CHAPTER

4 Employing CRISPR/Cas9 genome engineering to dissect the molecular requirements for mitosis

Kara L. McKinley[1]

Department of Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA, United States

[1]*Corresponding author: e-mail address: kara.mckinley@ucsf.edu*

CHAPTER OUTLINE

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Abstract

The faithful execution of cell division requires the coordinated action of hundreds of gene products. Precisely perturbing these gene products in cells is central to understanding their functions during normal cell division, and the contributions of their disruption to disease. Here, we describe experimental approaches for using CRISPR/Cas9 for gene disruption and modification, with a focus on human cell culture. We describe strategies for inducible gene disruption to generate acute knockouts of essential cell division genes, which can be modified for the chronic elimination of nonessential genes. We also describe strategies for modifying the genome to generate protein fusions to report on and modify protein behavior. These tools facilitate investigation of protein function, dissection of protein assembly networks, and analyses of disease-associated mutations.

1 INTRODUCTION

The precise disruption of gene function is a fundamental strategy for dissecting biological processes. Genetic approaches in systems such as budding and fission yeasts, flies, and the DT40 chicken B cell line have been central to identifying key players involved in cell division and defining their functions (a far-from-complete list includes Biggins et al., 1999; Hartwell, Mortimer, Culotti, & Culotti, 1973; Hoyt, Totis, & Roberts, 1991; Li & Murray, 1991; Meeks-Wagner, Wood, Garvik, & Hartwell, 1986; Stoler, Keith, Curnick, & Fitzgerald-Hayes, 1995; Sunkel & Glover, 1988; Takahashi, Yamada, & Yanagida, 1994). In contrast, the historically poor genetic tractability of human cells has driven the identification of major players in this system often through biochemical analyses (for example, the identification of many centromere proteins by Earnshaw & Rothfield, 1985; Foltz et al., 2006; Obuse et al., 2004; Okada et al., 2006). Robust perturbation of gene function is a critical complement to biochemical analyses to dissect the molecular mechanisms of cell division in human cells.

Until recently, the predominant strategy to disrupt gene function in human cells was RNA interference (RNAi). RNAi has played a critical role in the cell biological analyses of numerous mitotic functions in human cells as well as forward genetic-like screening to identify new cell division players (Ganem et al., 2014; Neumann et al., 2006, 2010). RNAi has produced similar important advances in studies of cell division in systems such as worms and fly cells (for example, Desai et al., 2003; Gonczy et al., 2000; Goshima et al., 2007; Oegema, Desai, Rybina, Kirkham, & Hyman, 2001). However, RNAi studies face several challenges, such as extensive variability in the efficiency of gene knockdown. Importantly, RNAi also frequently reduces the expression of genes other than the intended target, resulting in confounding off-target effects (Abudayyeh et al., 2017; Evers et al., 2016; Jackson et al., 2003; Sigoillot et al., 2012).

Recently, a bacterial adaptive immune system, the type II clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system, has been repurposed to allow for efficient and affordable gene disruption in human cells, as well as many other systems (Cong et al., 2013; Jinek et al., 2012, 2013; Mali, Yang, et al., 2013). The native composition of this system is reviewed elsewhere (Wright, Nunez, & Doudna, 2016). Here, we will focus on the engineered system for heterologous genome engineering (Fig. 1). For this system, a ribonucleoprotein complex comprised of the nuclease Cas9, and a chimeric RNA, termed the single guide RNA (sgRNA), generates double-strand breaks (DSBs) at specific sites in the genome. The sgRNA is composed of a variable 5′ targeting sequence (also known as the protospacer) followed by scaffold sequences. The targeting sequence (~20 nucleotides) is designed to be complementary to the region of the genome to be cleaved. The scaffold sequences allow the sgRNA to interact with Cas9. Thus, the researcher simply needs to clone an sgRNA containing a targeting sequence complementary to their gene of interest and introduce the sgRNA and Cas9 into their cells to generate DSBs at the specified site in the genome.

DSBs generated by Cas9 cleavage can be repaired by homology-directed repair (HDR) or nonhomologous end joining (NHEJ) (Fig. 1). HDR allows for the introduction of specified mutations or tags near the cut site (knock-in). In contrast, NHEJ repairs the break by the introduction of random nucleotides at the break site that can disrupt the ability of the gene to produce functional protein (knockout). In this chapter, we will use Cas9 to generate DSBs, although Cas9 variants exist to make modifications other than genomic DSBs. For example, fusion of transcriptional activators to a nuclease-deficient Cas9 that is directed to a gene promoter can stimulate transcription of endogenous genes for gain-of-function studies (Konermann et al., 2015; Maeder et al., 2013; Mali, Aach, et al., 2013; Tanenbaum, Gilbert, Qi, Weissman, & Vale, 2014).

