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Tamoxifen- and mifepristone-inducible versions of CRISPR effectors, Cas9 and Cpf1.

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ABSTRACT: Methods for making specific modifications to the genomes of living cells are powerful research tools. Two methods currently dominate, CRISPR and Cre recombinase. CRISPR has the advantage that it can act on unmodified target genes; Cre has the advantage of being available in drug-inducible versions, allowing temporal control, but it requires engineering ('floxing') of the target gene. Here, we have combined these advantages by constructing drug (tamoxifen / mifepristone)-inducible Cas9 and Cpf1 CRISPR effectors. We demonstrate their low background activity and robust activation with drugs, by using gRNAs to target them to TetR, in a cell carrying a Tet-repressed reporter gene. As well as being useful in their own right, the research tools generated here will pave the way to making further drug-controlled effector proteins.

Synthetic biology provides potentially useful technologies for materials synthesis, for sensing, and for construction of tissues, and provides new tools and perspectives for the study of basic biology. Among the most useful synthetic tools built so far for mammalian systems are inducible transcriptional switches that exert temporal and dose-dependent control over gene expression.

Engineered systems can be controlled through chemical, biological, and electromagnetic methods. The chemical option often involves the use of small molecules, which has several advantages: it offers a quick start, it is reversible, it can operate deep inside tissues that are inaccessible to light and, unlike biological agents such as viruses, most of the chemicals involved are too small to be immunogenic so they can be re-used indefinitely.

Figure 1. a) The T-REx system is a Tetracycline-Regulated Expression System for Mammalian Cells. In the absence of tetracycline, the Tet repressor binds to the TetO₂, repressing the transcription of the transgene. Upon addition, tetracycline binds to the Tet repressor rendering it unable to bind to the Tet operator. As a result, the gene of interest is transcribed. b) Cas9 and Cpf1 are components of the Class II CRISPR systems. They present three main differences: the PAM sequence, the type of cut and the number of gRNAs. c) In this paper, we engineer ligand-binding domains of nuclear hormone receptors without loss of function in Cas9 or Cpf1 to create the inducible CRISPR systems.

One of the best-known chemically inducible system for control of mammalian gene expression is probably the Tet on/off system, developed over 25 years ago from components of the bacterial Tn10-encoded Tet operon (1). Currently there are two broad classes of Tet-based gene regulation, one activates gene expression in the presence of tetracycline/ doxycycline and the other represses it. Ther-mofisher's T-REx system is based on the binding of the Tet repressor protein to a Tet operator-containing promoter that controls the gene of interest. This binding is inhibited by tetracycline/ doxycycline so, when these chemicals are present, so the promoter is not repressed, and the gene is activated (Figure 1a). Other systems use a fusion protein made from the Tet repressor and a viral transcriptional activator, which will activate transcription from a basal promoter with a Tet operator site unless tetracycline/doxycycline is present, when binding is blocked and the gene is off. The main advantages of the Tet system are its rapidity and reversibility. Although the system was invented some time ago, the number of studies using this system remains high (<https://www.ncbi.nlm.nih.gov/pub-med/?term=Tet+inducible>).

Other small molecule inducible systems, such as the rapamycin or the nuclear hormone receptor systems, are available (2). Nuclear hormone receptors exhibit a modular structure, having different physical protein domains that correspond closely to functional domains: these can be interchanged between related receptors without loss of function. Among them, the ERT2 is a truncated form of the estrogen steroid receptor that has the advantage of providing orthogonality because it binds to the steroid antagonist 4-Hydroxytamoxifen but not to the endogenous hormone estrogen. For example, 4-Hydroxytamoxifen has been combined with site-specific recombinases systems such the Cre-loxP to create the Cre-ERT2 system which has the speed advantage of regulating the Cre recombinase at the protein level (3). The ERT2 motif has also been fused to expression constructs of transcription factors involved in neurogenesis (i.e., ASCL1 and NEUROD1) for temporal control of virus-mediated gene expression in adult mouse Neural stem/progenitor cells. (4). A Zscan4c-ERT2 fusion protein has also been used to demonstrate an active role of Zscan4 in increasing the ability of mouse embryonic stem cells to contribute to a whole embryo (5).

