A piggyBac-based toolkit for inducible genome editing in mammalian cells

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ABSTRACT

We describe the development and application of a novel series of vectors that facilitate CRISPR-Cas9-mediated genome editing in mammalian cells, which we call CRISPR-Bac. CRISPR-Bac leverages the piggyBac transposon to randomly insert CRISPR-Cas9 components into mammalian genomes. In CRISPR-Bac, a single piggyBac cargo vector containing a doxycycline-inducible Cas9 or catalytically dead Cas9 (dCas9) variant and a gene conferring resistance to Hygromycin B is cotransfected with a plasmid expressing the piggyBac transposase. A second cargo vector, expressing a single-guide RNA (sgRNA) of interest, the reverse-tetracycline TransActivator (rtTA), and a gene conferring resistance to G418, is also cotransfected. Subsequent selection on Hygromycin B and G418 generates polyclonal cell populations that stably express Cas9, rtTA, and the sgRNA(s) of interest. We show that CRISPR-Bac can be used to knock down proteins of interest, to create targeted genetic deletions with high efficiency, and to activate or repress transcription of protein-coding genes and an imprinted long noncoding RNA. The ratio of sgRNA-to-Cas9-to-transposase can be adjusted in transfections to alter the average number of cargo insertions into the genome. sgRNAs targeting multiple genes can be inserted in a single transfection. CRISPR-Bac is a versatile platform for genome editing that simplifies the generation of mammalian cells that stably express the CRISPR-Cas9 machinery.

Keywords: CRISPR; piggyBac; genome editing; stem cells; regulatory element; IncRNA

INTRODUCTION

Within the last decade, the CRISPR (clustered regularly interspaced short palindromic repeat) bacterial immune system has provided researchers with multiple new methods to control gene expression in mammalian genomes. Coexpression of the Cas9 (CRISPR-associated protein 9) nuclease from Streptococcus pyogenes along with an engineered single guide RNA (sgRNA) that targets a protein-coding exon is an effective way to introduce frameshift mutations in proteins of interest, owing to the fact that repair of the DNA break introduced by Cas9 often results in small deletions surrounding the cut site. Coexpression of Cas9 and multiple sgRNAs can also be used to excise larger regions from genes of interest, or to excise DNA regulatory elements (Ran et al. 2013; Canver et al. 2014; Aparicio-Prat et al. 2015; Zhu et al. 2016; Gasperini et al. 2017). Expression of a catalytically dead Cas9 (dCas9) fused to a transcriptional activation or repression domain can be used to up- or down-regulate gene expression when sgRNAs are targeted to promoters or regulatory elements of interest (Hsu et al. 2014; Wright et al. 2016).

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RESULTS

Cloning of CRISPR-Bac

We modeled CRISPR-Bac (Fig. 1) after the pX330 plasmid system in which a humanized version of the Cas9 enzyme from Streptococcus pyogenes is coexpressed with a chimeric sgRNA driven by a U6 promoter (Cong et al. 2013). We cloned the Cas9 from pX330 into a doxycycline-inducible expression cassette in a piggyBac cargo vector that also expresses a gene conferring resistance to Hygromycin B. We then converted the dual BbsI sites in pX330, which are used to clone the sgRNA targeting sequence into that vector, into dual BsmBI sites. Like BbsI, BsmBI is a Type IIS restriction enzyme, and it generates overhanging ends that are identical to those generated by BbsI. We cloned the BsmBI-modified sgRNA expression cassette into a piggyBac cargo vector from Kirk et al. (2018) that expresses a bicistronic message which encodes the rtTA3 gene and a gene conferring resistance to G418 (originally cloned from Addgene plasmid #25735; Shin et al. 2006). The conversion of the pX330 BbsI sites, which are not unique in the rtTA-expressing vector, to BsmBI sites, allows the exact sgRNA design and cloning protocol for pX330 (Cong et al. 2013) to be used to clone sgRNAs into CRISPR-Bac.

Knockdown of a protein-coding gene using CRISPR-Bac

We tested whether CRISPR-Bac could be used to knock down a protein of interest in mouse embryonic stem cells (ESCs). We designed three sgRNAs targeting different exons of the Ezh2 gene (Supplemental Fig. S1; Supplemental Table S1) and cloned them into our sgRNA-rtTA-expressing vector using the protocol outlined in Cong et al. (2013). We then cotransfected our inducible Cas9-expressing piggyBac vector, a plasmid expressing the piggyBac transposase, and either each sgRNA-expressing vector separately, or a pool of all three sgRNAs into ESCs. As a control, we transfected an sgRNA-rtTA-expressing vector into which we did not clone a specific sgRNA-targeting sequence (our “no sgRNA” control). After selecting ESCs on Hygromycin B and G418 for 10 d, we removed the selection drugs and added 1 µg/mL of doxycycline to the media for 4 d to induce the expression of Cas9. To assess the extent of EZH2 knockdown, we performed western blot and immunofluorescence (IF). Relative to the control ESCs, we observed >60% reduction in EZH2 protein levels in the three lines expressing individual sgRNAs and more than 90% loss in the line expressing the pool of sgRNAs (Fig. 2A). In repeat transfections of the sgRNA pool, we consistently observed >90% loss of EZH2 protein levels (Fig. 2B). IF to EZH2 confirmed our western blot analysis (Fig. 2C). We compared the levels of EZH2 knockdown obtained via CRISPR-Bac to those