In this chapter, we will describe strategies to apply targeted DSBs generated by CRISPR/Cas9 to facilitate studies of mitosis. We will describe strategies to generate genetic knockouts and knock-ins in human tissue culture cells (Fig. 2). The techniques that we describe are useful for researchers to dissect the cellular functions of their proteins of interest by

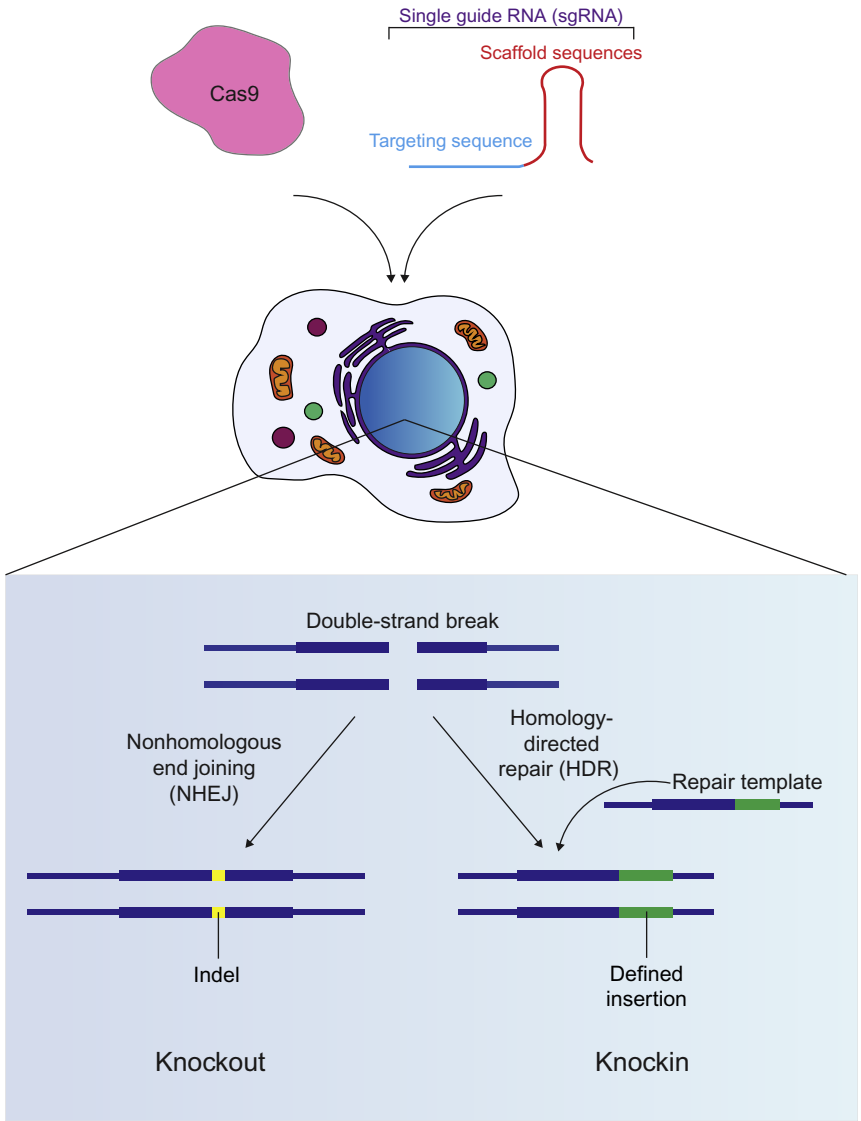

FIG. 1

Schematic of the applications of engineered CRISPR/Cas9 systems for gene editing. The sgRNA contains a scaffold sequence for interaction with the Cas9 and a targeting sequence designed by the researcher to direct Cas9 to a complementary sequence in the genome. DSBs generated by Cas9 cleavage are predominantly repaired by NHEJ, which can introduce a variety of indels that may disrupt the production of functional protein. In the presence of a repair template with homology to regions flanking the cut site, HDR can occur to introduce defined sequences.