Figure 2. Schematic of our system. Diagram of the system representing the tight control of mCherry expression by combining the TetR system and the inducible CRISPR system. The reversible action: In the presence of tetracycline or doxycycline, the TetR does not bind the tetO allowing the reporter to be expressed. Afterwards, by removing the tetracycline, the reporter expression can be again inhibited. The irreversible action: When a drug (4-hydroxytamoxifen or mifepristone) is added to the inducible CRISPR system, the CRISPR is transported to the nucleus where it disrupts its target gene, the TetR. With no more TetR being transcribed once the gene has been deleted, the inhibition exerted by the TetR system is not functional anymore and the reporter is permanently expressed.

Recent developments have created a tool that is even more popular than the TetR system (6). Also having a bacterial origin, in this case as a protective mechanism against exogenous DNA, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Type II system has been manipulated to be used in eukaryotic systems. The CRISPR system is currently the most commonly used RNA-Guided Endonuclease technology for genome engineering. Among the class II CRISPR effectors, Cas9 and Cpf1 (Figure 1b) have been remodelled for genome engineering (7) and, through deletion of nuclease domains and fusion with transcriptional activating or repressing domains, for RNA guided transcriptional regulation (8). They have also been modified for epigenetic regulation by fusing dCas9 to different epigenetic modifiers for targeted epigenetic modification at specific loci in the genome (9), and for imaging of chromatin within live cells (10).

The RNA-guided nature of Cas9 and Cpf1 brings some advantages over the operator-guided mechanism of Tet, because a gene of interest does not have to be placed artificially under the control of an operator-containing promoter and natural genes can be targeted in the context of their natural promoters and chromatin. The Tet system, though, has the advantage of being switchable using a small molecule, allowing gene control to be exerted at a time of an experimenter's choosing. We believe that a range of tools that combine both of these features would

be of interest for the research community. In this study we have therefore developed four small molecule-inducible CRISPR effectors for use in mammalian cells. We demonstrate their use in a system that combines the TetR system with the inducible CRISPR system. First, the transgene expression of a reporter was placed under the reversible control of a Tet operator, so that the expression of the reporter occurs only in the presence of tetracycline/ doxycycline. Next, a drug-inducible irreversible 'on' system was created by disrupting the Tet repressor with either of two drug-inducible CRISPR systems (HR-Cas9 and HR-Cpf1), each being made in varieties sensitive to one of two different drugs.

RESULTS AND DISCUSSION

Construction of the reporter system and verification of gRNAs

The system we have designed to demonstrate drug-controlled CRISPR action depends on CRISPR-mediated disruption of the gene encoding a TetR transcription inhibitor. In our test cells, this TetR inhibitor normally inhibits the expression of the mCherry fluorescent reporter gene (Figure 2). As a first step, we constructed a basic gene expression system in which mCherry was placed under the control of a CMV promoter to which the Tet operator site, TetO, had been added (Figure 3a, schematic). This was achieved by cloning the mCherry gene into the commercial expression vector, pcDNA4/TO, to create a pcDNA4/TO-mCherry construct and transferring it into T-REx-293 cells,

Figure 3. a) Tetracycline-regulated mCherry expression: The images show T-REx-293 cells stably expressing the TetR protein transfected with an inducible vector containing the reporter mCherry as gene of interest. After tetracycline addition, the transcription of the mCherry is initiated. It can be repressed again by tetracycline removal. b) Irreversible expression of mCherry on T-REx-293 -TO- mCherry cells co-transfected with Cas9 and Cpf1. Two weeks after transfection are required for the complete reporter expression. (10x objective; Scale bars: 200 μ m).

which already contain the TetR gene. After antibiotic selection, stable clones were tested for tetracycline-controlled mCherry expression. As expected, the cells did not express the reporter unless tetracycline was added to inhibit TetR (Figure 3a). When it was added they did express mCherry but ceased to do so on tetracycline removal. This confirmed an effective, reversible control of the mCherry reporter gene by TetR.