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Owing to the broad utility of CRISPR, multiple methods have been developed to deliver the CRISPR-Cas9 machinery to mammalian cells. Transient transfection of Cas9- and sgRNA-expressing plasmids, or of Cas9 protein and in vitro synthesized sgRNAs, are useful when the efficiency of transfection for the cell type of interest is high and when the desired endpoint can be reached via transient expression of Cas9 and the sgRNA. Lentiviral delivery of Cas9/sgRNA vectors is also possible, and provides distinct advantages when transfection efficiency is low, or when the desired endpoint requires stable expression and or integration of Cas9/sgRNAs into the genome, such as for studies performed in vivo or for genome-wide phenotypic screens (Hartenian and Doench 2015; Joung et al. 2017). However, delivery of the CRISPR machinery via lentivirus requires additional hands-on time, expertise, safety precautions, and cost relative to delivery via transient transfection.

The piggyBac transposon is a broadly used tool that allows DNA cargos up to 100 kilobases in length to be inserted into “AATT” sequences that are preferentially located in euchromatic regions of mammalian genomes (Ding et al. 2005; Cadinaños and Bradley 2007; Wilson et al. 2007; Wang et al. 2008; Li et al. 2011). Owing to its high efficiency of transposition, piggyBac has been used in a wide range of applications, including in the stable expression of multisubunit protein complexes, in the generation of transgenic mice and induced pluripotent stem cells, and in the large-scale production of recombinant proteins (Ding et al. 2005; Kaji et al. 2009; Yusa et al. 2009; Kahlig et al. 2010; Li et al. 2013). Most recently, piggyBac has begun to be used for CRISPR-based applications; piggyBac vectors have been used to study CRISPR off-target effects (Wu et al. 2014), to engineer mutations in human induced pluripotent stem cells (Wang et al. 2017), and to perform multiplexed activation of protein-coding and noncoding genes (Li et al. 2017).

Herein, we describe the creation and validation of a piggyBac-based system for inducible editing of mammalian genomes by CRISPR-Cas9. In the system, which we call “CRISPR-Bac,” two separate piggyBac cargo vectors, one that expresses an inducible Cas9 or dCas9 variant, and another that expresses an sgRNA and the reverse-tetracycline transactivator (rtTA; Gossen et al. 1995), are transfected into ESCs. We designed three sgRNAs targeting different exons of the Ezh2 gene (Supplemental Fig. S1; Supplemental Table S1) and cloned them into our sgRNA-rtTA-expressing vector using the protocol outlined in Cong et al. (2013). We then cotransfected our inducible Cas9-expressing piggyBac vector, a plasmid expressing the piggyBac transposase, and either each sgRNA-expressing vector separately, or a pool of all three sgRNAs into ESCs. As a control, we transfected an sgRNA-rtTA-expressing vector into which we did not clone a specific sgRNA-targeting sequence (our “no sgRNA” control). After selecting ESCs on Hygromycin B and G418 for 10 d, we removed the selection drugs and added 1 µg/mL of doxycycline to the media for 4 d to induce the expression of Cas9. To assess the extent of EZH2 knockdown, we performed western blot and immunofluorescence (IF). Relative to the control ESCs, we observed >60% reduction in EZH2 protein levels in the three lines expressing individual sgRNAs and more than 90% loss in the line expressing the pool of sgRNAs (Fig. 2A). In repeat transfections of the sgRNA pool, we consistently observed >90% loss of EZH2 protein levels (Fig. 2B). IF to EZH2 confirmed our western blot analysis (Fig. 2C). We compared the levels of EZH2 knockdown obtained via CRISPR-Bac to those...
obtained via transient transfection of the same sgRNA pool cloned into the widely used pX330 vector (Cong et al. 2013). Four days after transfection of pX330, we harvested cells and performed western blot and IF. Relative to pX330 lacking a gene-targeting sgRNA (“No sgRNA” control), we measured 25%–35% reduction in EZH2 protein (Fig. 2D,E). These results demonstrate that CRISPR-Bac can be used to inducibly knock down a protein-coding gene of interest.

