
IL1R2.4 (20 bp)	CCTATTTTTCTGTGACTCGC
ZFP42.3 (15 bp)	TTGAGCGCTCACCAC
ZFP42.3 (20 bp)	TTGAGCGCTCACCACGTGCC
ZFP42.4 (15 bp)	CGTGCGGGCCGGGTG
ZFP42.4 (20 bp)	CGTGCGGGCCGGGTGCCTGG

Transcriptional repression

Gene	Target Sequence
CHRM3.1	AGCCAACCCACCCCA
CHRM3.2	GTCTCAAGCACGCAG
FZD1.1	TGCCTGAGTAGTGCC
FZD1.2	CGCCGGGAAGCCGGA
PLCB1.1	CCAGCCAGTTGGGAT
PLCB1.2	CACGCTGGGTCAGGC
PRKC1.1	ACAGCTCGCGTGAAA
PRKC1.2	TCTGCCCCCGTAAGG
RAB11A.1	AAGAGGTAGTCGTAC
RAB11A.2	AAGGTGAGGCCATGG
RAB9A.1	GTACTCGCTGTCGCC
RAB9A.2	CCTGGCCCCGCCCCG
RAB7A.1	GTCTCCTCCTCGGCG
RAB7A.2	TAGCACGAGATCCAG
EEA1.1	ACAGAGGGTAAGAGA
EEA1.2	CAGCAGAAACTAGCA
PIK3C3.1	ACTACATCTATAGTT
PIK3C3.2	GGCGGGGAGTTCCGC
RAB5A.1	TCCTCCTCCGCCGCC
RAB5A.2	TTCGCGGGGCGGGGC

Transcriptional repression

Gene	Target Sequence
EEA1.3 (15 bp)	ACAGAGGGTAAGAGA
EEA1.3 (20 bp)	ACAGAGGGTAAGAGAGTGAA
EEA1.4 (15 bp)	ACCACCACCCGGCGC
EEA1.4 (20 bp)	ACCACCACCCGGCGCCGCCG
PIK3C3.3 (15 bp)	ACTACATCTATAGTT
PIK3C3.3 (20 bp)	ACTACATCTATAGTTGTGAC
PIK3C3.4 (15 bp)	CAGACGGTGCGATGG
PIK3C3.4 (20 bp)	CAGACGGTGCGATGGGGGAA
RAB5A.3 (15 bp)	TCCTCCTCCGCCGCC
RAB5A.3 (20 bp)	TCCTCCTCCGCCGCCGCCGC
RAB5A.4 (15 bp)	TTCGCGGGGCGGGGC
RAB5A.4 (20 bp)	TTCGCGGGGCGGGGCGAGGC
RAB5A.5 (15 bp)	GGTTCGTGAAGGAGC
RAB5A.5 (20 bp)	GGTTCGTGAAGGAGCCGGCG
RAB7A.3 (15 bp)	GTCTCCTCCTCGGCG
RAB7A.3 (20 bp)	GTCTCCTCCTCGGCGGGAGC
RAB7A.4 (15 bp)	TGGCCAAGACTCCAG
RAB7A.4 (20 bp)	TGGCCAAGACTCCAGGCCCG

Distance from promoter TSS

Gene	Target Sequence
HBB.1	CCAAAACCTAATAAGTAACT
HBB.2	TTAGCATGCATGAGCAAATT
HBB.3	GATTAAAACCTTCTGGTAAG
HBB.4	GTGCATCAACTTCTTATTTG
HBB.5	GAATCACAGCTTGGTAAGCA
HBB.6	AAGTCCAACTCCTAAGCCAG
HBB.7	CTTCTGACACAACTGTGTTC
HBB.8	TGATAGGCACTGACTCTCTC
HBB.9	TTGCCATGAGCCTTCACCTT
ASCL1.3	GAGCTGAATGGGACATTAGA
ASCL1.4	ACATAGTCCAGCACTTTTTT
ASCL1.5	CTCCAATTTCTAGGGTCACC
ASCL1.6	CTTCAAGTTCTTAGTAGAAT
ASCL1.7	GGAAGGGGGTGGGGGGCGTC
ASCL1.8	CAAGGAGCGGGAGAAAGGAA
ASCL1.9	AATGGGACACCCAGCCCCAC
ASCL1.10	ACTCGCCCTCCCTGGCCGGA
ASCL1.11	CTGCTGCTTCTGCTTTTTTT
RAB5A.1	TCCTCCTCCGCCGCCGCCGCCGC
RAB5A.2	TTCGCGGGGCGGGGCGAGGCAGG
RAB5A.4	GTACAGTAAAGAGCGAAGGGAAA
RAB5A.5	GGCTGGGGGGTCTCTGGGCTCCT
RAB5A.6	GTTCGTGAAGGAGCCGGCGGCTG
RAB5A.7	GGGACTGACTGAGGGAGCGACGG
RAB11A.1	AAGAGGTAGTCGTACTCGTCGTC
RAB11A.2	AAGGTGAGGCCATGGGCTCTCGC
RAB11A.4	GTCACTAAGTAATTGAACAACTA
RAB11A.5	CCCTTTGAGCCTCCTTTAGCGAC
RAB11A.6	GCGACTAAAGCTTGAAGCCCCAC
RAB11A.7	GCTCCTCGGCCGCGCAATGGGCA