Our next step was to test the ability of a constitutive CRISPR system to effectively disrupt the TetR gene. We co-transfected cells of the T-REx-293-TO-mCherry clone described above with a plasmid encoding the CRISPR effectors sp-Cas9, or hAsCpf1, and also with a plasmid encoding gRNA designed to target the effector to the TetR gene (Figure 3b schematic). Both CRISPR systems were able to disrupt TetR inhibition of mCherry reporter expression (Figure 3b).

The main drawback of our system was that gene editing was slow: It was not until 6 days post-transfection that the first evidence of mCherry expression was evident and 4 to 6 extra days were needed for maximal mCherry appearance in the cultures. After tetracycline addition, the mCherry expression was observable within hours, showing that the delay was in gene editing and disappearance of the TetR mRNA and protein, and not in transcription and translation of mCherry itself. For many applications, particularly disease modelling in in vivo mammalian systems, delays of a few days will not be important (it takes many weeks for gene loss to result in a tumour, cyst etc anyway). If faster timing were important, it may be possible to have a method to destroy the target protein at the same time than the gene expression was downregulated. Perhaps, for example, an auxin-inducible degron could be integrated in the system to completely degrade the target protein at the

Figure 4. 4-Hydroxytamoxifen (4-OHT) inducible Cas9. a) Schematic representation of the wild type Cas9 and three successful fusion proteins of Cas9 with the ligand binding domain of a mutated form of the estrogen receptor ERT2. The gRNA targets the TetR in all four constructs. b) Percentage of T-REx-293-mCherry positive cells after (+ 4-OHT) or without (- 4-OHT) TetR disruption with the three 4-OHT inducible Cas9 comparing to the number of mCherry positive cells after TetR disruption by the wild type Cas9. c) Micrographs of the T-REx-293-mCherry cells after TetR disruption either with the Cas9 or each of the inducible Cas9 (10x objective; Scale bars: 200 μ m).

same time than the gene is disrupted. This would, though, involve more work on modifying the genome and therefore abrogate the advantages of CRISPR over Cre.

Creation of ERT2- Cas9 fusion proteins

The need for inducible genome editing was appreciated soon after the generation of the first transgenic knockout mice (11). While some conventional knockouts were adequate for studying the function of a gene when that gene had a highly restricted pattern of expression or function, in many cases devastating effects of loss of gene function at early stages of development made it impossible to assess the function of the gene at later stages. An example is provided by the tumour suppressor, WT1, loss of which is implicated in the development of the human kidney nephroblastoma, Wilms' tumour (12). Attempts to create a mouse model of Wilms' tumour by making a homozygous knockout of the WT1 gene failed because, in the absence of the gene, no kidneys formed in the first place (13). Dissecting the role of WT1 in repressing tumour formation depended on removing gene function only once kidneys had begun to develop, either by RNAi in culture (14) or by exploiting conditional knockout techniques in vivo. The most common conditional knockout technique flanks the target gene of interest with loxP sites and places Cre recombinase, which recognizes and recombines these sites, under the control of a cell-specific promoter (15). Further control on this system was added by Feil and colleagues, who

made a fusion protein between Cre and ERT2 (16): this fusion protein was only active in the presence of 4-Hydroxytamoxifen, allowing control of Cre activity in time and space (17).

The Cre-ERT2 system works well but it involves extensive genetic modification because the target gene must be flanked by LoxP sites ('floxed'): it is therefore not a technique amenable to high-throughput studies of gene loss. CRISPR gene editing targets genes in their natural state, requiring only a gRNA for guidance, so can be applied to wild-type genomes and is suited to high-throughput studies, CRISPR being in all tests and only the gRNA changing between them. Conventional CRISPR does not, however, allow drug-mediated timed activation.

