Dual CRISPR interference and activation for targeted reactivation of X-linked endogenous FOXP3 in human breast cancer cells

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Abstract
Background: Unlike autosomal tumor suppressors, X-linked tumor suppressors can be inactivated by a single hit due to X-chromosome inactivation (XCI). Here, we argue that targeted reactivation of the non-mutated allele from XCI offers a potential therapy for female breast cancers.

Methods: Towards this goal, we developed a dual CRISPR interference and activation (CRISPRi/a) approach for simultaneously silencing and reactivating multiple X-linked genes using two orthogonal, nuclease-deficient CRISPR/Cas9 (dCas9) proteins.

Results: Using Streptococcus pyogenes dCas9-KRAB for silencing XIST and Staphylococcus aureus dCas9-VPR for activating FOXP3, we achieved CRISPR activation of FOXP3 in various cell lines of human female breast cancers. In human breast cancer HCC202 cells, which express a synonymous heterozygous mutation in the coding region of FOXP3, simultaneous silencing of XIST from XCI led to enhanced and prolonged FOXP3 activation. Also, reactivation of endogenous FOXP3 in breast cancer cells by CRISPRi/a inhibited tumor growth in vitro and in vivo. We further optimized CRISPRa by fusing dCas9 to the demethylase TET1 and observed enhanced FOXP3 activation. Analysis of the conserved CpG-rich region of FOXP3 intron 1 confirmed that CRISPRi/a-mediated simultaneous FOXP3 activation and XIST silencing were accompanied by elevated H4 acetylation, including H4K5ac, H4K8ac, and H4K16ac, and H3K4me3 and lower DNA methylation. This indicates that CRISPRi/a targeting to XIST and FOXP3 loci alters their transcription and their nearby epigenetic modifications.

Conclusions: The simultaneous activation and repression of the X-linked, endogenous FOXP3 and XIST from XCI offers a useful research tool and a potential therapeutic for female breast cancers.

Keywords: FOXP3, Breast cancer, CRISPR, X-linked gene, Transcript

Main text
Autosomal tumor suppressor genes can be inactivated by a two-hit Knudson mechanism. However, X chromosome-linked tumor suppressor genes, such as FOXP3 at Xp11.23 [1], can be inactivated by a single-hit mechanism, because of X-chromosome inactivation (XCI). In female breast cancer cells, all identified gene deletions of
**FOXP3** are heterozygous, and mice with a Foxp3-heterozygous mutation develop spontaneous breast cancers, suggesting that the active allele may be the only allele affected [1]. Thus, for females with cancer, it may be possible to reactivate the non-mutated, inactivated allele for therapeutic purposes. We recently developed, for complex gene regulation, a flexible endonuclease-deficient CRISPRi/Cas9 (dCas9)-based platform that independently controls the expression of various genes (repression and activation) within the same cell [2]. Thus, for therapeutic purposes, targeted reactivation of XCI-endogenous tumor suppressor genes may be an effective strategy to restore their function in female cancer cells. The X-linked FOXP3 gene has dual roles in tumor cells and immune cells. As a master transcriptional regulator of regulatory T cells, FOXP3 limits antitumor immunity [3], whereas, in breast cancer cells, it is an epithelial cell-intrinsic tumor suppressor and is implicated in a tumor-suppressive function in the inhibition of tumor initiation and progression [1, 4–6]. Thus, using FOXP3 as an X-linked model gene, we aimed to develop, for human female breast cancer cells, a tunable and reversible, targeted reactivation of X-linked tumor suppressor genes. In the present study, using CRISPR interference and activation (CRISPRi/a), we achieved, for human female breast cancer cells, targeted reactivation of X-linked endogenous FOXP3, at least a partial reactivation from XCI. Next, we investigated the potential epigenetic mechanism during CRISPRi/a-mediated reactivation of FOXP3.