**Targeted deletion of genetic elements using CRISPR-Bac**

An important use of CRISPR-Cas9 is to create targeted deletions of regulatory elements (Ran et al. 2013; Canver et al. 2014; Aparicio-Prat et al. 2015; Wright et al. 2016; Zhu et al. 2016; Gasperini et al. 2017). To test the utility of CRISPR-Bac in this application, we cloned into CRISPR-Bac pairs of sgRNAs that flank multiple different regulatory elements (RE1, RE2, RE3, RE4), to delete 2331 bp, 2480 bp, 1222 bp, and 2609 bp regions, respectively (Supplemental Fig. S1; Supplemental Table S1). We created ESCs that stably express the different sgRNA pairs along with doxycycline-inducible Cas9. We induced expression of Cas9 for 4 d, collected genomic DNA, and performed quantitative PCR (qPCR) using amplicons within the deleted regions. By comparing qPCR results between the sgRNA-expressing ESCs and nontargeting sgRNA control ESCs, we approximated the extent that each targeted region was deleted in a polyclonal cell population. For four of four deletions, we observed more than 40% reduction in signal, indicating that close to half of the alleles in the cell population were deleted (Fig. 3A; two-sided t-test).

To assess deletion efficiency in single cells, we isolated 36 individual colonies from cells transfected with sgRNAs to the RE3 element and extracted their genomic DNA. To assess whether a deletion occurred on at least one allele, we performed PCR using primers that flanked the expected deletion. Twenty-one of 36 clones (58%) showed a band within the expected size range for a deletion (310–426 bp; Fig. 3B), signifying that these clones were at least heterozygous for the deletion. To distinguish between clones that were heterozygous versus homozygous for the deletion, we used a pair of primers that amplify inside the deletion. Twelve out of the 21 clones (33% of the 36 clones) did not show a band, indicating that no wild-type allele was present, and the cells were homozygous for the deletion (Fig. 3C). Many homozygous clones showed weak wild-type bands, which we presume were due to genomic DNA from MEF feeder cells and not due to the presence of a wild-type allele in the clones. In support of this notion, we performed qPCR to detect the...
wild-type allele on seven total clones: two clones classified as wild-type, two as heterozygous, and three as homozygous. Relative to heterozygous and wild-type clones, all three homozygous clones showed a 10 and 11 cycle difference, respectively, signifying that the clones that we genotyped as homozygous indeed lacked wild-type alleles (Fig. 3D). These data demonstrate that CRISPR-Bac can be used to generate targeted genomic deletions with high efficiency.

**Activation and repression of protein-coding gene transcription using CRISPR-Bac**

In addition to creating targeted genomic deletions, the CRISPR-Cas9 system can be used to up- or down-regulate genes from their endogenous promoters, by targeting dCas9 fused to effector domains that recruit transcriptional coactivators or corepressors. We cloned one such transcriptional activator fusion, dCas9-VP160 from Cheng et al. (2013), and one such transcriptional repressor fusion, dCas9-KRAB from Kearns et al. (2014), into the same piggyBac-based inducible expression vector that we used to express catalytically active Cas9 (Fig. 1). We then tested our ability to up-regulate Ascl1, a silent gene in ESCs, with dCas9-VP160, and we tested our ability to down-regulate Oct4, an active gene in ESCs, with dCas9-KRAB (Fig. 4A). Using CRISPR-Bac, we routinely observed 350-fold up-regulation of Ascl1 relative to nontargeting sgRNA control cells (Fig. 4B). This level of activation was similar to that obtained using transient transfection of dCas9-VP160 and SpgRNA vectors from Cheng et al. (2013) and Perez-Pinera et al. (2013) (Fig. 4B). Using multiple sets of published and in-house-designed sgRNAs, the maximum level of Oct4 down-regulation we achieved was two- to threefold (Fig. 4C and data not shown). Relative to the 4 d used for protein knockdown and genomic deletions, we observed that for transcriptional modulation experiments, 2 d of doxycycline treatment was sufficient to detect maximal effects induced by dCas9-VP160 and -KRAB. These data show that CRISPR-Bac can be used to up- and down-regulate transcription of protein-coding genes of interest.

**Activation and repression of IncRNA transcription using CRISPR-Bac**

We next examined whether we could use CRISPR-Bac to activate and repress transcription of a IncRNA using dCas9-VP160 and dCas9-KRAB, respectively. We chose to target a IncRNA called Aim in two cell types: mouse ESCs, in which Aim is expressed at low levels, and mouse trophoblast stem cells (TSCs), in which Aim is more highly expressed and active (Fig. 5A; Latos et al. 2009; Calabrese et al. 2015; Andergassen et al. 2017). In ESCs, we were able to activate Aim ~15-fold above its levels in nontargeting sgRNA control cells (Fig. 5B), but we were not able to repress Aim, likely due to its low endogenous expression (data not shown; Latos et al. 2009). Compared to transient transfection of dCas9-VP160 and SpgRNA vectors from Cheng et al. 2013; Perez-Pinera et al. 2013), we achieved a greater level of activation with CRISPR-Bac (Fig. 5B). In TSCs, we were able to repress Aim to 10% of its normal expression and activate Aim 2.5-fold relative to
nontargeting sgRNA control cells (Fig. 5C). Therefore, CRISPR-Bac can be used to activate and repress transcription of lncRNAs.