qPCR Primer

Gene	sequence
ADRB2_forward	TTGCTGGCACCCAATAGAAGC
ADRB2_reverse	CAGACGCTCGAACTTGGCA
ASCL1_forward	CCCAAGCAAGTCAAGCGACA
ASCL1_reverse	AAGCCGCTGAAGTTGAGCC
CCR4_forward	TCTCGCCAAGACACTGAACAG
CCR4_reverse	GGCCCTGCATTCCTCAAGAAG
CHRM3_forward	GGCCTGTGCCGATCTGATTAT
CHRM3_reverse	CGGCCTCGTGATGGAAAAG
CHRM4_forward	CAGCTCGGGCAATCAGTCC
CHRM4_reverse	GCCTATGATGAGATCAGCACAC
EEA1_forward	AGCAACTCCTATAAACACAGTGG
EEA1_reverse	AGCAAGATTAGACTCTCCTCCAT
FZD1_forward	AGCCATCCAGTTGCACGAG
FZD1_reverse	GAGTCGGGCCACTTGAAGTT
HBB_forward	AGGAGAAGTCTGCCGTTACTG
HBB_reverse	CCGAGCACTTTCTTGCCATGA
HTR6_forward	GCAACACGTCCAACTTCTTCC
HTR6_reverse	TGCAGCACATCACGTCGAA
IL1B_forward	AAACAGATGAAGTGCTCCTTCC
IL1B_reverse	AAGATGAAGGGAAAGAAGGTGC
IL1R2_forward	ATGTTGCGCTTGTACGTGTTG
IL1R2_reverse	CCCGCTTGTAATGCCTCCC
PIK3C3_forward	CCTGGAAGACCCAATGTTGAAG
PIK3C3_reverse	CGGGACCATACACATCCCAT
PLCB1_forward	GGAAGCGGCAAAAAGAAGCTC
PLCB1_reverse	CGTCGTCGTCACTTTCCGT
PRKC1_forward	GACAACGAACAGCTCTTCACC
PRKC1_reverse	CCAGGACGTTCTGGTACACA
RAB5A_forward	AGACCCAACGGGCCAAATAC
RAB5A_reverse	GCCCCAATGGTACTCTCTTGAA
RAB7A_forward	GTGTTGCTGAAGGTTATCATCCT
RAB7A_reverse	GCTCCTATTGTGGCTTTGTACTG
RAB9A_forward	AGGGACAACGGCGACTATC
RAB9A_reverse	TCTGACCTATCCTCGGTAGCA
RAB11A_forward	CAACAAGAAGCATCCAGGTTGA
RAB11A_reverse	GCACCTACAGCTCCACGATAAT
ZFP42_forward	AGAAACGGGCAAAGACAAGAC
ZFP42_reverse	GCTGACAGGTTCTATTTCCGC

NGS primer

CD47_forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNAAGATCTTACAGTACAGACTTC
CD47_reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC NNCAGGACAAATAAAAAAAGAAGC

Indel analysis primer

ASCL1_forward	CGAGGAACCCGAAGAGAATAACAGTGAG
ASCL1_reverse	CCGCTGGCGCCTTCTTGTTTCTA
CD47_forward	TATCAGTTCAGCAAGTTCTATTTAGCAGTGTGTT
CD47_reverse	AGTACATTACCCCAGGACCAATCAGCCAAA
DNMT1_forward	CTGGGACTCAGGCGGGTCAC
DNMT1_reverse	CCTCACACAACAGCTTCATGTCAGC
EMX1_forward	TGGATGCCCGTGTCATTAAGAGAGAGACTTT
EMX1_reverse	CCCTTCTGTGAATGTTAGACCCATGGGAGC
FANCF_forward	GTATTAGGGCTTTTAAGTTGCCCAGAGTCAAGGA
FANCF_reverse	GTCTGTTAGCAGACCCAGATAGACAGGAGAC
GRIN2B_forward	GAATGCAGGGCTTGTGTACTTATAGCCCC
GRIN2B_reverse	ACAAATGCATGGTTTAGTCCTCAGCACAAAC
IL1B_forward	CCATGAGATTGGCTAGGGTAACAGCA
IL1B_reverse	AGAGACAGAGAGACTCCCTTAGCACC
PIK3C3_forward	GTGGGCGCCTTGTGCACATGC
PIK3C3_reverse	CACCTCCCGTGCTAATACACCATGTGCTC
RAB5A_forward	CGCCCCGCGAACAAACCTAGGC
RAB5A_reverse	CCAGGACGGAGACCAGGCGGAACC
VEGFA_forward	AAACTCCCCCCACCCCCTTTCC
VEGFA_reverse	ATTCCAGCACCGAGCGCCC