**Results and discussion**

**Transcription regulation of XIST and FOXP3 by CRISPRi/a in human breast cancer cells**

Since most human female breast cancer cell lines have low or no FOXP3 expression [1], accompanied by heterozygous gene deletions but rare FOXP3 mutations (Table S1), it may be possible to reactivate FOXP3 from XCI. We determined the expression levels of X-inactive specific transcript (XIST) in various female breast cancer cell lines, including MDA-MB-231, MCF7, and HCC202. As shown in Fig. S1, expression of XIST was high in HCC202 cells, but low in MCF7 and MDA-MB-231 cells. To achieve simultaneous transcriptional repression of XIST and reactivation of FOXP3 in the same cells, we utilized two orthogonal CRISPRi/Cas9 systems [2], Streptococcus pyogenes (Sp) dCas9-Krüppel-associated box (KRAB) (SpdCas9-KRAB) for silencing XIST and Staphylococcus aureus (Sa) dCas9-tripartite VP64-p65-Rta proteins (VPR) (SadamCas9-VPR) for reactivation of FOXP3 (Figs. S2A and B). We established the CRISPRi/a MDA-MB-231 cell model stably expressing SadCas9-VPR and SpdCas9-KRAB (Table S2). Then, we transiently co-transduced XIST- and FOXP3-single guide RNAs (sgRNAs) into the CRISPRi/a MDA-MB-231 cells (Figs. S2C–E). The efficacy of transduction of sgRNAs in the cells was validated by fluorescence microscopy (Fig. 1A) and Western blots (Fig. 1B). After transduction of sgRNAs, quantitative real-time PCR (qPCR) analysis showed that levels of the FOXP3 transcript were increased 8-fold by FOXP3-sgRNA with XIST-sgRNA at day 4 and up to 8 days (Fig. 1C). However, after doxycycline (Dox) addition, levels of the XIST transcript were reduced by XIST-sgRNA, but this difference was not statistically significant (Fig. 1D). Likewise, levels of the FOXP3 transcript in MDA-MB-231 cells were not significantly different after addition of Dox (Fig. 1C), suggesting a dominant activation of the FOXP3 transcript from an active X-linked allele. Furthermore, CRISPRi/a (with FOXP3/XIST-sgRNAs) and scrambled (CRISPRi/a without FOXP3/XIST-sgRNAs) MDA-MB-231 cells were injected into the fourth mammary fat pads of 8-week-old NSG female mice followed by Dox injection weekly for 28 days. As shown in Fig. S2F–H, xenograft tumor growth of CRISPRi/a MDA-MB-231 cells was slower than that of scrambled MDA-MB-231 cells. Also, reactivation of FOXP3 in xenograft tumors by CRISPRi/a was validated by qPCR (Fig. S2I). Next, we established the CRISPRi/a MCF7 cell model and transiently co-transduced both XIST- and FOXP3-sgRNAs into these cells (Fig. S3A and B and Table S2). After transduction of sgRNAs, levels of the FOXP3 transcript were increased more than 8-fold by the FOXP3-sgRNA with XIST-sgRNA (Fig. S3C). However, levels of the FOXP3 transcript in the cells were not changed by Dox, although levels of the XIST transcript were reduced by Dox (Fig. S3D), suggesting activation of the FOXP3 transcript from an active X-linked allele.

