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LETTER TO THE EDITOR

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Casilio: a versatile CRISPR-Cas9-Pumilio hybrid for gene regulation and genomic labeling

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Dear Editor,

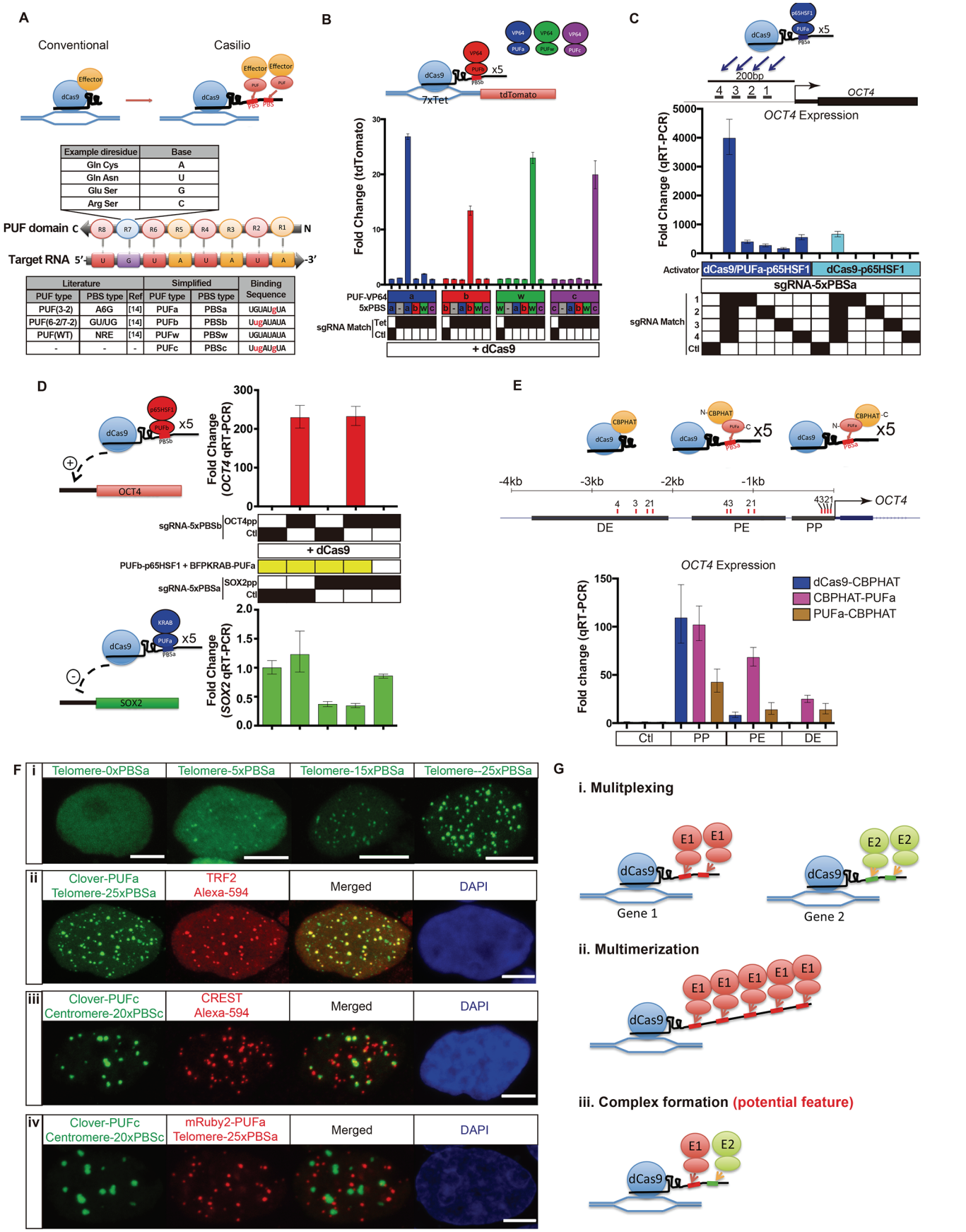
The CRISPR-Cas9 system has recently been widely adopted in genome editing due to its simplicity [1-3]. Nuclease-deficient mutant dCas9 protein can be fused to effector domains and the fusion proteins can be guided by sgRNAs to genomic sites to regulate gene expression or label chromosomes [4-10]. However, only one type of effector is applied in most experiments due to the exclusive sgRNA:Cas9 pairing. Moreover, multimerization by directly fusing multiple copies of effectors with dCas9 protein to achieve sufficient effector activity is technically challenging. RNA aptamer approaches utilizing viral RNA sequences such as MS2 and PP7 have been combined with the CRISPR-Cas9 system to provide tools with improved multiplexing and multimerization functionalities [11, 12]. However, there are a limited number of well-characterized RNA aptamers. Furthermore, incorporation of three or more copies of these structured aptamers onto sgRNA reduces sgRNA expression, thus limiting the number of effector proteins that can be recruited. We established here the Casilio system by combining CRISPR-Cas9 and Pumilio RNA-binding protein. Pumilio and FBF proteins share a conserved Pumilio/FBF (PUF) RNA-binding domain which is programmable to bind a specific 8-mer RNA sequence (PUF-binding site, PBS) [13, 14] (Figure 1A). The Casilio system consists of the dCas9 protein, an sgRNA appended with one or more PUF-binding site(s) (sgRNA-PBS), and an effector fused with a PUF domain (PUF fusion) (Figure 1A). The sgRNA-PBS specifies both DNA binding via its spacer sequence and effector recruitment via PBS.

First, to test whether insertion of PBS at the 3′ end of sgRNA affects its function, we generated a series of TetO-targeting (sgTetO) and control (sgCtl) sgRNAs with 0-47 copies of PBSa or PBSb, and tested their ability to direct dCas9-VP64 to activate tdTomato expression in a HEK293T/TetO::tdTomato cell line (Supplementary information, Figure S1A). All of the sgTetO-PBS but none of the sgCtl-PBS directed dCas9-VP64 to activate TetO::tdTomato, demonstrating that dCas9-VP64/sgRNA can tolerate insertion of at least 47 copies of PBS. Next, we tested whether sgRNAs with different copy numbers of PBS can recruit PUF-VP64 to activate TetO::tdTomato reporter (Supplementary information, Figure S1B). For both PUFa-VP64/sgRNA-PBSa and PUFb-VP64/sgRNA-PBSb, the modules with 5-10 copies of PBS appended to the sgRNA activated the TetO::tdTomato most efficiently, whereas those with 15, 20, and 47 copies of PBS led to lower albeit substantial activation (Supplementary information, Figure S1B).

We then tested whether specificity of PUF-PBS pairs provides independence between Casilio modules. In addition to PUFa-VP64/sgRNA-PBSa and PUFb-VP64/sgRNA-PBSb, we constructed two additional activator modules, PUFw-VP64/sgRNA-PBSw and PUFc-VP64/sgRNA-PBSc (Figure 1A), and tested their ability to activate TetO::tdTomato expression. As shown in Figure 1B, PUF-VP64 activated TetO::tdTomato expression only when the sgRNA with the cognate PBS was provided. This demonstrates that the Casilio modules with different PUF domains can operate independently with high specificity.

A recent paper showed p65HSF1 as a potent transcriptional activation domain [15]. We constructed Casilio activator module PUFa-p65HSF1 and compared it with PUFa-VP64 for the ability to activate TetO::tdTomato. PUFa-p65HSF1 showed an increase in TetO::tdTomato activation by approximately 3-fold compared with PUFa-VP64 and even enabled activation with the presence of one copy of PBSa in the sgRNA (Supplementary information, Figure S1C). We thus switched to PUF-p65HSF1 for subsequent experiments.