To confer control by 4-Hydroxytamoxifen on CRISPR activity, we engineered several designs of fusion protein between Cas9 and the ligand binding domain of a mutated form of the estrogen receptor (ERT2) that binds to steroid antagonist 4-Hydroxytamoxifen but not to the endogenous hormone estrogen (Figure 1c). The constructs differed with respect to five features: 1) the position of the ERT2 with respect to the Cas9 (amino or carboxy terminal ERT2 fusion proteins, or ERT2 embed in an insertion point inside the Cas9, as Savage et al. 2016 showed was effective for a catalytically dead dCas9), 2) the number of ERT2 copies inserted, 3) the presence/ absence of a SV40 nuclear localization signal (NLS), 4) the incorporation of a 3-amino-acid

Figure 5. a) Irreversible expression of mCherry on T-REx-293 -TO- mCherry cells co-transfected with a 4-Hydroxytamoxifen inducible Cas9 (upper picture). ERT2 was changed by a different nuclear hormone receptor, the hPR LBD resulting likewise, in mCherry appearance because of the TetR disruption (lower picture). 13 days after transfection (11 days after 4-OHT or mifepristone removal) are required for the complete reporter expression with both inducible Cas9. b) Flow cytometry analysis showing the percentage of mCherry positive T-REx-293 cells after co-transfection with the 4-Hydroxytamoxifen and the mifepristone Cas9 with or without drug addition.

linker sequence (LEP) and 5) a myristoylation signal. The idea behind using a myristoylation signal was to promote the binding of ERT2 to the membrane until it is activated by 4-Hydroxytamoxifen, when it should dimerize and be imported into the nucleus along with the Cas9 (18).

Our criteria for having made a successful drug-inducible Cas9 effector were that it should express low basal activity in the absence of drug and robust activation of the effector in the presence of the drug. Three of these constructs met these two criteria (Figure 4a): 1) 'ECES' - Cas9 flanked by 2

copies of ERT2, one on the amino terminal and the other one on the carboxy terminal, 2) 'ECEC', being the same as ECES except that it had a nuclear localization signal between the carboxy-terminal of Cas9 and the ERT2, and 3) 'CEC', the construct with the ERT2 embedded within the Cas9 sequence (at amino acid 231). The induction of mCherry reporter expression by these constructs, in the presence or absence of 1 μ M 4-Hydroxytamoxifen, is shown quantitatively in Figure 4b and qualitatively in Figure 4c. Of the three constructs, 'ECES' showed the maximum activation but also had the highest basal activity. ECE, the construct that had ERT2 inside the Cas9 sequence, showed

Figure 6. Cas9 and Cpf1 three dimensional structures (PDB 4008 and PDB 5B43 sequences visualized with Geneious 3D structure viewer). The Insertion points for the ligand binding domains of the hormone receptors ERT2 and hPR are the spaces located between the amino acids indicated in cyan and magenta

the highest ratio of activation over basal activity (1:49). For that reason, we kept ECE as our best 4-Hydroxytamoxifen-inducible Cas9 fusion protein.

Substitution of the 4-Hydroxytamoxifen-sensitive ligand binding domain with a mifepristone-sensitive one

To further evaluate the capacity of the ECE 4-Hydroxytamoxifen-inducible Cas9 construct to be adapted for control by alternative drugs, we replaced the ERT2 sequence with that of a different nuclear hormone receptor. We used the human progesterone ligand binding domain hPR-LBD, which binds to the synthetic steroid, mifepristone. The resulting fusion protein system was able to disrupt the TetR in response to mifepristone in a manner very similar to the ECE 4-Hydroxytamoxifen-regulated construct (Figure 5). Furthermore, the basal activities for both the 4-Hydroxytamoxifen-controlled and the mifepristone-controlled Cas9 were less than 1% of that of wild-cas9. (Suppl. Figure1).