**CRISPRi/a-mediated targeted reactivation of X-linked FOXP3 from XCI in human breast cancer cells**

The HCC202 cell line expresses a synonymous heterozygous mutation (p.L266L c.798G>C) in the coding region of FOXP3 (Table S1), enabling us to determine, by cDNA sequencing, if FOXP3 is reactivated from one or both alleles. Also, in HCC202 cells, the two alleles of FOXP3 showed no deletion, but there were low expression levels of FOXP3 (Table S1). Thus, we established the CRISPRi/a HCC202 cell model (Fig. S4A and Table S2). After being transiently co-transduced with both XIST- and FOXP3-sgRNAs, the sgRNA-transduced cells were sorted by flow cytometry (Fig. 1E). After sgRNA transductions, levels of the FOXP3 transcript were increased approximately 6-fold by the FOXP3-sgRNA at day 4 and up to day 10 (Fig. 1G and H). Simultaneously, levels of the FOXP3 transcript
Fig. 1 | Assessment of CRISPRi/a in activation of endogenous FOXP3 and repression of XIST in human breast cancer cells. A efficacy of co-transduction of the XIST (mIFP) and FOXP3 (mCherry) sgRNAs in CRISPRi/a MDA-MB-231 cells before and after Dox addition at days 0, 2, 4, and 6 as determined by fluorescence microscopy. The expression of SpdCas9-KRAB (GFP) is induced by Dox in CRISPRi/a cells. BF, bright field. Scale bar, 1,000 μm. B protein expression of SpdCas9 and SadCas9 in CRISPRi/a MDA-MB-231 cells before and after sgRNA transduction and Dox treatment at days 0 and 4 as determined by Western blots with specific anti-SadCas9 and anti-SpCas9 antibodies. C, D quantitative expression analysis of FOXP3 and XIST before and after sgRNA transduction and Dox treatment of CRISPRi/a MDA-MB-231 cells at days 0, 2, 4, 6, 8, and 10 as determined by qPCR. The fold change in mRNA expression was calculated using the 2^(-ΔΔCt) method with GAPDH mRNA as an internal control. E targeted cell sorting of the CRISPRi/a HCC202 cells after transduction of XIST (mIFP) and FOXP3 (mCherry) sgRNAs and addition of Dox at day 4 as determined by flow cytometry. F, G quantitative expression analysis of FOXP3 and XIST before and after sgRNA transduction and Dox addition in CRISPRi/a HCC202 cells at days 0, 2, 4, 6, 8, and 10 as determined by flow cytometry. H expression of XIST in CRISPRi/a cells after Dox addition for 4 days as determined by RNA fluorescence in situ hybridization analysis. Cells were hybridized to the XIST probe (green). DAPI (blue) was used as a nuclear counterstain. DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 50 μm. I mutation analysis of a 798G/C mutation (red arrow) of the human FOXP3 transcript in CRISPRi/a HCC202 cells before and after activation of FOXP3 as determined by cDNA sequencing. J effect of CRISPRi/a-induced endogenous FOXP3 on growth of HCC202 cells. Cell proliferation was measured after Dox treatment. K bioluminescent and optical images of CRISPRi/a (with FOXP3/XIST sgRNAs) and scrambled (CRISPRi/a without FOXP3/XIST sgRNAs) HCC202 cells using the In Vivo Bio-luminescence Imaging System. Eight-week-old NSG female mice were performed by intratibial injection with 1x10^5 CRISPRi/a and scrambled HCC202 luciferase cells, respectively, followed by Dox injection (2.5 mg/kg weekly). L Quantitative analysis of the ex vivo bioluminescent imaging results from panels in K. M Representative optical images of lung metastasis in mice with CRISPRi/a and scrambled control cells at day 28 after bone transplantation. Red down arrows indicate Dox injection. N Quantification of lung metastatic nodules. The number of surface tumor lesions over all lobes of the lungs was scored for metastatic nodules. Data are presented as the means ± standard deviation. p values were determined by ANOVA followed by Tukey’s post hoc test, Mann Whitney test or by two-way ANOVA test vs. control group. All experiments were repeated three times.
were elevated approximately 2-fold at day 4 after Dox addition (Fig. 1F). Of note, by cDNA sequencing, a heterozygous 798G/C mutation of FOXP3 was identified in Dox-treated CRISPRi/a-FOX3/XIST HCC202 cells but not in untreated CRISPRi/a-FOX3/XIST HCC202 cells (Fig. 1I), suggesting that, in HCC202 cells, CRISPRi/a-induced activation of the FOXP3 transcript is at least partially reactivated from XCI under XIST downregulation. In addition, to test the effect of CRISPRi/a-induced FOXP3 reactivation on FOXP3 target genes in HCC202 cells, we assayed the expression of p21 (CDKN1A) and SKP2, which are prominent transcriptional targets of FOXP3 in breast cancer cells [7, 8]. Our data revealed that, for HCC202 cells, reactivation of endogenous FOXP3 by CRISPRi/a induced the transcription of p21 but reduced the transcription of SKP2 (Fig. S4B and C). Of note, Dox-induced reactivation of FOXP3 from XCI enhanced the transcriptional regulation of p21 and SKP2 in HCC202 cells.