To test the possibility that recruitment of multiple molecules of p65HSF1 by sgRNA-PBS might increase transactivation activity, we compared activation of endogenous genes *OCT4* and *SOX2* in HEK293T cells induced by the Casilio system with that induced by a direct dCas9-p65HSF1 activator using either a cocktail of 4 sgRNA-PBS per gene or individual sgRNA-PBS. Using either the sgRNA-PBS cocktail or a single sgRNA-PBS, we observed higher activation using the Casilio system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Casilio
Effector
dCas9
PUF
PBS
Example diresidue
Base
Gln Cys
A
Gln Asn
U
Glu Ser
G
Arg Ser
C
PUF domain C
R8 R7 R6 R5 R4 R3 R2 R1 N
Target RNA 5′
U G U A U A U A
-3′
Literature
Simplified
PUF type
PBS type
Ref
PUF type
PBS type
Binding Sequence
PUF(3-2)
A6G
[14]
PUFa
PBSa
UGUAUgUA
PUF(6-2/7-2)
GU/UG
[14]
PUFb
PBSb
UugAUAUA
PUF(WT)
NRE
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PUFw
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UGUAUAUA
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PUFc
PBSc
UugAUgUA
B
VP64
PUFa
PUFb
PUFc
x5
7xTet
tdTomato
Fold Change (tdTomato)
PUF-VP64
5xPBS
sgRNA Match
Tet
Ctl
+ dCas9
C
p65HSF1
200bp
4 3 2 1
OCT4
OCT4 Expression
Fold Change (qRT-PCR)
Activator
dCas9/PUFa-p65HSF1
dCas9-p65HSF1
sgRNA-5xPBSa
sgRNA Match
D
OCT4
Fold Change (OCT4 qRT-PCR)
sgRNA-5xPBSb
OCT4pp
Ctl
+ dCas9
PUFb-p65HSF1 + BFPKRAB-PUFa
sgRNA-5xPBSa
SOX2pp
Ctl
KRAB
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Fold Change (SOX2 qRT-PCR)
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CBPHAT
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4321
OCT4
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OCT4 Expression
dCas9-CBPHAT
CBPHAT-PUFa
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Fold change (qRT-PCR)
Ctl
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i
Telomere-0xPBSa
Telomere-5xPBSa
Telomere-15xPBSa
Telomere--25xPBSa
ii
Clover-PUFa
Telomere-25xPBSa
TRF2
Alexa-594
Merged
DAPI
iii
Clover-PUFc
Centromere-20xPBSc
CREST
Alexa-594
Merged
DAPI
iv
Clover-PUFc
Centromere-20xPBSc
mRuby2-PUFa
Telomere-25xPBSa
Merged
DAPI
G
i. Mulitplexing
dCas9
E1
E2
Gene 1
Gene 2
ii. Multimerization
iii. Complex formation (potential feature)

compared to the direct fusion for both *OCT4* and *SOX2* activation. We observed little to no activation by single targeting of the dCas9-p65HSF1 fusion protein to *OCT4* and *SOX2* while robust activation was observed in the corresponding Casilio experiments (Figure 1C and Supplementary information, Figure S1D). These results demonstrate the superior activity of Casilio activator over the direct fusion approach. We then sought to determine the optimal number of PBS for activation of endogenous genes and found that 5 copies of PBS produced the highest activation, resembling results from the TetO::tdTomato activation experiment (Supplementary information, Figure S1E).

To test the feasibility of the Casilio system to recruit various effectors to distinct targets, we tested repression of eGFP by a KRAB-PUFa repressor and activation of tdTomato by a PUFc-p65HSF1 activator simultaneously in a HEK293T/TetO::tdTomato/SV40::EGFP cell line (Supplementary information, Figure S1F). When both modules were applied, simultaneous activation of TetO::tdTomato and repression of SV40::EGFP were achieved (Supplementary information, Figure S1F). Next, we tested whether the expression of multiple endogenous genes can be independently regulated using this strategy. We directed the Casilio activator and repressor modules to *OCT4* and *SOX2* promoters, respectively, and observed simultaneous activation of *OCT4* and repression of *SOX2* when two sets of sgRNA-5×PBS individually targeting *OCT4* and *SOX2* were introduced into cells together with dCas9 and PUF fusions (Figure 1D).

A recent study used dCas9-tethered histone acetyltransferase (HAT) to activate enhancers [10]. We tested whether the Casilio system could recruit multiple molecules of HAT domain to increase the efficiency of epigenetic editing. We targeted the proximal promoter (PP), proximal enhancer (PE) and distal enhancer (DE) of *OCT4* with cocktails of 4 sgRNA-PBS for each region (Figure 1E). We constructed fusions of the HAT domain from CREB-binding protein (CBP) attached to the C-terminus of dCas9 (dCas9-CBPHAT), and N-terminus (CBPHAT-PUFa) and C-terminus (PUFa-CBPHAT) of PUFa and tested their ability to activate *OCT4* expression. dCas9-CBPHAT and CBPHAT-PUFa modules have similar activity at PP. Interestingly, the Casilio modules have higher efficiency in activating *OCT4* gene via PE and DE compared to dCas9-CBPHAT, with CBPHAT-PUFa giving the highest activation (Figure 1E). In addition, CBPHAT-PUFa could mediate significant *OCT4* activation when directed by a single sgRNA-5×PBSa (Supplementary information, Figure S1G).

Another important application of dCas9 is to label genomic loci for live cell imaging [9]. We first tested whether the Casilio system can label telomeric repeats using sgTelomere (Supplementary information, Figure S2A) appended with 0, 5, 15 or 25 copies of PBSa to recruit Clover-PUFa (Figure 1F). Expression of sgTelomere-5×PBSa, -15×PBSa and -25×PBSa with dCas9 and Clover-PUFa produced fluorescent foci at telomeres (Figure 1Fi), as confirmed by their overlap with immunostaining signals of telomeric repeat-binding factor 2 (TRF2) (Figure 1Fii). Interestingly, the strength of telomere labeling increased as more copies of PBS were appended to sgTelomere-PBS (Figure 1Fi). Quantification of foci number and signal-to-noise ratio in cells expressing sgRNA with 5, 15 or 25 copies of PBSa confirmed such increase (Supplementary information, Figure S2C and S2D), suggesting that the multimerization feature of the Casilio system allows titration of labeling intensity at target loci. We further tested the ability of the Casilio system to simultaneously label centromeres and telomeres. By using sgRNA to target alpha-satellite repeats

Figure 1 The Casilio system enables multiplexing and multimerization of effectors for gene regulation, epigenetic editing and chromosomal labeling. **(A)** Schematics of the Casilio system. Top: comparison of conventional dCas9 fusion and Casilio tethering of effectors. Center: the 8-repeat PUF domain and the corresponding 8-mer target RNA. Table shows PUF diresidues and the corresponding RNA base. Bottom: annotation for the 4 PUF isotypes and PBS used in this study. **(B)** The specificity of the Casilio system. Column plot shows mean fold changes (mean ± SEM; *n* = 3) of tdTomato fluorescence of cells transfected with the indicated constructs. **(C)** Multimerization of activators by the Casilio system enables robust activation of endogenous *OCT4* gene. Upper: schematic showing positions of 4 sgRNA-PBS used to activate *OCT4*. Lower: mean fold changes (mean ± SEM; *n* = 3) measured by qRT-PCR of *OCT4* expression induced by dCas9/PUFa-p65HSF1 or dCas9-p65HSF1 activator with the indicated cocktail of *OCT4* or control sgRNAs-5×PBSa. **(D)** The Casilio system can activate and repress different genes simultaneously. Column plots show mean fold changes of *OCT4* (upper) and *SOX2* gene expression (lower) in cells transfected with the indicated constructs. **(E)** Recruitment of the HAT domain by the Casilio system to enhancers to activate *OCT4* expression. HEK293T cells were overexpressed with different HAT fusions and cocktails of 4 sgRNA-5×PBSa targeting PP, PE or DE as indicated. qRT-PCR was performed to evaluate the fold changes of *OCT4* expression. **(F)** Simultaneous labeling of telomeres and centromeres by the Casilio system (scale bars: 5 μm). (i) Labeling of telomeres by Clover-PUFa and sgTelomere with 0, 5, 15 or 25 copies of PBSa. (ii) TRF2 immunostaining to validate labeling of telomeres by dCas9/Clover-PUFa/sgTelomere-25×PBSa. (iii) CREST immunostaining to validate labeling of centromeres by dCas9/Clover-PUFc/sgCentromere-20×PBSc. (iv) Co-labeling of centromeres and telomeres by the Casilio system. **(G)** Cartoon highlighting three major features of the Casilio system.

in centromeres (sgCentromere-20×PBSc; Supplementary information, Figure S2B), we observed specific centromere labeling as confirmed by CREST immunostaining (Figure 1Fiii). Although not all centromeres marked by CREST were detected by the Casilio system, likely due to the low number of sgCentromere-binding sites in some centromeres (Supplementary information, Figure S2B), most of the Casilio signal overlapped with CREST signals. When we co-introduced Clover-PUFc/sgCentromere-20×PBSc, Ruby-PUFa/sgTelomere-25×PBSa and dCas9 into HEK293T cells, we observed simultaneous labeling of both centromeres and telomeres in the same cells (Figure 1Fiv), demonstrating that the Casilio system can be used to independently label multiple genomic loci.