Under normal physiological conditions, the Aim lncRNA is monoallelically expressed due to a process called genomic imprinting that leads to methylation of its promoter and gene silencing specifically on the maternally inherited allele (Stoger et al. 1993; Lee and Bartolomei 2013). To assess whether activation of Aim via CRISPR-Bac led to mono- or biallelic activation of the lncRNA, we performed RNA fluorescence in situ hybridization (FISH) in ESCs stably expressing dCas9-VP160 and either a nontargeting sgRNA or an Aim-targeting sgRNA. We performed a two-color RNA FISH experiment where one probe was complementary to the Aim lncRNA, and the other probe was complementary to the Kcnq1ot1 lncRNA. Kcnq1ot1, like Aim, is also imprinted and monoallelically expressed (Lee and Bartolomei 2013). Unlike Aim, Kcnq1ot1 is robustly expressed in ESCs under normal conditions (Umlauf et al. 2004). Kcnq1ot1 therefore served as a control to gauge the extent of Aim monoallelism upon activation by CRISPR-Bac. After taking z-stacks on a widefield microscope and deconvolving the resultant images, we used an automated pipeline to identify puncta whose RNA FISH signal surpassed a specified threshold. In two images taken of cells expressing the nontargeting sgRNA control, we counted zero puncta of Aim relative to 100 puncta of Kcnq1ot1, confirming prior data that show Kcnq1ot1 is robustly expressed in ESCs while Aim is not (Umlauf et al. 2004; Latos et al. 2009). In contrast, in two images taken of cells expressing the Aim-targeting sgRNA, we counted 97 puncta of Aim relative to 130 puncta of Kcnq1ot1 (Fig. 5D). These data support the notion that CRISPR-Bac activates expression of Aim on the unmethylated paternal allele, and that the methylated maternal allele of Aim remains resistant to activation (Stoger et al. 1993).
The CRISPR-Bac platform relies on simultaneous delivery of two cargo vectors: one vector expressing the sgRNA and rtTA/G418 resistance genes, and the other vector expressing the Cas9/dCas9 variant and hygromycin resistance genes (Fig. 1). We sought to determine whether the extent of activation of a target gene of interest could be altered by altering the ratios of sgRNA, Cas9, and piggyBac transposase vectors in transfections. We tested a range of sgRNA-to-dCas9-VP160-to-transposase ratios, using the Airn IncRNA as our target gene for activation (Supplemental Table S3). We found modest but significant differences in the level of Airn activation when we transfected higher amounts of sgRNA and dCas9-VP160 plasmids relative to the piggyBac transposase plasmid (Fig. 6A; see table of adjusted P-values from Tukey’s HSD test), and these differences were accompanied by increased numbers of sgRNA and dCas9-VP160 cargo insertions per cell (Fig. 6B). Thus, the extent of target gene activation using CRISPR-Bac can be partly controlled by changing the ratios of sgRNA/rtTA, Cas9, and transposase plasmids in transfections.

Simultaneous up-regulation of two genes via CRISPR-Bac

By coexpression of multiple sgRNAs, CRISPR can be used to activate or repress multiple genes simultaneously (Cheng et al. 2013). To test if CRISPR-Bac is capable of multiplexed gene activation, we created ESCs expressing dCas9-VP160 and sgRNAs targeting the Ascl1 and Aim promoters (same sgRNAs as in Figs. 4B, 5B,C, 6A). Relative to nontargeting sgRNA controls, qPCR demonstrated simultaneous 411-fold activation of Ascl1 and 9.5-fold activation of Aim when sgRNAs for both targets were cotransfected (Fig. 6C). This confirms that CRISPR-Bac can be used to target multiple genes in a single experiment.

CRISPR-Bac can be used in human cells

To determine if the CRISPR-Bac system could be used in human cells, we tested Cas9-mediated knockdown and dCas9-VP160-mediated up-regulation in SUM-159 cells, a commonly used cell line in breast cancer research (Grigoriadis et al. 2012). Similar to our experiments in ESCs, we cotransfected the inducible Cas9-expressing piggyBac vectors into SUM-159 cells, a plasmid expressing the piggyBac transposase, and a pool of four sgRNAs targeting human EZH2 exons, then selected the cells with Hygromycin B and G418 for at least 10 d, and induced Cas9 expression with 1 µg/mL of doxycycline for 4 d. Via western blot, we detected >70% reduction in EZH2 protein levels in both replicates, which was confirmed via IF (Fig. 7A,B). In parallel, we cotransfected the dCas9-VP160, transposase, and a pool of two sgRNAs targeting the promoter of Il1RN, drug selected for at least 10 d, and induced dCas9-VP160 expression for 2 d. Il1RN was activated ~54-fold relative to the no dCas9 control (Fig. 7C). In these experiments, the Cas9/EZH2 sgRNA cells served as a negative control in the dCas9-VP160/Il1RN sgRNA experiment, and vice versa. These data show that the CRISPR-Bac system can be used in human cells.