Using the established CRISPRi/a HCC202 cells, we determined the effect of CRISPRi/a-induced endogenous FOXP3 on cell growth. We transiently transduced XIST sgRNA, FOXP3 sgRNA, or both into CRISPRi/a HCC202 cells for 48 hours, and then added Dox to the cells for 5 days. As shown in Figs. S4D and E, levels of the FOX3 transcript were gradually elevated in the CRISPRi/a cells with FOX3 sgRNA, whereas levels of XIST were reduced in the CRISPRi/a cells with XIST sgRNA from days 1 to 5 after addition of Dox. Cell growth was slower for CRISPRi/a cells with FOX3 sgRNA and slowest for CRISPRi/a cells with FOX3/XIST sgRNAs relative to CRISPRi/a control cells, but there was no difference between cells with XIST sgRNA alone and control CRISPRi/a HCC202 cells (Fig. 1I), suggesting that CRISPRi/a-induced endogenous FOX3 inhibits growth of HCC202 cells. Furthermore, we injected CRISPRi/a HCC202 cells with or without FOX3/XIST-sgRNAs into the fourth mammary fat pads of 8-week-old NSG female mice. However, we failed to generate orthotopic xenograft tumors using CRISPRi/a and scrambled HCC202 cells until day 28 after injection. Since bone is a common metastatic site for patients with breast cancer [9], we injected the luciferase-transduced CRISPRi/a and scrambled HCC202 cells into the tibia bones of 8-week-old NSG female mice followed by weekly Dox injections. As shown in Fig. 1K and L, luciferase imaging analysis showed that xenograft tumor growth in the bone was slower for CRISPRi/a cells (CRISPRi/a with FOX3/XIST-sgRNAs) compared to scramble cells (CRISPRi/a without FOX3/XIST-sgRNAs) for 28 days after tumor cell injection, supporting tumor growth inhibition by CRISPRi/a-induced endogenous FOX3 in vivo. Likewise, lung metastases were evident in 60% of the mice (3/5) with CRISPRi/a cells but were not observed in mice (0/5) injected with scramble cells (Fig. 1M). However, due to a small sample size, quantitative analysis of tumor nodules in the lung showed no significant difference between mice with CRISPRi/a cells and scramble cells (p = 0.1667, Fig. 1N).

To exclude off-target effects of our CRISPRi/a, we evaluated the potential off-target genes of our designed sgRNAs using the Cas-Offinder (Table S3a-d). For FOX3-sgRNA, the three nucleotide mismatched genes, CFAP61, ERI3, and ZFAT, were assessed by qPCR. Although expression levels of the potential off-target genes undulated in the CRISPRi/a cells with FOX3 sgRNAs, overall changes in these genes were not significant from days 0 to 10 after Dox addition (Fig. S5A-C). Likewise, for XIST-sgRNA, the three nucleotide mismatched genes, ASXL2, IGF2BP2, and VAMP4, were not significantly changed from days 0 to 10 after Dox addition (Fig. S5D-F).

Effect of DNA demethylation on CRISPRi/a-mediated activation of FOX3 in human breast cancer cells