In conclusion, here we show that the Casilio system can achieve multiplexing and multimerization of proteins such as transcriptional regulators, epigenetic modifiers and fluorescent proteins at defined genomic loci (Figure 1G). The main advantages of the Casilio system include: (I) Multiplexing. Different Casilio modules can be simultaneously delivered into a cell and each can operate at their defined target sites with independent function. Since PUF domains can be easily programmed to recognize any 8-mer RNA motifs, this greatly expands the potential number of independent Casilio modules. (II) Multimerization. Simplicity of the linear 8-mer PBS motif allows extensive multimerization of PUF fusions on sgRNA-PBS without hindering sgRNA transcription or dCas9/sgRNA DNA-binding activity. This feature allows multiple molecules of PUF fusions to be assembled on the sgRNA, achieving localized concentration of effectors or protein tags. This is particularly beneficial for fluorescent imaging and transcriptional regulation. This feature may facilitate labeling of non-repeat sequences where tiling of > 30 sgRNAs were required [9]. (III) Complex formation. With further development and optimization, the sgRNA-PBS has the potential to act as an RNA scaffold for PUF-directed assembly of stoichiometrically defined protein complexes. Specifically, different numbers of PBS with varying specificities can be appended to the sgRNA to enable the assembly of multiprotein complex with defined stoichiometry and defined ordering along the sgRNA-PBS (Figure 1G). Considering these features, we believe that the Casilio system will become a powerful tool for studying gene function and chromosome structure.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)

Cheng et al Supplementary Information

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Materials and Methods

Vectors in this study are listed in Table S1. For additional information and updates, please visit http://casil.io/

Cloning

PUFa [PUF(3-2)] and PUFb [PUF(6-2/7-2)] with N-terminal Nuclear Localization Signal (NLS) [1] were amplified from constructs containing these coding sequences with primers containing SgrAI and PacI sites and were used to replace SgrAI-dCas9-FseI from pAC164:pmax-dCas9Master_VP64 to create pAC1355:pmax-NLSPUFa_VP64 and pAC1356:pmax-NLSPUFb_VP64. A fusion PCR with 5' fragment up to repeat 4 of NLSPUFb and 3' fragment from repeat 5 to the end of NLSPUFa was used to create pAC1357:pmax-NLSPUFw_VP64. A fusion PCR of 5' fragment of NLSPUFa with 3' fragment of NLSPUb was used to to create pAC1358:pmax-NLSPUFc_VP64.

p65HSF1 activator ORF was amplified from MS2-P65-HSF1_GFP (Addgene: 61423) with FseI PacI sites to replace VP64 fragment in pAC164 to create pAC1410:pmax-

dCas9_p65HSF1, and replace VP64 in pAC1355 and pAC1358 to create pAC1393: pmax-NLSPUFa_p65HSF1 and pAC1411:pmax-NLSPUFc_p65HSF1, respectively.

Clover and mRuby2 were amplified from pcDNA3-Clover (Addgene #40259) and pcDNA3-mRuby2 (Addgene #40260) respectively with primers containing SgrAI and FseI cloning site, ligated with various FseI-PUF-PacI amplified from the above pAC1356~1358 and vector digested from pAC149:pCR8-dCas9VP160 (Addgene #48221) to create gateway donor vectors pAC1402, pAC1403 and pAC1404 containing ORFS of Clover_PUFa and Clover_PUFc, mRuby2_PUFa, respectively. These ORFs are then transferred to PB3-neo vector by recombining with pAC1119:PB3-neo(-)-pmaxDEST(+) by LR Clonase (Invitrogen) to create expression vectors pAC1360 (Clover_PUFa), pAC1381 (Clover_PUFc) and pAC1362 (mRuby2_PUFa).

NLSKRAB repressor domain was amplified from SOX2 TALE Repressor (KRAB 1-75) (Addgene #42945) with primers containing AgeI-ClaI sites and ligated with NLSPUFa amplified with primers containing AclI PacI and with pAC1360 digested with SgrAI-PacI as vector to create pAC1412: PB3-neo(-)-pmax-NLSKRAB_NLSPUFa.

The FseI-p65HSF1-PacI fragment was released from pAC1393 and ligated with SgrAI-NLSPUMb fragment released from pAC1356 and pAC1360 digested with SgrAI-PacI as vector to create pAC1413: PB3-neo(-)-pmax-NLSPUFb_p65HSF1. The BFPKRAB fragment was amplified from pHR-SFFV-dCas9-BFP-KRAB (Addgene #46911) and was used to replace Clover fragment from pAC1360 to create pAC1414: PB3-neo(-)-pmax-

BFPKRAB_NLSPUFa. Then, an NheI-CAGGS-NLSPUFb_p65HSF1-NheI fragment was amplified from pAC1413 and inserted into pAC1414 digested with NheI to create a dual expression vector for BFPKRAB-NLSPUFa and NLSPUFb-p65HSF1 (pAC1414: PB3-NLSPUFb_p65HSF1(-)neo(-)-BFPKRAB2_NLSPUFa).

Four gateway donor vectors with improved linker sequences and three extra NLS on the N-terminal and one additional NLS on the C-terminal of PUF as well as cloning sites for N-terminal (SgrAI,ClaI) and C-terminal (FseI-PacI) insertions were created (pAC1404~1408). HAT sequence was amplified from mouse Crebbp gene using mouse cDNA with primers containing FseI-PacI site and inserted into pAC164 to create pAC1364: pmax-dCas9Master_CBPHAT and into pAC1405 to create pAC1415: pCR8-4xNLSPUFa_2xNLS_CBPHAT. HAT sequence was amplified with another pair of primers containing SgrAI-AclI site and cloned into SgrAI-ClaI site of pAC1405 to create pAC1416: pCR8-CBPHAT_4xNLSPUFa_2xNLS. pAC1415 and pAC1416 were recombined into pAC90:pmax-DEST (Addgene #48222) to create expression vectors pAC1417: pmax-4xNLSPUFa_2xNLS_CBPHAT and pAC1418: pmax-CBPHAT_4xNLSPUFa_2xNLS, respectively. FseI-mCherry-PacI fragment was amplified from a plasmid containing mCherry sequence and ligated with SgrAI-dCas9-FseI to PB3-neo(-)-pmax to generate pAC1419: PB3-neo(-)-pmax-dCas9Master_mCherry.