Conclusions

In Mus musculus-derived ESCs and TSCs, we have shown that CRISPR-Bac can be used to knock down proteins through frameshift/deletion, to delete kilobase-sized...
regulatory elements with high efficiency, and to up- and down-regulate the transcription of protein-coding genes and an imprinted lncRNA. Levels of CRISPR-induced activation could partly be controlled through delivery of different ratios of CRISPR-Bac vectors. It seems likely that the use of different promoter elements within CRISPR-Bac (for example, a constitutive CMV promoter driving dCas9-VP16 instead of a TRE) might afford additional levels of control. It may also be possible to engineer CRISPR-Bac vectors that express multiple sgRNAs, as has been done elsewhere (Kabadi et al. 2014; Sakuma et al. 2014; Albers et al. 2015). Although in this work we only tested CRISPR-Bac in a limited number of cell types, it seems reasonable to presume that the CRISPR-Bac vectors or their modified derivatives would be functional in other mammalian cell types, given the broad activity of the piggyBac transposase (Ding et al. 2005; Cadinaños and Bradley 2007; Wilson et al. 2007; Wang et al. 2008; Kaji et al. 2009; Yusa et al. 2009; Kahlig et al. 2010; Li et al. 2011, 2013). Indeed, CRISPR-Bac facilitated efficient knockdown and transcriptional up-regulation in at least one human cell line, SUM159 (Grigoriadis et al. 2012). In our view, the main utility of CRISPR-Bac over other genome editing platforms is that CRISPR-Bac allows the generation of stable cell lines without the need to package CRISPR-Cas9 components into lentiviral delivery systems. It also preserves the sgRNA cloning strategy from the widely used pX330/335 systems, facilitating horizontal transfer of sgRNAs between the two platforms (Cong et al. 2013). Relative to prior studies that have used piggyBac to carry out CRISPR in mammalian cells (Wu et al. 2014; Li et al. 2017; Wang et al. 2017), our study describes a single platform with interchangeable functionalities that has been optimized for protein knockdown, regulatory element deletion, and the up- and down-regulation of protein-coding and noncoding gene transcription.

MATERIALS AND METHODS

Construction of CRISPR-Bac vectors

To create the doxycycline-inducible Cas9, dCas9-VP160, and dCas9-KRAB piggyBac vectors, a parent piggyBac vector was created in which a bGH-polA signal and an EF1 alpha promoter driving expression of a hygromycin resistance gene were ligated into the cumate-inducible piggyBac transposon vector from System Biosciences after its digestion with HpaI and SpeI, which cut just downstream from each chicken beta-globin insulator sequence and removed all other internal components of the original vector. The TRE from pTRE-Tight (Clontech) was cloned upstream of the bGH-polA site, and Cas9 from pX330 (Addgene plasmid # 42230; Cong et al. 2013; gift from Feng Zhang), dCas9-VP160 from Addgene plasmid # 48225 (Cheng et al. 2013; gift from Rudolf Jaenisch), and dCas9-KRAB from Addgene plasmid #
were each cloned behind the TRE by digestion with AgeI and SalI (NEB) followed by Gibson Assembly (NEB), to generate piggyBac cargo vectors capable of inducibly expressing Cas9, dCas9-VP160, and dCas9-KRAB, respectively, upon addition of doxycycline.

To create the rtTA-sgRNA expressing piggyBac vector, the dual BbsI sites in pX330 were converted to BsmBI sites using oligonucleotides, and the entire U6 expression cassette was cloned via Gibson assembly into the PacI site upstream of the rtTA3-IRES-Neo cassette in the rtTA-piggyBac-Cargo vector described in Kirk et al. (2018). The rtTA3-IRE-Neo cassette was originally cloned from pSLIK-Neo and was a gift from Iain Fraser (Addgene plasmid # 25735). Oligonucleotides used for cloning are in Supplemental Table S1.

We have submitted four plasmids to Addgene: (1) PB_rtTA_BsmBI, #126028, (2) PB_tre_Cas9, #126029, (3) PB_tre_dCas9_KRAB, #126030, and (4) PB_tre_dCas9_VP160, #126031.

Oligonucleotides used for sgRNA cloning are listed in Supplemental Table S1, and their location relative to gene features are shown in Supplemental Figure S1. Protein knockdown sgRNAs were designed using Desktop Genetics, and all other sgRNAs were designed using the CRISPOR program or taken from published sources (Supplemental Table S1; Haeussler et al. 2016).