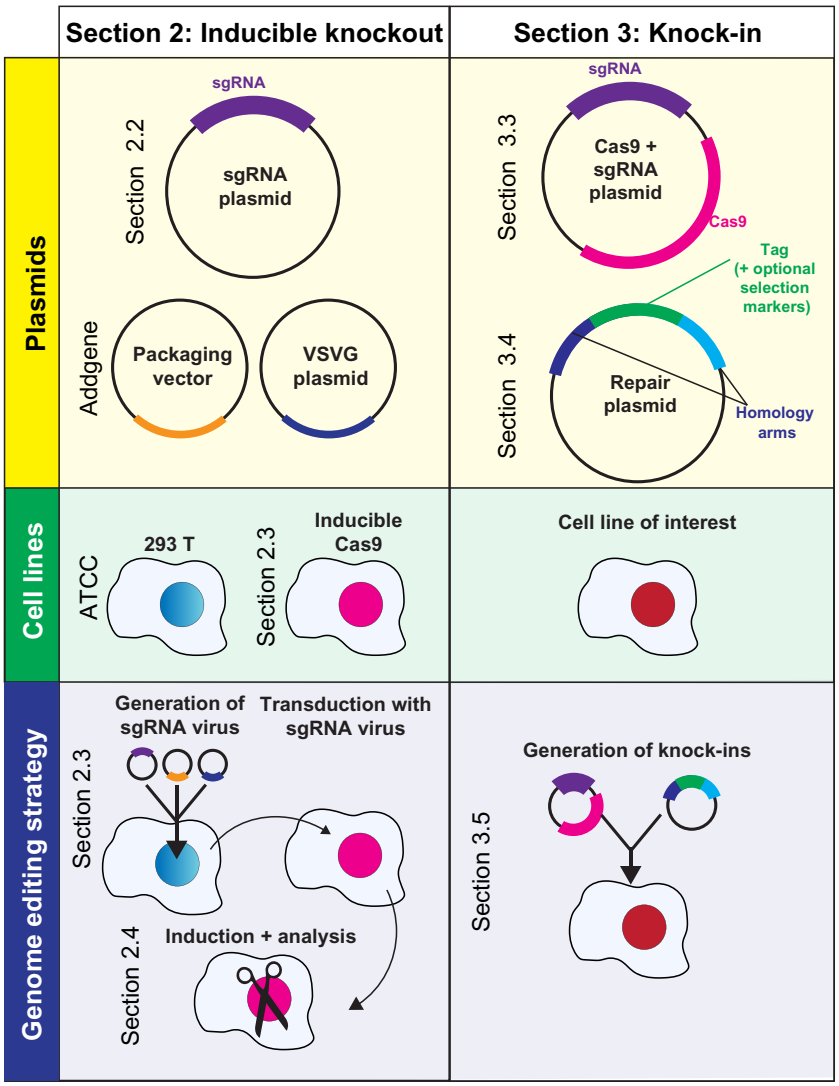

FIG. 2

Overview of the protocols presented in this chapter. Section 2 covers the generation and analysis of inducible knockout cell lines. Section 3 covers the production of knock-in cell lines.

(1) monitoring endogenous protein localization and behavior in living cells
(2) determining the phenotypic consequences that arise from absence of the protein
(3) testing the phenotypes associated with structural or disease mutations
(4) genetically defining the relationships between proteins, for example, hierarchies of protein recruitment to a cellular site.

2 CRISPR/Cas9 GENE DISRUPTION IN HUMAN CELLS FOR ACUTE AND CHRONIC PROTEIN ELIMINATION

2.1 OVERVIEW

The CRISPR/Cas9 system generates DSBs at sites in the genome specified by the sgRNA sequence (Fig. 1). DSBs are predominantly repaired by NHEJ (Mao, Bozzella, Seluanov, & Gorbunova, 2008). If DSBs are introduced into protein-coding exons, errors introduced during NHEJ can result in insertion/deletion mutations (indels) that disrupt the production of functional protein, for example by altering the translational reading frame (Cong et al., 2013; Wang et al., 2013). If a break is repaired without errors, the product can be cleaved again, such that multiple cycles of DSB generation and repair can occur until mutations are introduced in the gene that disrupt complementarity with the sgRNA sequence. In this manner, generation of specific DSBs by CRISPR/Cas9 can result in efficient disruption of protein production from all targeted alleles, resulting in a null phenotype (Koike-Yusa, Li, Tan, Velasco-Herrera Mdel, & Yusa, 2014; Shalem et al., 2014; Wang, Wei, Sabatini, & Lander, 2014).