Adding drug control to the alternative CRISPR effector, Cpf1

The CRISPR effector Cpf1 is an RNA-guided nuclease broadly similar to Cas9, but with some properties that make it more suitable for editing AT-rich regions and for generating cuts with overhangs. We therefore wondered whether our techniques for making Cas9 drug-controlled could be applied to Cpf1. The insertion site for ERT2 in our ECE Cas9 construct was suggested from the work of Savage et al. (2016), who identified insertion sites within Cas9 that can accept synthetic functional domains by creating and

screening an unbiased Cas9 insertion library using randomized transposition. When they mapped the crystal structure of the Cas9 they realised that ‘hotspots’ of useful sites were often located in flexible loops and the ends of helices. (19) This provided a good guide for the engineering of Cpf1 but Patel et al. (2016) showed that there is very poor sequence alignment between individual domains of Cas9 their functional counterparts in Cpf1. Use of the same site to confer drug control on Cpf1 could not therefore be expected to work. In view of this, an analysis of the three-dimensional structure of both Cas9 and Cpf1 was done to predict a possible insertion point for the CRISPR on the Cpf1. We applied the general principles identified by Savage et al. (2016) and sought a location within the 3D structure of Cpf1 that would allow extra amino acids to be added without any conformational interaction with the rest of the Cas9 structure, the gRNA or the DNA. Cpf1 has a bi-lobed scaffold with a ‘crab claw’ shape (Figure 6). (20), so we chose the flexible loop around the joint at the top of the “crab claw”, a protrusion of Cpf1 which comprises the AA 584 (Glu) and 585 (Lys) represented on cyan and magenta in Figure 6, as a location most likely to be suitable. We believe this to be the first time that an active Cpf1 has been engineered with ligand-binding domains of hormone receptors.

We created two fusion proteins, one with ERT2 in this location within Cpf1, and the other with hPR-LBD there. We then tested the 4-Hydroxytamoxifen- and mifepristone-inducibility of Cpf1 and we compared it to the drug-controlled Cas9. The number of mCherry positive cells after the addition or not of 4-Hydroxytamoxifen or mifepristone was quantified by FACS sorting and compared with

Figure 7. a) Schematic representation of the tamoxifen- and mifepristone-inducible versions of CRISPR effectors, Cas9 and Cpf1. b) mCherry quantification by flow cytometry after the addition or not of 4-Hydroxytamoxifen (to the ERT2 controlled CRISPR) or mifepristone (to the hPR controlled CRISPR).

the number of cells expressing mCherry after TetR disruption with the wild type Cas9 or Cpf1 (Figure 7: as negative controls we used cells that expressed the TetR but that were not transfected with the CRISPR systems). In the experimental groups, both Cpf1 fusion proteins were able to efficiently disrupt the TetR, and they showed lower basal activity and higher activity than both inducible Cas9 systems. Mutations of the TetR were confirmed using the T7 assay (Supplementary Figure 2).

To summarize, our objective was to add extra layers of control to the CRISPR/Cas9 and CRISPR/Cpf1 gene-editing systems to allow them to be activated only in the presence of an exogenously applied drug. Our results demonstrate that we have produced 4-Hydroxytamoxifen- and mifepristone-inducible versions of both Cas9 and Cpf1 with a very low basal activity but with strong activity following activation.

We expect that over the next years we will experience an increase on the strategies to develop efficient inducible systems for mammalian gene expression in parallel with further crystallographic studies of well-studied and new proteins. In-depth studies of the three-dimensional structure of proteins may lead to improvements in the field of protein engineering by creating new fusion proteins capable of transcriptional activation or repression, which in turn can be used to develop new synthetic biology tools for mammalian gene expression systems.

CONCLUSIONS:

For this paper, we engineered two different drug-sensitive domains into Cas9 and Cpf1. In principle, the drug-controllability could be customized further by replacing the ligand binding domain of the fusion protein. Also, the targeted genes can be customized by changing the gRNAs. Among the advantages of the system, we can highlight the

fact that the target proteins were modified to be responsive to an already existing drug, that it showed low basal activity and high induction capacity, that the system is compatible with mammalian cells, and features modularity to allow fine-tuning the system. All these features are highly desirable for control of mammalian gene expression.