Embryonic stem cell (ESC) culture

ESCs were grown on gelatin coated plates at 37°C in a humidified incubator at 5% CO2. Media were changed daily and consisted of DMEM high glucose plus sodium pyruvate, 0.1 mM nonessential AA, 100 µ/mL penicillin-streptomycin, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, 15% ES-qualified FBS, and 1:500 LIF conditioned media produced from Lif-1Cα (COS) cells. ESCs were split at an approximate ratio of 1:6 every 48 h.

Trophoblast stem cell (TSC) culture

TSCs were cultured as in Quinn et al. (2006). Briefly, TSCs were cultured at 37°C on preplated irradiated MEF feeder cells in TSC media (RPMI [Invitrogen], 20% Qualified FBS [Invitrogen], 100 µ/mL penicillin-streptomycin, 1 mM sodium pyruvate [Invitrogen], 100 µM β-mercaptoethanol [Sigma], and 2 mM l-glutamine) supplemented with Fgf4 (25 ng/mL; Invitrogen) and Heparin (1 µg/mL; Sigma) just before use. At passage, TSCs were trypsinised with 0.125% Trypsin (Invitrogen) for 3 min at room temperature and gently dislodged from their plate with a sterile, cotton-plugged Pasteur pipette (Thermo Fisher). To deplete MEF feeder cells from TSCs prior to RNA isolation, TSCs were preplated for 40 min and cultured for 3 d in 70% MEF-conditioned TSC media supplemented with Fgf4 (25 ng/mL; Invitrogen) and Heparin (1 µg/mL; Sigma).
To generate stable CRISPR-Bac SUM-159 cells, 5 × 10^5 cells were seeded per well of a six-well plate and the next day transfected with 2.5 µg of plasmid DNA at a 1:1:2 ratio of rtTA-sgRNA to dCas9 to transposase. Cells were selected on Hygromycin (150 µg/mL; Gibco) and G418 (200 µg/mL; Gibco) for 9 d.

To generate stable CRISPR-Bac SUM-159 cells, 5 × 10^5 cells were seeded per well of a six-well plate and the next day transfected with 2.5 µg of plasmid DNA at a 1:1:2 ratio of rtTA-sgRNA to Cas9 to transposase using Lipofectamine 3000 (Invitrogen). Cells were subsequently selected on Hygromycin (250 µg/mL; Gibco) and G418 (600 µg/mL; Gibco) for at least 10 d.

Transient transfections

For transient transfections using pX330, 5 × 10^5 ESCs were seeded in a single well of a six-well plate and transfected the next day using Lipofectamine 3000 with 2.5 µg of the pX330 empty vector ("no sgRNA") or a pool of pX330 vectors expressing the 3 sgRNAs to mouse Ezh2. Cells were harvested 4 d after transfection. For transient transfections using VP160, 5 × 10^5 ESCs were seeded in a single well of a six-well plate and transfected the next day using Lipofectamine 3000 with 1.25 µg of dCas9-VP160 (Cheng et al. 2013) and 1.25 µg of the SpgRNA empty vector ("no sgRNA"); Perez-Pinera et al. 2013) or SpgRNA containing sgRNAs targeting either Airn or Ascl1. Cells were harvested 2 d after transfection.

Protein isolation and western blotting

To isolate protein for western blotting, cells were washed with PBS, and then lysed with RIPA buffer (10 mM Tris–Cl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with 1 mM PMSF (Fisher Scientific) and 1 µg/mL protease inhibitor cocktail (Sigma) for 15 min at 4°C, 4 d after induction with 1 µg/mL doxycycline. Prior to western blotting, protein levels were quantified using the DC assay from Bio-Rad. For western blotting, primary and secondary antibody incubations were done for 1 h at room temperature. Antibodies used were EZH2 (Cell Signaling #5246, 1:1000 dilution), ERK2 (Santa Cruz; sc-1647; 1:500), donkey anti-mouse IgG-HRP secondary (Santa Cruz; sc-2314; 1:2500), and donkey anti-rabbit IgG-HRP secondary (Santa Cruz; sc-2313; 1:2500).

Genomic DNA isolation and qPCR

To isolate genomic DNA, 400 µL of ESC lysis buffer (100 mM Tris-HCl, pH 8.1, 5 mM EDTA, pH 8.0, 200 mM NaCl, 0.2% SDS)

Stable transfections of CRISPR-Bac components

To generate stable CRISPR-Bac E14 ESC lines, 5 × 10^5 cells were seeded in a single well of a six-well plate, and the next day transfected with piggyBac cargo vectors and pUC19-piggyBac transposase from Kirk et al. (2018), totaling 2.5 µg of plasmid DNA (see exact amounts in Supplemental Table S3), using Lipofectamine 3000 (Invitrogen) according to manufacturer instructions. Cells were subsequently selected on Hygromycin (150 µg/mL; Gibco) and G418 (200 µg/mL; Gibco) for 7 to 12 d. Due to the efficiency of piggyBac cargo integration and the rapidity of Hygromycin selection, most observable death from drug selection occurred within ~3 d after addition of Hygromycin and G418 (i.e., cells with Hygromycin resistance were invariably resistant to G418).