Expression vectors for sgRNA-PBS were constructed as follows: First, a sgRNA scaffold based on sgF+E [2] with BbsI for oligo cloning of guide sequence and with 3’ BsaI (right

upstream of the terminator) for insertion of PBS were ordered as a gBlock (IDT), and were cloned into pX330 (Addgene #42230) replacing the AflIII-NotI region to create vector pAC1394: pX-sgRNA-0xPBS. Then, oligos encoding 5xPBSa sites each separated by ggc-spacer flanked by 5'-AGAT-3' overhangs on one side and 5'-ATCT-3' on the other side were treated with T4PNK and annealed and ligated into pAC1394 digested with BsaI (to create compatible overhangs). Clones were then screened for 1 copy (5xPBS), 2 copies (10xPBS), etc of the oligo insertions for the different number of PBS. For 1xPBS and 2xPBS vectors, they were constructed using oligo containing one PBS site. Guide sequence for each target were then cloned onto the sgRNA-PBS expression vectors via BbsI. For sgRNA expression vectors with GFP expression markers, they were constructed by transferring the sgRNA-PBS expression cassette from the pX vectors onto a PB-GFP vector via AscI site. The sgRNA spacer sequences are: Ctl (Control), GTTCTCTTGCTGAAAGCTCGA; Tet, GCTTTTCTCTATCACTGATA, SV40-P1, GCATACTTCTGCCTGCTGGGGAGCCTG; OCT4-PP-1, GGCCCCGCCCCCTGGATGGG; OCT4-PP-2, GGGGGGAGAAACTGAGGCGA; OCT4-PP-3, GGTGGTGGCAATGGTGTCTG; OCT4-PP-4, GACACAACTGGCGCCCCTCC; OCT4-PE-1, GGCCCCTACTTCCCCTTCAA; OCT4-PE-2, GAGTGATAAGACACCCGCTT; OCT4-PE-3, GCCTGGGAGGGACTGGGGGA; OCT4-PE-4, GGACAATCCCGGTCCCCAGA; OCT4-DE-1, GGTCTGCCGGAAGGTCTACA; OCT4-DE-2, GGCAGGTAGATTATGGGGCC; OCT4-DE-3, GAAGACGGCCTCTCAGAGGA; OCT4-DE-4, GTATTTCTGGCCTGGGCAAG; SOX2-PP-1, GCATGTGACGGGGGCTGTCA; SOX2-PP-2, GCTGCCGGGTTTTGCATGAA;

SOX2-PP-3, GCCGGCCGCGCGGGGGAGGC; SOX2-PP-4, GGCAGGCGAGGAGGGGGAGG; Telomere, GTTAGGGTTAGGGTTAGGGTTA; Centromere, GTTGAGGCCTTCGTTGGAAAC.

Cell culture and transfection

HEK293T cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)(Sigma) with 10% fetal bovine serum (FBS)(Lonza), 4% Glutamax (Gibco), 1% Sodium Pyruvate (Gibco) and penicillin-streptomycin (Gibco). Incubator conditions were 37 °C and 5% CO_2. For activation experiments, cells were seeded into 12-well plates at 100,000 cells per well the day before being transfected with 200ng of dCas9 construct, 100ng of modified sgRNA and 100ng of PUF-fusion with Attractene tranfection reagent (Qiagen). After transfection, cells were grown for 48h and harvested for either RNA extraction or fluorescence-activated cell sorting (FACS). For dual activation-repression experiments, transfection remained the same, however cells were seeded into 12-well plates at 150,000 cells per well and were grown for 72 h before being harvested for FACS. For experiments with *OCT4* and *SOX2* (or *B4GALNT1*) dual activation-repression, cells were triple-sorted by BFP (for the activator-repressor module PUFb-p65HSF1/BFPKRAB-PUFa), mCherry (for dCas9mCherry) and GFP (for the sgRNA-PBS on vectors co-expressing EGFP) before RNA extraction. For imaging experiments, cells were seeded into 6-well plates with 22x22x1 microscope cover glass at 300,000 cells per well the day before being transfected with 50ng of dCas9 construct, 500ng of modified sgRNA and 50ng of a PUF-fluorescent fusion with Attractene transfection reagent. After transfection, cells were grown for 48 h then immunostained.

Quantitative RT-PCR analysis

Cells were harvested with trypsin, washed with Dulbecco's phosphate-buffered saline (dPBS), centrifuged at 125g for 5 mins and then RNA was extracted using RNeasy Plus Mini Kit (Qiagen). A cDNA library was made using Applied Biosystems High Capacity RNA-to-cDNA kit with 1 μg of RNA. TaqMan Gene expression assays (Applied Biosystems) were designed using GAPDH (Hs03929097, VIC) as endogenous control and OCT4 (Hs00999632, FAM) and SOX2 (Hs01053049, FAM) as targets. TaqMan Universal Master Mix II, with UNG (Applied Biosystems) was used for Quantitative PCR (qPCR), with 2 μl of 1:10 diluted cDNA used for each reaction. Activation was analyzed with the Applied Biosystems ViiA7 instrument. Gene expression levels were calculated by "delta delta Ct" algorithm and normalized to control samples.

Fluorescence-activated cell sorting

Cells were trypsinized and fixed for 10 min with 2% paraformaldehyde. Afterwards, the cells were centrifuged at 125g for 5 min and resuspended in dPBS. Samples were analyzed on a FACScalibur flow cytometer using CellQuest Pro software (BD Bioscience). Fifty thousands events were collected in each run.

Immunostaining and microscopy

While adherent to a cover glass, cells were fixed in 2% paraformaldehyde, washed with 0.1% Triton X-100 in dPBS, permeabilized with 0.4% Triton X-100 in dPBS for 5 min at 4 °C, blocked in 5% Blotting-grade blocking buffer (BIO-RAD) for 30 min, incubated with the primary antibody in blocking buffer at 4 °C overnight, washed three times with

dPBS, then incubated in the dark with a respective Alexa Fluor-conjugated secondary antibody at room temperature for 3 hours, washed again, and stained with DAPI. The cover glass was mounted on a slide with glycerol before imaging. Immunostaining of telomeres was performed with a 1:100 dilution of an anti-TRF2 primary antibody (Novus Biologicals: NB110-57130) and a 1:500 dilution of an Alexa fluor 594-conjugated anti-Rabbit IgG secondary antibody (Invitrogen, A11037). A 1:100 dilution of CREST antibody (Antibodies Incorporated: 15-235-0001) was used in conjunction with a 1:500 diltuion of an Alexa fluor 594-conjugated anti-Human IgG secondary antibody (Invitrogen, A11014) to detect centromeres. Analysis and processing of confocal images were done using Bitplane Imaris and Fiji.

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Supplementary figure legends

Figure S1. Supporting data figure for Casilio-mediated gene regulation. (A) Top: A schematic for the experiment to test the ability of dCas9-VP64 to bind and activate a tdTomato transgene after inserting varying number of PBS at the 3' end of the sgRNA. Bottom: Column plot showing the mean fold changes (±S.E.M.; n=3) in tdTomato fluorescence of cells transfected with the different constructs indicated in the legend below the plot. (B) Top: A schematic describing the experiment to test activation of a TetO::tdTomato transgene by Casilio-VP64 activator with different number of PBS. Bottom: Column plot showing the mean fold changes (±S.E.M.; n=3) of tdTomato fluorescence of cells transfected with the different constructs indicated in the legend below the plot. (C) Comparison of Casilio activator based on VP64 (PUFa-VP64; red columns) and p65HSF1 (PUFa-p65HSF1; blue columns) domains for activation of TetO::tdTomato in conjunction with control sgRNA with 5xPBSa or TetO-targeting sgRNA with 0,1,5,15 or 25 copies of PBSa. Columns show mean fold change (with S.E.M.; n=3) of tdTomato fluorescence relative to experiments using control sgRNA (sgCtl). (D) Top: A gene model showing the relative match positions (Strokes labeled 1~4) of sgRNA-PBS used to activate SOX2 gene. Bottom: Mean fold changes (95% C.I.; n=3) measured by qRT-PCR for activation of SOX2 expression using dCas9/PUFa-p65HSF1 activator or dCas9-p65HSF1 activator with the indicated cocktail of SOX2 targeting sgRNA-5xPBSa or control sgRNA-5xPBSa. (E) Activation of *OCT4* and *SOX2* by sgRNA-PBS with different numbers of PBS. (i) Mean fold changes (95% C.I.; n=3) of OCT4 expression with the indicated single or cocktails of *OCT4*-targeting sgRNA-PBSa with 1,5,15 or 25 copies of PBSa. (ii) Mean fold changes (95% C.I.; n=3) of *SOX2*

expression with the indicated single or cocktails of *SOX2*-targeting sgRNA-PBSa with 1,5,15 or 25 copies of PBSa. (F) Casilio activator and repressor modules can independently and simultaneously activate and repress TetO::tdTomato and SV40::EGFP transgenes. Top: Schematic diagram illustrating the experiment to achieve simultaneous activation and repression of TetO::tdTomato and SV40::EGFP by PUFc-p65HSF1 and KRAB-PUFa, respectively. Bottom: Upper column plot shows mean fold changes (with S.E.M.; n=3) of tdTomato fluorescence; Lower column plot shows mean fold changes (with S.E.M.; n=3) of EGFP fluorescence of cells transfected with constructs indicated in the central legend. (G) Activation of *OCT4* expression by CBPHAT-PUFa with individual guides targeting PP, PE and DE. Column plot shows mean fold changes (95% C.I.; n=3) of *OCT4* expression after transfection of dCas9/CBPHAT-PUFa and single or cocktails of sgRNAs targeting PP, PE, DE of *OCT4*.