MATERIAL AND METHODS

Plasmid construction:

pTrex-mCherry: The mCherry gene was cloned from pCherryPicker2 (Clontech) and PCR amplified with primers containing attachment (att) sites for Gateway® recombination according to manufacturer's instructions and recombined by BP reaction in vector pDONR-221 (see supplementary table 1 for primer sequences). The mCherry was subsequently inserted from the pENTR vector into pT-Rex-DEST30 (Thermo Fisher Scientific), by LR reaction according to manufacturer's (Thermo Fisher Scientific) instructions.

Fusion proteins: pCas9_GFP (Plasmid #44719 addgene) was used to make all the Cas9-containing plasmids. Briefly, gblocks from IDT were created for the modified ligand binding domain of the mutated estrogen receptor (ERT2), the ligand binding domain of the human progesterone receptor, a nuclear localization signal, a 3 aa peptide linker and a myristoylation signal (all sequences in supplementary Table 1). Using Gibson assembly to assemble the fragments resulted in the following constructs: ERT2-Cas9; Cas9-ERT2-NLS; Cas9-ERT2; Cas9-ERT2-NLS-LEP; Cas9-ERT2-LEP; ERT2-Cas9-ERT2-LEP; ERT2-Cas9-ERT2-NLS-LEP, ERT2-Cas9-ERT2; ERT2-Cas9-ERT2-NLS; Myr-Cas9-ERT2-NLS-LEP; Myr-Cas9-ERT2-LEP; Myr-Cas9; Cas9-ERT2-Cas9; Myr-Cas9-ERT2-Cas9; Cas9-hPR-Cas9; Myr-Cas9-hPR-Cas9, where 'NLS' is a SV40 nuclear localisation sequence, 'Myr' is a myristoylation site and 'LEP' is the 3AA linker L= Leucine, E=Glutamic acid, P=Proline

pYo10 (pcDNA3.1-hAsCpfi) (Plasmid #69982 addgene) was used along with gblocks to make all the Cpfi-containing plasmids. Constructs: Cpfi-ERT2, Cpfi-ERT2-LEP; Cpfi-ERT2-Cpfi; Cpfi-hPR-cpfi.

For both, the Cas9 and the Cpfi, all the fragments were cloned by Gibson assembly Hifi assembly master mix (NEB, REF: E2621S) and transformed in NEB 5 alpha competent *E. coli* cells (NEB REF: C2987H). Plasmids were purified using the maxi prep kit from Qiagen (Ref: 12163).

gRNA-carrying plasmids: For designing gRNAs that would target the TetR gene, the genomic sites of the form 5'-NGG-3' (for the cas9) and 5'-TTTN-3' (for the Cpfi) in our intended target site, the TetR, that were more likely to have high on target effects and low off target activity were chosen with the CRISPR design tool of Benchling (<https://benchling.com/>). Following the recommendations of the gRNA Synthesis Protocol (21), a 455 bp or 399 bp fragment for the Cas9 and Cpfi respectively bearing the U6 promoter + target sequence + guide RNA scaffold + termination signal were synthesized as a gBlock from IDT and cloned with the Zero blunt cloning kit (Thermo Fisher Scientific ref: K270020) into the empty backbone vector pCR-Blunt II-TOPO from the kit. One Shot™ TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific ref: C404003) were used for transformation of the plasmids and maxi prep kits (Qiagen ref: 12163) used for purification. All the constructs were stored at -20 °C until transfection on mammalian cells.