SUM-159 cell culture

SUM-159 cells were maintained in DMEM/F12 medium (Gibco, Thermo Fisher Scientific) supplemented with 5% FBS, 5 µg/mL insulin, 1 µg/mL hydrocortisone, and antibiotic/antimycotic cocktail (Gemini Bio Products) as in Zawistowski et al. (2017).

FIGURE 7. The CRISPR-Bac system functions in human cell lines. See Supplemental Table S2 for details on replicates and experimental design for each figure panel. (A) Western blots to EZH2 and ERK2 loading control from two replicate CRISPR-Bac experiments in SUM-159 cells. “No Cas9” refers to measurements taken from dCas9-VP160/rtta-II1RN sgRNA expressing SUM-159 cells that were cultured in parallel to those expressing Cas9 and the EZH2 sgRNA pool. Values underneath blots represent knockdown of EZH2 relative to No Cas9 control and normalized for loading with ERK2 protein levels. (B) Representative IF images showing EZH2 knockdown from “No Cas9” and “EZH2 pool, rep #1” SUM-159 cells in A. Image #1 shows a cell with partial knockdown next to a cell with full knockdown, and image #2 shows two cells with full knockdown. Scale bar, 10 µm. (C) qPCR results showing transcriptional activation of Il1RN in SUM-159 cells. In panel C, “No dCas9” refers to measurements taken from Cas9/rtta-EZH2 sgRNA expressing SUM-159 cells that were cultured in parallel to those expressing dCas9 and the EZH2 sgRNA pool, and “No sgRNA” refers to measurements taken from Cas9/rtta-EZH2 sgRNA expressing SUM-159 cells that were cultured in parallel to those expressing dCas9 and the EZH2 sgRNA pool. Data from the nontargeting sgRNA control (No sgRNA) and sgRNA expressing cells are plotted relative to the average of the signal in the No sgRNA control cells. Individual qPCR data points are shown in box-and-whisker format. (***) P < 0.001 from a two-sided t-test between no sgRNA and sgRNA-expressing cells.

SUM-159 cell culture

SUM-159 cells were maintained in DMEM/F12 medium (Gibco, Thermo Fisher Scientific) supplemented with 5% FBS, 5 µg/mL insulin, 1 µg/mL hydrocortisone, and antibiotic/antimycotic cocktail (Gemini Bio Products) as in Zawistowski et al. (2017).
supplemented with 80 μL proteinase K (Denville) was used per 24-well of ESCs, four days after induction with 1 μg/mL doxycycline. Lysed ESCs were incubated at 55°C overnight, cells were boiled at 100°C for 1 h to degrade RNA, and DNA was precipitated by addition of 2 volumes of 100% ethanol. DNA was pelleted and resuspended in 1 x TE (10 mM Tris-HCl 1 mM EDTA pH 8.0) overnight at room temperature prior to qPCR. qPCR was performed using 100 ng of DNA per reaction and iTaq Universal SYBR Green Supermix (Bio-Rad), with primers specified in Supplemental Table S1. All related plots were generated using R version 3.4.1 (The R Development Core Team 2017).

qPCR for DNA copy-number analysis

Genomic DNA was prepared as in Genomic DNA isolation and qPCR section above. qPCR signal (SsoFast, Bio-Rad) from the genomic DNA was compared to signal from a molar standard amplified from increasing amounts of the corresponding dCas9-VP160 and rtaTA plasmids. Primers used are listed in Supplemental Table S1. All related plots were generated using R version 3.4.1 (The R Development Core Team 2017).

Generation of clonal ESCs with targeted genomic deletions and genotyping

After 4 d of dox induction, RE3 deletion E14 cells were cultured 2 d in the absence of dox to ensure that Cas9 was fully depleted. Then, 2000 cells were plated on a 10 cm plate with preplated irradiated MEF feeder cells. After 4 d, individual colonies were picked and plated on irMEFs. Clonal lines were passaged twice off of MEFs before genomic DNA was prepared as in Genomic DNA isolation and qPCR section above.

Genotyping PCR reactions were performed with gDNA using Apex Taq DNA Polymerase (Genesee Scientific). The first set of primers flanked the deletion and identified clonal lines with at least one allele deleted. The second set only amplified a wild-type allele, with both primers sitting inside the deletion. Many clones showed weak wild-type bands, likely due to MEF gDNA contamination and not due to the presence of a wild-type allele in the ESC clone. Primers used are listed in Supplemental Table S1.