The ability to generate genetic loss-of-function alleles using CRISPR presents numerous advantages over strategies that target mRNA to disrupt protein production, such as RNAi. In particular, targeting the gene itself can completely abrogate protein production, rather than generating a partial knockdown. Importantly, gene knockouts are also irreversible, allowing for the long-term maintenance of stable knockout cells completely lacking the protein of interest without ongoing intervention from the researcher. However, a subset of genes in the genome are essential for cellular proliferation and/or viability, such that cells bearing knockout mutations in these genes cannot be propagated long-term. To dissect the functions of essential genes, knockout mutations must be generated in a temporally controlled manner to allow for analyses of the acute consequences of protein disruption. Inducible gene knockouts are of particular value for mitosis researchers, whose genes of interest are strongly enriched for genes essential for cellular proliferation.

Here, we will describe the generation of inducible knockout cell lines in which indels in the gene of interest are generated upon addition of doxycycline to the medium (Fig. 3). Cells carry a doxycycline-inducible Cas9 and a constitutively expressed sgRNA targeting a protein-coding exon in the gene of interest. Upon addition of doxycycline to the system, Cas9 is expressed and the Cas9–sgRNA complex cuts the specified DNA sequence. Errors introduced during repair of this break can disrupt the production of functional protein.

2.2 GENERATION OF sgRNA-EXPRESSING PLASMIDS

2.2.1 Overview

In this section, we will describe the process of selecting a targeting sequence and introducing it into a plasmid to express as a fusion with the scaffold. The strategy described here relies on the introduction of indels that disrupt protein production.

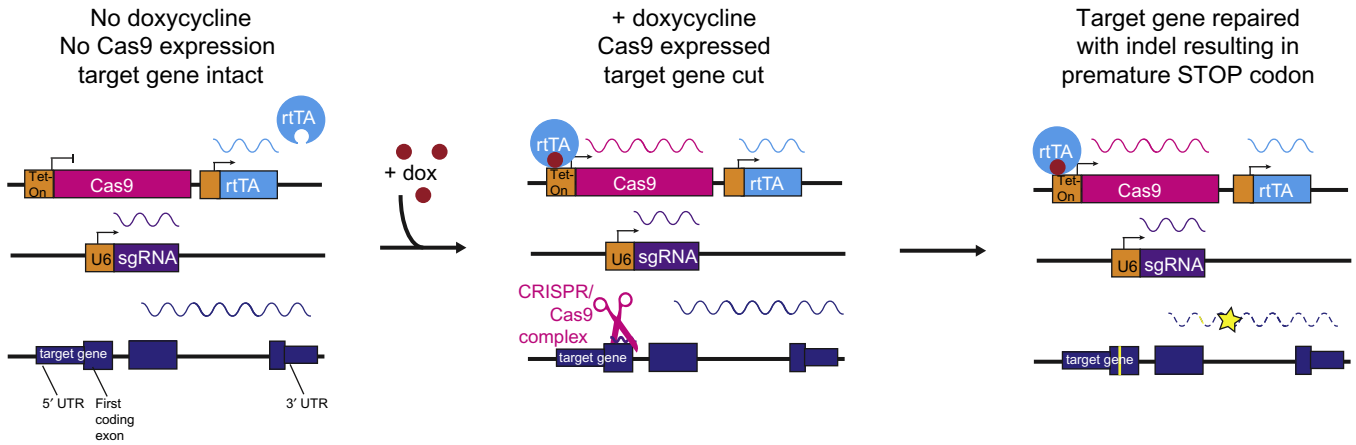

FIG. 3

Schematic of the inducible knockout system. Cells carry Cas9 under control of a TRE3G (Tet-On) promoter, and a reverse tetracycline-controlled transactivator (rtTA). In addition, they express a single guide RNA (sgRNA) from a U6 promoter. Following addition of doxycycline, the rtTA activates transcription of Cas9. Cas9 is directed by the sgRNA to cut the gene of interest in an early coding exon. Error-prone NHEJ introduces indels that disrupt the reading frame (*yellow bars*), resulting in premature stop codons (*yellow star*). *Wavy lines* indicate transcripts.

Therefore, we will design sgRNAs to generate DSBs in protein-coding sequences in the gene of interest. However, it is also possible to generate large defined deletions by using two sgRNAs that flank the sequence to be excised (Han et al., 2014). Large deletions may be particularly valuable to study the functions of noncoding gene products such as long noncoding RNAs.