Figure S2. Supporting data figure for Casilio-mediated chromosomal imaging. (A) Bar graph showing the number of perfect matches (allowing NGG PAM) for sgTelomere per hg19 chromosome. (B) Bar graph showing the number of perfect matches (allowing NGG PAM) for sgCentromere per hg19 chromosome. (C) Quantification of the number of fluorescent foci and signal-to-noise ratio of labeling of telomeres by Casilio directed by sgRNA-PBS with different number of PBS. (D) Quantification of the number of fluorescent foci in HEK293T cells transfected with dCas9/PUFa-Clover and a telomere-targeting sgRNA with 0,5,15 or 25 PBSa sites. (n=20; Mann-Whitney statistics: ***=$p<0.0005$, ****=$p<0.0001$) (B) Quantification of signal-to-noise ratio as a proportion of total signal at foci over the total nuclear signal by Casilio with 5,15 or 25x

PBSa on the sgRNA targeting telomeres. (n=20; Mann-Whitney statistics:

****=p<0.0001)

Supplementary Tables

Table S1. List of vectors and their Addgene accession numbers.

Citable ID	Descriptive name	Description	Addgene ID
pAC164	pmax-dCas9Master_VP64	dCas9-VP64 driven by CAGGS promoter in expression vector pmax (Clontech)	71879
pAC1119	PB3-neo(-)-pmaxDEST(+)	PB gateway destination vector with neo selectable marker and pmax cassette (Clonetech)	71880
pAC1355	pmax-NLSPUFa_VP64	NLSPUFa_VP64 in transient expression vector pmax	71881
pAC1356	pmax-NLSPUFb_VP64	NLSPUFb_VP64 in expression vector pmax	71882
pAC1357	pmax-NLSPUFw_VP64	NLSPUFw_VP64 in expression vector pmax	71883
pAC1358	pmax-NLSPUFc_VP64	NLSPUFc_VP64 in expression vector pmax	71884
pAC1360	PB3-neo(-)-pmax-Clover_NLSPUFa	Clover_NLSPUFa in pAC1119	71885
pAC1362	PB3-neo(-)-pmax-	mRuby2_NLSPUFa in	71886

	mRuby2_NLSPUFa	pAC1119	
pAC1364	pmax-dCas9Master_mCBPHAT	dCas9Master_mCBPHAT in pmax expression vector	71887
pAC1371	pX-sgRNA-5xPBSa	Cloning vector for expression of sgRNA-5xPBSa	71888
pAC1372	pX-sgRNA-15xPBSa	Cloning vector for expression of sgRNA-15xPBSa	71889
pAC1373	pX-sgRNA-25xPBSa	Cloning vector for expression of sgRNA-25xPBSa	71890
pAC1374	pX-sgRNA-5xPBSb	Cloning vector for transient expression of sgRNA-5xPBSb	71891
pAC1375	pX-sgRNA-15xPBSb	Cloning vector for expression of sgRNA-15xPBSb	71892
pAC1376	pX-sgRNA-25xPBSb	Cloning vector for expression of sgRNA-25xPBSb	71893
pAC1379	pX-sgRNA-5xPBSw	Cloning vector for expression of sgRNA-	71894

		5xPBSw	
pAC1380	pX-sgRNA-5xPBSc	Cloning vector for expression of sgRNA-5xPBSc	71895
pAC1381	PB3-neo(-)-pmax-Clover_NLSPUFc	Clover_NLSPUFc in pAC1119	71896
pAC1393	pmax-NLSPUFa_p65HSF1	NLSPUFa_p65HSF1 in pmax expression vector	71897
pAC1394	pX-sgRNA-0xPBS	Cloning vector for expression of sgRNA without PBS. It contains extra sequences for BsaI digestion for insertion of PBS	71898
pAC1399	pX-sgRNA-20xPBSc	Cloning vector for expression of sgRNA-20xPBSc	71899
pAC1402	pCR8-Clover_NLSPUFa	Clover_NLSPUFa in pCR8 gateway donor vector	71900
pAC1403	pCR8-Clover_NLSPUFc	Clover_NLSPUFc in pCR8 gateway donor vector	71901
pAC1404	pCR8-mRuby2_NLSPUFa	mRuby2_NLSPUFa in pCR8 gateway donor vector	71902

pAC1405	pCR8-4xNLS_PUFa_2xNLS	NLSPUFa pCR8 gateway donor vector for insertion of N-terminal domain (SgrAI or AgeI with ClaI) and C-terminal domain (FseI PacI). Grow in dcm- cells (e.g., NEB C3040) to prepare vector for ClaI digestion	71903
pAC1406	pCR8-4xNLS_PUFb_2xNLS	NLSPUFb pCR8 gateway donor vector for insertion of N-terminal domain (SgrAI or AgeI with ClaI) and C-terminal domain (FseI PacI). Grow in dcm- cells (e.g., NEB C3040) to prepare vector for ClaI digestion	71904
pAC1407	pCR8-4xNLS_PUFw_2xNLS	NLSPUFw pCR8 gateway donor vector for insertion of N-terminal domain (SgrAI or AgeI with ClaI) and C-terminal domain (FseI	71905

		PacI). Grow in dcm- cells (e.g., NEB C3040) to prepare vector for ClaI digestion	
pAC1408	pCR8-4xNLS_PUFc_2xNLS	NLSPUFc pCR8 gateway donor vector for insertion of N-terminal domain (SgrAI or AgeI with ClaI) and C-terminal domain (FseI PacI). Grow in dcm- cells (e.g., NEB C3040) to prepare vector for ClaI digestion	71906
pAC1410	pmax-dCas9Master_p65HSF1	dCas9Master_p65HSF1 in pmax expression vector	71907
pAC1411	pmax-NLSPUFc_p65HSF1	NLSPUFc_p65HSF1 in pmax expression vector	71908
pAC1412	PB3-neo(-)-pmax-NLSKRAB_NLSPUFa	NLSKRAB_NLSPUFa in pAC1119	71909
pAC1413	PB3-neo(-)-pmax-NLSPUFb_p65HSF1	NLSPUFb_p65HSF1 in pAC1119	71910
pAC1414	PB3-NLSPUFb_p65HSF1-neo(-)-	Dual expression vector for NLSPUFb_p65HSF1 and	71911

	BFPKRAB_NLSPUFa	BFPKRAB_NLSPUFa	
pAC1415	pCR8-4xNLS_PUFa_2xNLS_mCBPHAT	4xNLS_PUFa_2xNLS_mCBPHAT in pCR8 Gateway donor vector	71912
pAC1416	pCR8-mCBPHAT_4xNLS_PUFa_2xNLS	mCBPHAT_4xNLS_PUFa_2xNLS in pCR8 Gateway donor vector	71913
pAC1417	pmax-4xNLS_PUFa_2xNLS_mCBPHAT	4xNLS_PUFa_2xNLS_mCBPHAT in pmax expression vector	71914
pAC1418	pmax-mCBPHAT_4xNLS_PUFa_2xNLS	mCBPHAT_4xNLS_PUFa_2xNLS in pmax expression vector	71915
pAC1419	PB3-neo(-)-pmax-dCas9Master_mCherry	dCas9Master_mCherry in pAC1119	71916
pAC1420	pX-sgRNA-1xPBSa	Cloning vector for expression of sgRNA-1xPBSa	71917
pAC1421	pX-sgRNA-2xPBSa	Cloning vector for expression of sgRNA-2xPBSa	71918
pAC1422	pX-sgRNA-1xPBSb	Cloning vector for expression of sgRNA-	71919