RNA isolation and qPCR

RNA was isolated using TRIzol (Invitrogen). For RT-qPCR assays, 1–2 μg of RNA was reverse transcribed using MultiScribe RT (Applied Biosystems), and qPCR was performed using iTaq Universal SYBR Green (Bio-Rad) and primers specified in Supplemental Table S1. All related plots were generated using R version 3.4.1 (The R Development Core Team 2017).

RNA FISH

Fosmid W1-2156F18 (Airn) and BAC RP23-101N20 (Kcnq1ot1) were ordered from the BACPAC resource center and fingerprinted with restriction digestion prior to use to verify inserted DNA. Fluorescent labeling was performed using BioPrime (Invitrogen). ESCs were fixed on coverslips for 10 min in 4% paraformaldehyde/PBS, followed by a 10-min permeabilization on ice in 0.5% TritonX-100 in PBS and 1:200 Ribonucleoside Vanadyl Complex (NEB). Coverslips were stored at −20°C in 70% ethanol until use.

To initiate the RNA FISH protocol, coverslips were dehydrated by serial 3-min incubations with 75%, 85%, 95%, and 100% ethanol, and air-dried for 5 min. RNA FISH probes were added, and coverslips were placed cell-side down in a chamber humidified with 50% formamide/2 x SSC overnight at 37°C. After overnight incubation, coverslips were washed 3 x with 50% formamide/2 x SSC at 42°C and 3 x with 1 x SSC at 50°C. Each wash was 5 min long. Coverslips were then rinsed 1 x with PBS before a 2 min incubation in DAPI stock diluted 1:1000 in water. Coverslips were rinsed twice more and affixed to glass slides using Vectashield (VectorLabs), then sealed with nail polish.

Four-dimensional data sets were acquired by taking multichannel Z-stacks on an Olympus BX61 widefield fluorescence microscope using a Plan-Apochromat 60X/1.4 objective and a Hamamatsu ORCA R2 camera, controlled by Velocity 6.3 software. Excitation was provided by a mercury lamp, and the following filters were used for the three fluorescent channels that were imaged: 377/25 ex, 447/30 em for DAPI (DAPI-5060B Semrock filter); 482/17 ex, 536/20 em for AlexaFluor 488 (Semrock FITC-3540B filter); 562/20 ex, 642/20 em for Cy3 (Semrock TRED-4040B filter).

Pixel size was 0.108 μm, z spacing was 0.2 μm, and images had 1344 × 1024 pixels. Between 46–49 Z-stacks were acquired for each image. Z-stacks were deconvolved using the iterative-constrained algorithm (Mediacy AutoQuantX3) with default algorithm settings. Sample settings for the deconvolution were: peak emissions for dyes (570, 519, 461 nm for Cy3, AlexaFluor 488 and DAPI, respectively), widefield microscopy mode, NA = 1.4, RI of oil = 1.518, and RI of sample = 1.45. After deconvolution, RNA FISH signals were located using the “Spots” function in Imaris software (version 8.3.1) and marked with equal sized spheres. To initially call spots on all images, spot detection values were set at 0.5 μm for xy and 1.5 μm for z, and background subtraction and auto quality settings were used. We manually optimized the quality/sensitivity setting to call Kcnq1ot1 spots, and then used the same quality threshold to call Airn spots for the same image. Images are shown as maximum intensity projections made using ImageJ (Schindelin et al. 2012).

Immunofluorescence (IF)

Cells were fixed on coverslips the same as for RNA FISH (see above). To initiate the IF protocol, coverslips were washed twice in PBS and blocked for 30 min at room temperature in blocking solution (1 x PBS with 0.2% Triton X-100, 1% goat serum, and 6 mg/mL IgG-free BSA). Then, coverslips were washed in 0.2% Triton/1 x PBS and incubated with EZH2 antibody (Cell Signaling #5246; 1:200 in block solution) for 1 h at RT. Coverslips were washed 3 x with 0.2% Triton/1 x PBS for 4 min each and incubated with secondary antibody (AlexaFluor 647 goat anti-rabbit, A-21245; 1:1000 in block solution for ESCs and AlexaFluor 488 goat anti-rabbit, A-11034; 1:1000 in block solution) for 30 min at RT. After incubation, coverslips were washed 3 x with 0.2% Triton/1 x PBS for 4 min each and rinsed 1 x with PBS before a 2 min incubation in DAPI stock diluted to 5 ng/mL in water. Coverslips were rinsed twice more and mounted to glass slides using Prolong Gold (Thermo Fisher Scientific P10144). Imaging
and deconvolution were performed the same as described in the RNA FISH section with the following excitation for dyes: 670, 519, and 461 nm for AlexaFluor 474, AlexaFluor 488, and DAPI, respectively. Images are shown as maximum intensity projections made using ImageJ (Schindelin et al. 2012).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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