XIST RNA works in concert with DNA methylation and histone modifications to maintain XCI [10, 11]. In regulatory T cells, DNA demethylation of the conserved non-coding sequence (CNS) of the FOX3 intron 1 is specific for inducing or stabilizing transcription of FOX3 [12–15]. First, we determined whether treatment with the DNA methylation inhibitor, 5-aza-2’-deoxycytidine (5-Aza-CdR), enhanced CRISPRi/a-mediated activation of FOX3 in human breast cancer cells. As shown in Fig. S6A and B, we transiently transduced FOX3-sgRNAs into CRISPRa HCC202 cells, followed by treatment with or without 5-Aza-CdR. For the 10 CpG sites of conserved CNS of FOX3 intron 1, pyrosequencing analysis showed deregulation of DNA methylation by CRISPRa, 5-Aza-CdR, or both, in 7/10 CpG sites, but these changes appeared to be not statistically significant (Figs. 2A and S7A-C). Likewise, FOX3 was induced in the cells after FOX3-sgRNA transduction, but levels of the FOX3 transcript were not changed by treatment with 5-Aza-CdR (Fig. 2B). Next, we transiently co-transduced FOX3/XIST-sgRNAs into CRISPRi/a HCC202 cells, followed by treatment with or without 5-Aza-CdR and Dox addition (Fig. S6C and D). Treatment of FOX3/XIST-sgRNAs-transduced CRISPRi/a cells with 5-Aza-CdR enhanced levels of the FOX3 transcript approximately 2-fold at days 2 and 4 (Fig. 2C), suggesting that induction of FOX3 by 5-Aza-CdR from XCI was under XIST downregulation. Likewise, pyrosequencing analysis revealed deregulation of DNA methylation by CRISPRi/a, 5-Aza-CdR, or both in most CpG sites (Figs. 2A and S7D).
The ten-eleven translocation (TET) family of DNA demethylase proteins converts cytosine methylated at C5 (5mC) to 5hmC, 5fC, and 5caC, and finally to cytosine with the aid of thymine-DNA glycosylase [16]. These changes are associated with elevated gene transcription [17]. Thus, we constructed SadCas9-TET1 (Fig. S8A and B) for targeted DNA demethylation in the FOXP3 CNS locus to enhance the activation of FOXP3 in human breast cancer cells. However, we failed to transfect SadCas9-TET1 into the CRISPRa HCC202 cells due to the large construct size. Next, we transfected the SadCas9-TET1 and transduced the FOXP3-sgRNA into CRISPRa HCC202 cells.
HEK 293T cells (Fig. S6E). For these cells, the efficacies of transfection and transduction were validated by fluorescence microscopy (Fig. 2D). After 2 days of transfection, Western blots confirmed protein expression of SadCas9-TET1 in the transfected cells (Fig. S8C). On days 2 and 4, levels of the FOXP3 transcript were elevated approximately 3-fold in SadCas9-TET1-transfected and FOXP3-sgRNA transduced cells relative to cells transduced with FOXP3-sgRNA (Fig. 2E), suggesting, for HEK 293T cells, synergistically enhanced activation of FOXP3 by co-expression of TET1 and VPR. Likewise, pyrosequencing analyses validated, for most CpG sites, deregulation of DNA methylation by CRISPRa with VPR, TET1, or both (Figs. 2A and S7E).

**Histone modifications during CRISPRi/a-mediated activation of FOXP3 in human breast cancer cells**

Histone methylation and acetylation either repress or activate transcription [18]. First, using established the CRISPRi/a HEK 293T cell models (Fig. S9A-D and Table S2), we performed chromatin immunoprecipitation (ChIP)-qPCR assays with a SadCas9-specific antibody (Fig. 2F). As shown in Fig. 2G, binding of SadCas9-VPR to the intron 1 CNS locus of FOXP3 was elevated more than 3-fold in cells transduced with FOXP3/XIST-sgRNAs relative to cells without sgRNAs; this binding was enhanced after addition of Dox to the cells. Although SadCas9-VPR also bound to FOXP3 neighbor genes, PPP1R3F and CCDC22, these bindings were not elevated after transduction of FOXP3/XIST-sgRNAs or addition of Dox to the cells (Fig. 2G). Further, expressions of PPP1R3F and CCDC22 in the cells were not changed after the transduction of FOXP3/XIST-sgRNAs and addition of Dox (Fig. S10A and B). In addition, no specific binding of SadCas9-VPR was evident in the XIST locus (Fig. 2G). These data suggest a FOXP3-sgRNA-guided specific binding of SadCas9-VPR to the intron 1 CNS locus of FOXP3.

To address the histone modification in the intron 1 CNS locus of FOXP3 during activation of FOXP3, we performed a ChIP-qPCR assay using CRISPRa-FOXP3/XIST-sgRNAs HEK 293T cells. As shown in Fig. 2H-N, in CRISPRi/a cells after FOXP3/XIST-sgRNAs transduction and Dox addition, H4K5ac, H4K8ac, and H4K16ac were enriched in the intron 1 CNS locus of FOXP3 but not in the promoter regions of PPP1R3F and CCDC22. Of note, during activation of FOXP3, H4K8ac and H4K16ac were elevated more than 2-fold in the intron 1 CNS locus (Fig. 2H and I), but there were no significant changes after Dox addition. H3K4me3, H3K9me3, H3K27me3, and H3K27ac were minimally changed in the FOXP3, PPP1R3F, and CCDC22 loci after FOXP3/XIST-sgRNAs transduction (Fig. 2K-N). However, H3K4me3 was elevated more than 3-fold in the intron 1 CNS locus after addition of Dox (Fig. 2L). These data suggest that, during activation of FOXP3, FOXP3-sgRNA guided specific H4 acetylation at active alleles and H3K4 methylation at inactive alleles in the intron 1 CNS locus of FOXP3.