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pAC1423	pX-sgRNA-2xPBSb	Cloning vector for expression of sgRNA-2xPBSb	71920
pAC1424	pX-sgRNA-10xPBSb	Cloning vector for expression of sgRNA-10xPBSb	71921
pAC1425	pX-sgRNA-20xPBSb	Cloning vector for expression of sgRNA-20xPBSb	71925
pAC1426	pX-sgRNA-47xPBSb	Cloning vector for expression of sgRNA-47xPBSb	71926
pAC1427	pX-sgRNA-10xPBSw	Cloning vector for expression of sgRNA-10xPBSw	71927
pAC1428	pX-sgRNA-15xPBSw	Cloning vector for expression of sgRNA-15xPBSw	71928
pAC1429	pX-sgRNA-10xPBSc	Cloning vector for expression of sgRNA-10xPBSc	71929
pAC1430	pX-sgRNA-15xPBSc	Cloning vector for	71930

		expression of sgRNA-15xPBSc	
pAC1431	PB3-LGFPL(-)-sgSOX2PP1-5xPBSa	Vector for expression of sgSOX2PP1-5xPBSa with a GFP marker flanked by loxP sites	71931
pAC1432	PB3-LGFPL(-)-sgSOX2PP2-5xPBSa(-)	Vector for expression of sgSOX2PP2-5xPBSa with a GFP marker flanked by loxP sites	71932
pAC1433	PB3-LGFPL(-)-sgSOX2PP3-5xPBSa	Vector for expression of sgSOX2PP3-5xPBSa with a GFP marker flanked by loxP sites	71933
pAC1434	PB3-LGFPL(-)-sgSOX2PP4-5xPBSa	Vector for expression of sgSOX2PP4-5xPBSa with a GFP marker flanked by loxP sites	71934
pAC1435	PB3-LGFPL(-)-sgOCT4PP1-5xPBSb	Vector for expression of sgOCT4PP1-5xPBSb with a GFP marker flanked by loxP sites	71935
pAC1436	PB3-LGFPL(-)-sgOCT4PP4-	Vector for expression of	71936

	5xPBSb	sgOCT4PP4-5xPBSb with a GFP marker flanked by loxP sites	
pAC1437	PB3-LGFPL(-)-sgOCT4PP3-5xPBSb	Vector for expression of sgOCT4PP3-5xPBSb with a GFP marker flanked by loxP sites	71937
pAC1438	PB3-LGFPL(-)-sgOCT4PP2-5xPBSb	Vector for expression of sgOCT4PP2-5xPBSb with a GFP marker flanked by loxP sites	71938