Mammalian cell culture and transfection

Human Embryonic kidney cells (HEK-293 cells) that stably express the tetracycline repressor for the T-REx™ system (T-REx™-293 Cell Line, Thermo Fisher Scientific ref: R71007) were maintained in a humidified incubator at 37°C and 5% CO₂, in complete medium. This consisted of Dulbecco's minimum essential medium (DMEM, Gibco 41966) supplemented with 10% FBS (Biosera ref: FB-1090). 24 hours prior to transfection, T-REx-293 cells were harvested by trypsinization and seeded at a density of 80,000 cells in 500 µl of complete medium per well on 24 well plates. Cells were transfected using 1 µl lipofectamine 3000 (Invitrogen ref: L3000008) and the pTrex-mCherry plasmid (800 ng) and kept in selection medium. This consisted of complete medium + 800 µg/ml G418 (Thermo Fisher Scientific 11811023) + 5 µg/ml blasticidin (Thermo Fisher Scientific R21001). They were maintained under this selection for 2 weeks and clones were isolated using cloning rings. Each resulting clone was tested with 1 µg/ml tetracycline to verify that mCherry expression was under tetracycline control and was afterwards transfected with wild-type Cas9/Cpfi, or our engineered drug-inducible Cas9/Cpfi, and the corresponding gRNA. After 24 hours, the medium was replaced by fresh selection medium, with or without the inducing drugs (1 µM 4-Hydroxytamoxifen or 10 nM mifepristone).

mCherry imaging/ fluorescence microscopy

After transfection and treatment, cells were passaged twice a week and maintained for 2 weeks (without addition of inducing drugs) before image acquisition. Images were acquired using a Zeiss Axio Observer D1 inverted microscope with an AxioCam MRm camera and 10x objective.

Flow Cytometric analysis

After image acquisition, mCherry expression was also detected by flow cytometry. Cells were washed with PBS (Phosphate-buffered saline Sigma ref: P4417), dissociated with trypsin- EDTA (sigma ref: T4174), re-suspended in PBS with 0.1% BSA at a density of 15x10⁶ cells/ml and filtered using BD blue capped filter tubes (BD 352235) in order to obtain single-cell suspensions, which were applied immediately to a BD LSRFortessa™ Cell Analyzer and analysed using the BD Biosciences FACSDiva Software.

T7 assay

Genomic DNA was isolated from the T-REx-293 cells using a DNA extraction kit protocol (DNeasy Blood & Tissue Kit Qiagen ref: 69504) according to the manufacturer's instructions. After that, the target region was amplified by PCR with Q5® Hot Start High-Fidelity 2X Master Mix (NEB ref: M0494S). All PCR reactions used the same conditions: 35 cycles [98°C, 30 s; (98°C, 5 s; 58°C, 10 s; 72°C 20 s); 72°C, 2 min; 4°C hold]. After amplification, PCR products were analysed by 1% agarose gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen ref: 28104).

To generate heteroduplex DNA, the following conditions were used: 95°C for 5 min, 95°C to 85°C cooling (at a rate of -2°C/s), 85°C to 25°C cooling (at a rate of -0.1°C/s), hold at 4°C. Following denaturation/renaturation of the genomic PCR samples, 1 µl of T7E1 (New England BioLabs.) was added to the samples and digestion reactions were incubated for 1 h at 37°C. After digestion 1 µl of 0.25 M EDTA was used to stop the enzymatic reaction. Undigested PCR samples (as a control) and T7E1-digested PCR products were electrophoresed on a 2% agarose gel.

Statistical analysis

The means ± SEM of multiple independent measurements were calculated. Normality was tested through the Anderson-Darling Normality Test. Homoscedasticity with Bartlett's Test. A One-way ANOVA with Tukey Pairwise Comparisons was used to determine whether there were differences between some groups. A two-tailed Student's t-test was used to test the null hypothesis that there was no difference between groups.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website

Supplementary Figure 1: Non-treated inducible CRISPR systems. Supplementary Figure 2: T7 Endonuclease I mismatch cleavage assay. Supplementary table 1: Sequences.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Tet: Tetracycline, ERT2: a truncated form of the estrogen steroid receptor, hPR: human Progesterone receptor

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