We have previously generated sgRNA-expressing plasmids for 209 targets of interest to cell division researchers (McKinley & Cheeseman, 2017), which are available from Addgene (https://www.addgene.org/Iain_Cheeseman/). Below, we describe the strategies for generating additional sgRNA plasmids.

2.2.2 Considerations for selection of targeting sequences

(1) *Basic requirements*

The targeting sequence is a sequence of approximately 20 nucleotides that directs Cas9 to cut the complementary sequence in the genome (Fig. 4). The targeting sequence should satisfy three requirements:

A. Feasibility:

Cas9 will only cleave a genomic sequence complementary to the targeting sequence if the genomic sequence is immediately followed by a short nucleotide sequence called the protospacer adjacent motif (PAM) (Fig. 4). Although the PAM sequence varies according to the species from which the Cas9 is derived, we will focus on the most commonly used variant, *Streptococcus pyogenes* Cas9 or spCas9, for which the PAM sequence is NGG where N is any nucleotide.

B. Specificity: the targeting sequence should not bear significant sequence similarity to sites within the genome other than the gene of interest (off-target sites).

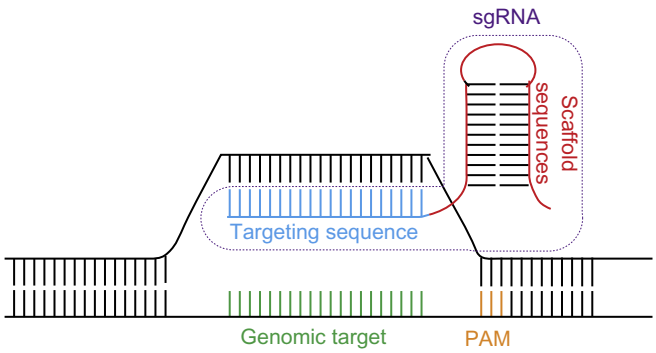

FIG. 4

Schematic of an sgRNA interacting with an endogenous gene. Note that the targeting sequence contains the sequence up to, but not including, the PAM.

Mutations generated at sites other than the gene of interest will confound any analyses of the desired knockout. Therefore, targeting sequences should be selected that have as many mismatches as possible with other sites within the genome. Targeting sequences that exhibit extensive sequence similarity within annotated genes should particularly be avoided due to the increased likelihood that indels generated in these sequences would have phenotypic effects. Also see Consideration 2, on- and off-target effects, below.

Numerous online tools are available to identify targeting sequences. These tools report the possible targeting sequences, their specificity relative to a selected genome and, in some cases, measures of the efficiency with which the sgRNA is expected to cut the desired sequence and/or generate out-of-frame deletions. We have used crispr.mit.edu from the Zhang lab for our analyses, but numerous alternatives are also available, including e-crisp.org (Heigwer, Kerr, & Boutros, 2014).

C. Efficiency: the targeting sequence should maximally disrupt the production of functional protein. There are several ways to maximize protein disruption:

i. For essential genes: use a published sgRNA that produced potent negative selection.
Recent work has employed genome-wide CRISPR knockout screens to identify essential genes in the human genome by negative selection (Hart et al., 2015; Wang et al., 2015). For these analyses, depletion of an sgRNA from the population after a defined period of population doublings indicates a strong fitness cost associated with gene disruption by that sgRNA. For essential genes, the sgRNA exhibiting the strongest negative selection is likely to most potently disrupt the function of the corresponding protein. Although multiple sgRNAs per gene were averaged for these analyses, the depletion of each individual sgRNA can be found in Wang et al.'s table S2, with the corresponding targeting sequence found in table S1. Thus, these data can be mined to select the most potent sgRNA(s) for an essential gene of interest.

ii. Target an early (5′) protein-coding exon.
A general strategy to disrupt protein function is to generate mutations that disrupt the reading frame. The altered reading frame would likely result in a premature stop codon and nonsense-mediated mRNA decay, or, at a minimum, produce sequence that can no longer execute the protein's function. Targeting an early coding exon increases the probability of disrupting protein function.

iii. Target an important protein domain.
Importantly, NHEJ generates a wide variety of different indels. Thus, each allele in each cell in the population can harbor different

mutations of the targeted gene. Insertions or deletions of nucleotides in multiples of three will maintain the reading frame. However, these indels will locally disrupt the amino acid sequence. Therefore, if indels can be targeted to regions in which the precise amino acid sequence is important for protein folding or function (for example, enzymatic domains), both frameshifting and nonframeshifting indels will produce loss-of-function mutants. Thus, sgRNAs targeting enzymatic domains are more efficient than those targeting 5′ exons (Shi et al., 2015).