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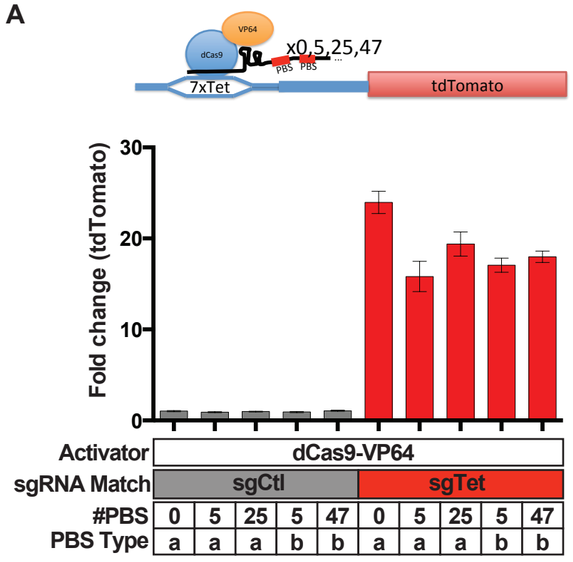
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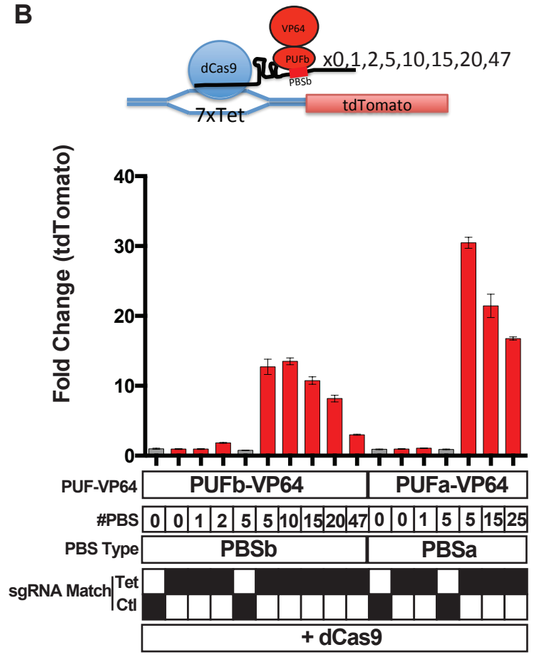
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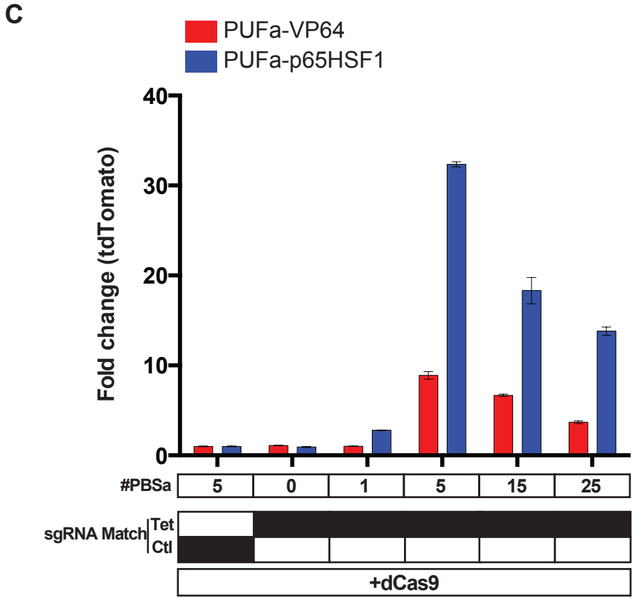
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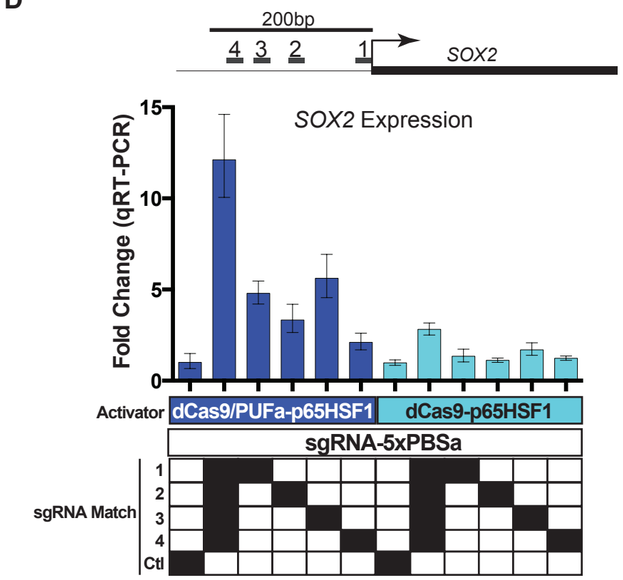
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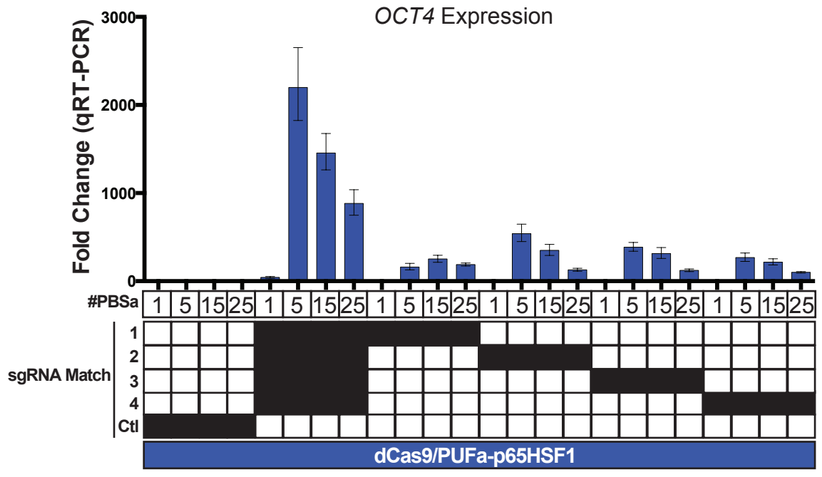
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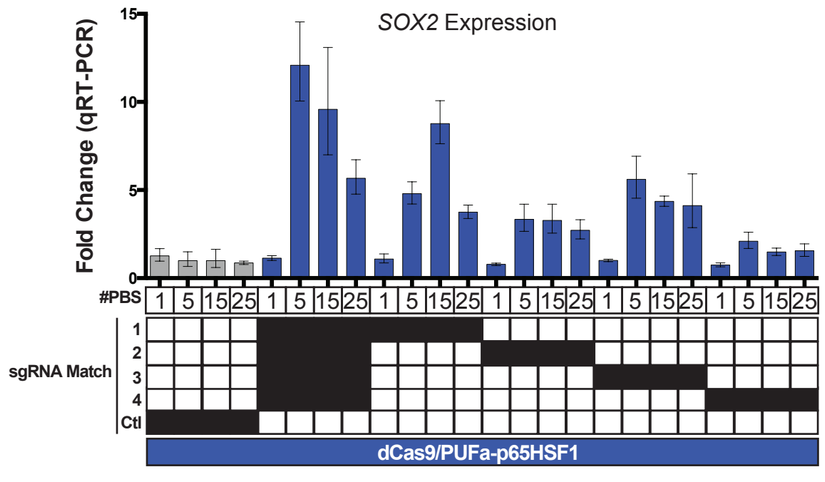
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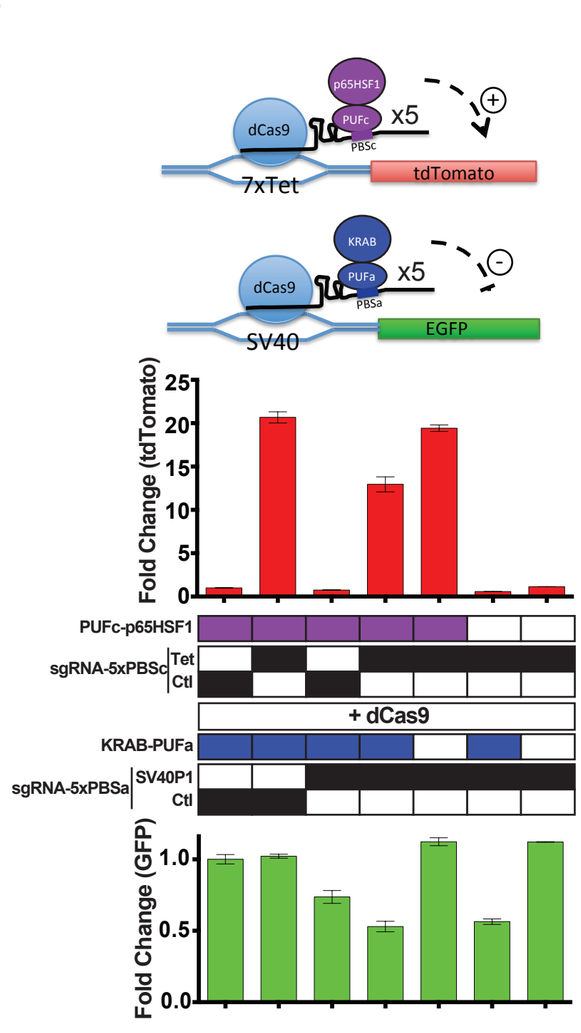
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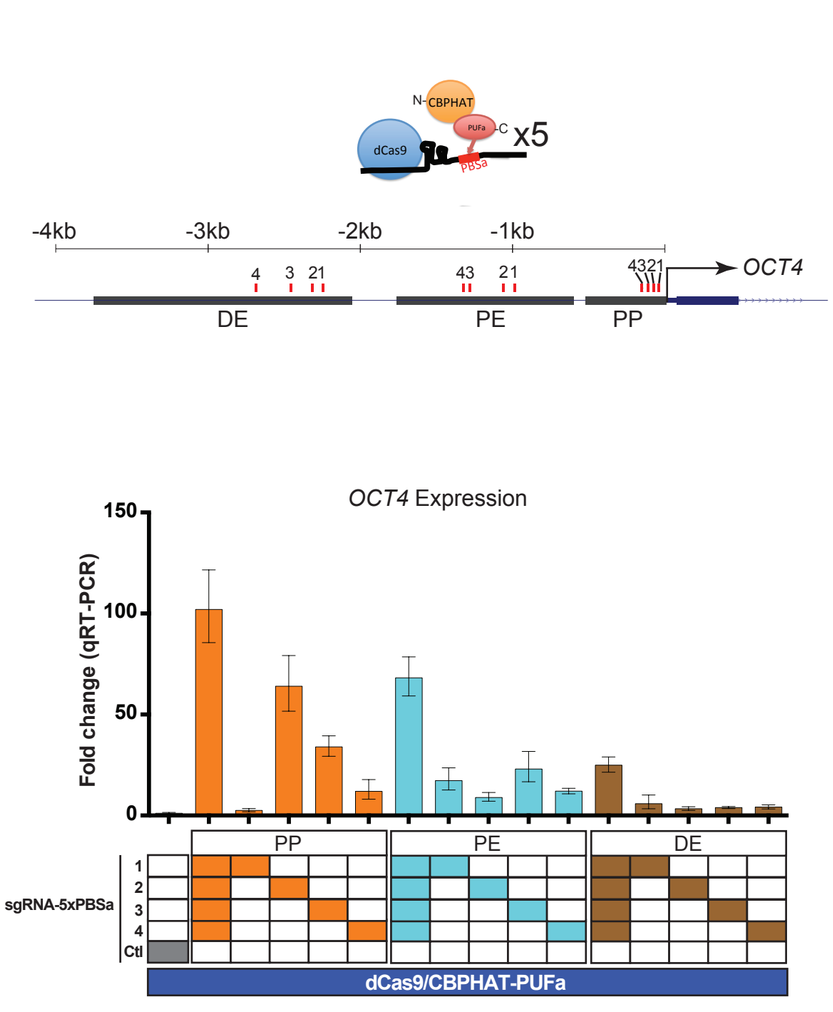
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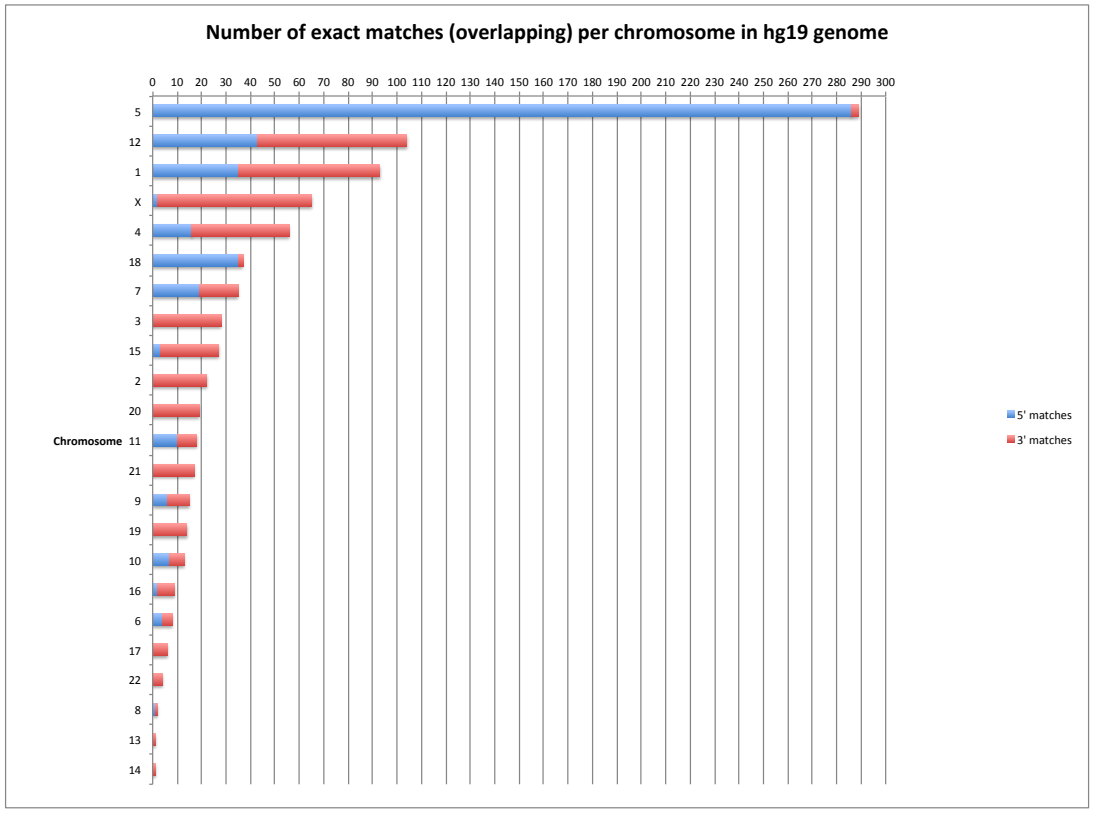
A sgTelomere