As reported here, we developed, for human female breast cancer cells, a CRISPRi/a approach for targeted transcriptional regulation of specific X-linked FOXP3, using two orthogonal dCas9-fusion systems, including SpdCas9-KRAB for CRISPRi to the XIST locus and SadCas9-VPR for CRISPRa to the FOXP3 locus (Fig. S11). The targeted reactivation of endogenous FOXP3 from XCI was achieved by simultaneous use of CRISPRi/a. Of note, targeted reactivation of FOXP3 inhibited growth of human female breast cancer cells. Furthermore, we optimized our CRISPRa system with the TET1 catalytic domain to enhance the transcriptional activation of FOXP3. The CRISPRi/a-mediated activation of FOXP3 was accompanied by H4 acetylation at active alleles, including H4K5ac, H4K8ac, and H4K16ac, and H3K4 methylation at inactive alleles in the intron 1 CNS locus of FOXP3, indicating a CRISPRi/a-mediated epigenetic mechanism during activation of FOXP3.

**Conclusions**

The present study provides a better understanding of the CRISPRi/a-mediated activation of X-linked endogenous FOXP3 and its regulatory mechanism in human female breast cancer cells. Also, our identification of the reactivation of the X-linked FOXP3 from XCI moves beyond an incremental advance in breast cancer therapy by a targeted reactivation of X-linked tumor suppressor genes. Since epithelial FOXP3 is inactivated in 70% of breast cancer samples [1], our results may lead to the design of preclinical studies to develop more effective treatments for female breast cancers with FOXP3 dysfunction. In addition, this concept and tools may provide new routes of targeted therapy for other X-chromosome-linked genetic disorders.

**Abbreviations**

- S-Aza-CdR: 5-aza-2’-deoxycytidine; ChIP: Chromatin immunoprecipitation; CNS: Conserved non-coding sequence; CRISPRa: CRISPR activation; CRISPRi: CRISPR interference; dCas9: Endonuclease-deficient CRISPR/Cas9 protein; Dox: Doxycycline; H3K4me3: Tri-methylation at the 4th lysine residue of the histone H3 protein; H3K9me3: Tri-methylation at the 9th lysine residue of the histone H3 protein; H3K27me3: Tri-methylation at the 27th lysine residue of the histone H3 protein; H3K27ac: Acetylation at the 27th lysine residue of the histone H3 protein; H3K27me3: Tri-methylation at the 27th lysine residue of the histone H3 protein; H4K5ac: Acetylation at the 5th lysine residue of the histone H4 protein; H4K16ac: Acetylation at the 16th lysine residue of the histone H4 protein; H4K20me3: Tri-methylation at the 20th lysine residue of the histone H4 protein; H4K20me3: Tri-methylation at the 20th lysine residue of the histone H4 protein; H3K4me3: Tri-methylation at the 4th lysine residue of the histone H3 protein; H3K9me3: Tri-methylation at the 9th lysine residue of the histone H3 protein; H3K27me3: Tri-methylation at the 27th lysine residue of the histone H3 protein; H3K27ac: Acetylation at the 27th lysine residue of the histone H3 protein; H3K27me3: Tri-methylation at the 27th lysine residue of the histone H3 protein; H4K5ac: Acetylation at the 5th lysine residue of the histone H4 protein; H4K16ac: Acetylation at the 16th lysine residue of the histone H4 protein; H4K20me3: Tri-methylation at the 20th lysine residue of the histone H4 protein; XCI: X-chromosome inactivation; XIST: X-inactive specific transcript.
The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01472-x.