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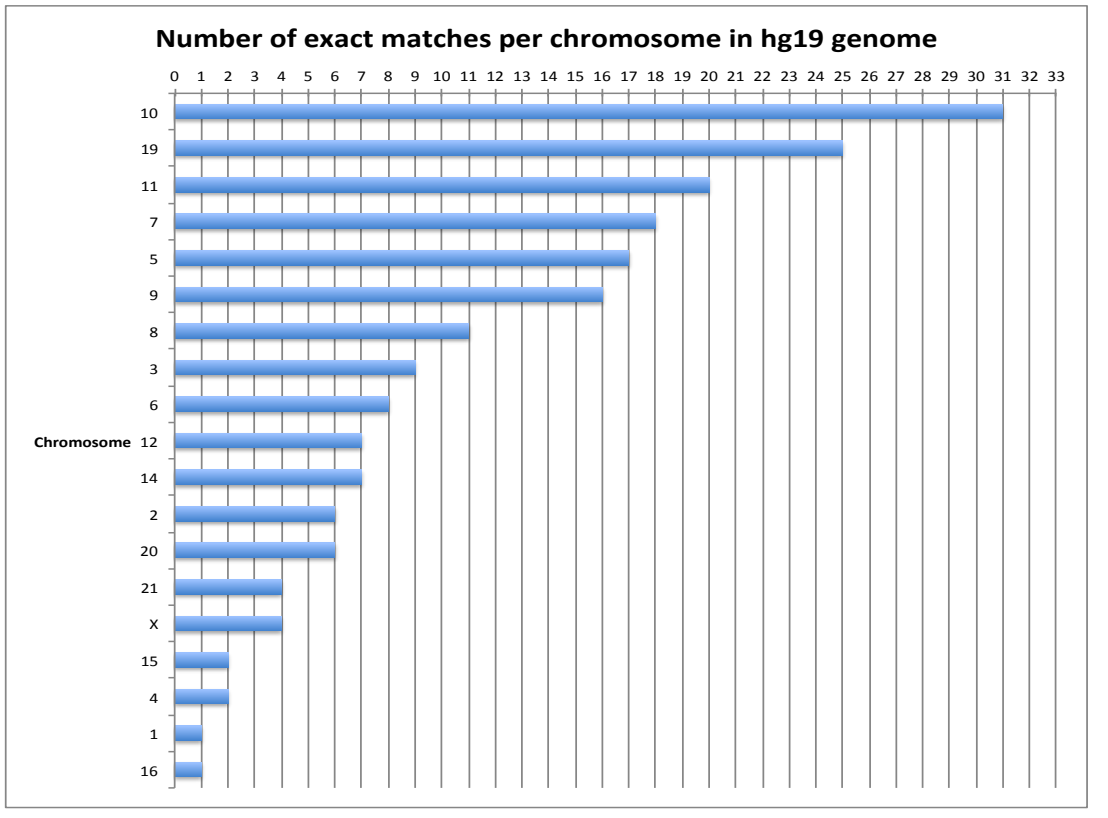

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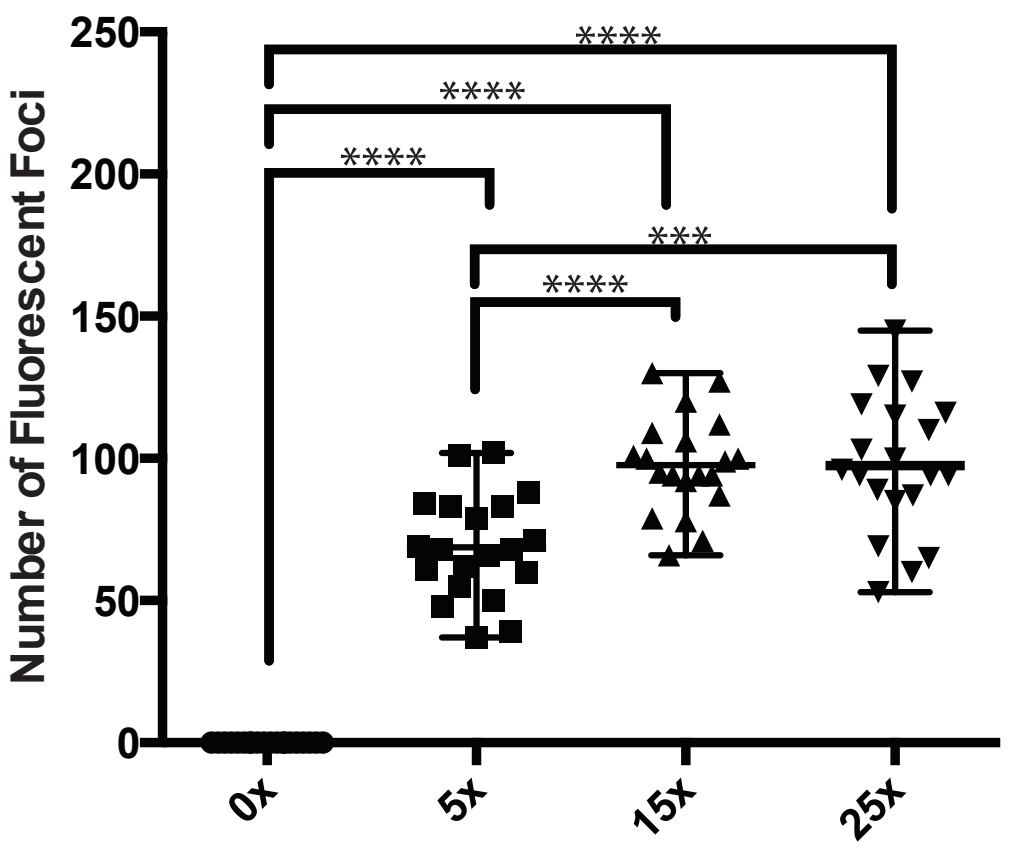

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