Supplementary Information

**Figure S1.** Expression of XIST in human embryonic kidney (HEK) 239T cells and breast cancer cell lines. The expression levels of XIST were assessed by qPCR. The fold change in expression was calculated using the \(2^{-\Delta\Delta Ct}\) method with GAPDH mRNA as an internal control. Data are presented as the means ± SD. * indicates a significant change in expression compared to the inactive X chromosome. DNA binding by SadCas9-VPR (VP64/p65/Rta) and repression of XIST in human embryonic kidney (HEK) 293T cells. All experiments were repeated three times.

**Figure S5.** Assessment of potential off-target genes of FOXP3 sgRNA (mCherry) with or without 5-Aza and Dox treatment at days 0, 2, 4, 6, 8, and 10 as determined by qPCR. The fold change in expression was calculated using the \(2^{-\Delta\Delta Ct}\) method with GAPDH mRNA as an internal control. Data are presented as the means ± SD. * indicates a significant change in expression compared to the inactive X chromosome. DNA binding by SadCas9-VPR (VP64/p65/Rta) and repression of XIST in human embryonic kidney (HEK) 293T cells. All experiments were repeated three times.

**Figure S7.** DNA methylation status of the rich CpG sites in conserved CNS of XIST in day 0 and day 10 Cpg sites in the conserved CNS of XIST in day 1 using the PyroMark Q96 ID pyrosequencing analysis. B DNA methylation analysis by pyrosequencing for CRISPRa HCC202 cells, CRISPRi/a HCC202 cells, and CRISPRa HEK 293T cells with various treatments. Pyrosequencing was performed to measure the methylation levels at 10 CPG sites in the conserved CNS of XIST intron 1 using the PyroMark Q96 ID pyrosequencing.

**Figure S9.** Targeted activation of FOXP3 and inactivation of XIST in human embryonic kidney (HEK) 293T cells. A CRISPRi experimental procedure for the transduction of XIST sgRNA (mCherry) with or without 5-Aza and Dox treatment at days 2, 4, and 7 as determined by fluorescence microscopy. Scale bar, 1,000 μm. C Protein expression of TET1 after transfection into HEK 293T cells. The SadCas9-TET1 vector was transiently transacted into HEK 293T cells. The red arrow indicates the size of the SadCas9-TET1 catalytic domain. The blue arrow indicates the full size of the endogenous TET1 protein. I8 Immunoblotting.

**Figure S10.** Effect of CRISPRa-mediated activation of FOXP3 on expression of its neighboring genes in HCC202 cells. PPP1R3F and CCDC22 are two FOXP3 neighboring genes at Xp11.23. Quantitative expression analysis of CCDC22 (A) and PPP1R3F (B) before and after sgRNA transduction and Dox induction in the CRISPRa/a HCC202 cells. All experiments were repeated three times. All experiments were repeated three times.

**Figure S11.** Targeted reactivation of the X-linked endogenous FOXP3 gene from X chromosome inactivation (XCI) in female cells. PCR (polymerase chain reaction) of 1 or 2 recruit rat XIST primer and promotes epigenetic modifications that block X-linked FOXP3 gene transcription on the inactive X chromosome. DNA binding by SadCas9-VPR (VP64/p65/Rta) and SadCas9-TET to the XIST promoter, reactivates X-linked FOXP3 gene transcription from XCI.
Acknowledgements

We thank Dr. Donald Hill for editorial assistance in preparing this manuscript.

Authors’ contributions

LW, RL and LSQ designed the research approach. XC, ZX, SW, XL and RL performed the experiments. XC, RL and LW analyzed data. XC, SB and LW performed statistical analyses. LZ, DZ and LSQ provided key resources. XC made a draft of the paper. XC, ESR, RL, LSQ and LW revised and edited the paper. The author(s) read and approved the final manuscript.

Funding

This work was supported by grants from the OOD (W81XWH-17-1-0017 for L. Wang and W81XWH-17-1-0018 for L.S. Qi) and the Breast Cancer Research Foundation of Alabama (L. Wang).

Availability of data and materials

Results are based, in part, upon data generated by Cas-OFFinder (http://www.genome.net/cas-offinder).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have agreed to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

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