(2) *On- and off-target effects and number of targeting sequences to select*

Design 2 or more sgRNAs per gene of interest. Targeting sequences that nominally satisfy the requirements described above may nonetheless be suboptimal for analyses, because numerous poorly defined features can affect specificity and efficiency. Comparing multiple knockout cell lines with distinct sgRNAs that satisfy the requirements outlined for Consideration 1 will maximize the likelihood of observing an on-target phenotype and minimize the likelihood of confounding off-target effects, as follows:

A. On-target effects:

Cleavage efficiency varies between sgRNAs with different targeting sequences, based on contextual features that remain incompletely understood. Therefore, multiple sgRNAs for each gene of interest should be compared to define a targeting sequence that robustly generates DSBs. The efficiency of the knockout in each cell line can be compared at the cellular level if the knockout results in a robust phenotypic effect, at the protein level by quantitative immunofluorescence, or at the DNA level by mismatch-detection assays such as T7 endonuclease I or Surveyor. Using a published and validated sgRNA sequence (Consideration 1 part C i, above) will also enrich for efficient targeting sequences.

B. Off-target effects:

If multiple knockout cell lines targeting the same gene with distinct sgRNA sequences generate the same phenotype, it is likely that the phenotype is due to disruption of the gene, and not disruption of an unintended target. Therefore, multiple sgRNAs can provide a valuable control for off-target effects. As with RNAi studies, the gold standard for confirmation of an on-target effect is the ability to fully rescue the knockout phenotype by expressing a CRISPR-resistant copy of the gene of interest. The CRISPR-resistant gene should contain synonymous mutations in the PAM sequence and/or the sequence complementary to the targeting sequence, such that it generates functional protein but is no longer a substrate for the Cas9–sgRNA complex (see Section 2.6).

(3) *Isoform specificity*

It is important to note that the strategy described here will only affect proteins produced from mRNAs that retain the mutated exon. Isoforms that skip the

mutated exon, or are produced from start sites downstream of the mutated exon, will remain unaffected and may provide relevant functions. Targeting alternative exons can be a valuable tool to dissect the function of specific isoforms. When aiming to disrupt all isoforms, it is important to target a constitutive exon. We use the Ensembl genome browser to select exons annotated as constitutive, but existing annotations may contain errors or may not fully reflect tissue-specificity of isoform expression (Kern, Nicholls, Page, & Cheeseman, 2016). We have also encountered unannotated downstream start sites that produce truncated protein (McKinley & Cheeseman, 2017). Although it is challenging to detect potential residual isoforms, where possible, immunofluorescence with an antibody against a C-terminal epitope may detect truncations generated by alternative start sites (McKinley & Cheeseman, 2017).

2.2.3 Protocol to clone targeting sequences into lentiviral vectors

We introduce the sgRNAs into the cell lines of interest by lentiviral transduction using two plasmids, pLenti-sgRNA (available from Addgene, #71409) (McKinley et al., 2015; Park et al., 2017), which also confers puromycin resistance, and/or its blasticidin-resistant counterpart, pKM808 (McKinley & Cheeseman, 2017) (deposited at Addgene). These plasmids express the sgRNA from the U6 promoter. They are derived from plasmids generated by Feng Zhang's laboratory (Sanjana, Shalem, & Zhang, 2014; Shalem et al., 2014) and allow for introduction of targeting sequences simply by annealing oligos and ligating them into a vector with complementary overhangs. A modification of the Zhang laboratory's protocol for this cloning is as follows:

(1) Design oligos:

a. Identify two or more targeting sequences per gene, as described in Section 2.2.2. Note that the targeting sequence does not include the PAM sequence found in the target (Fig. 4). For example, the following sequence is present in the coding region of Aurora kinase A (*AURKA*)

ATTCTGGAATATGCACCACTtgg

where tgg is the PAM

The corresponding targeting sequence is:

ATTCTGGAATATGCACCACT

For each targeting sequence, you will order two oligos (Oligo 1 and Oligo 2).

b. To design Oligo 1, append CACC to the 5′ end of the targeting sequence. If the targeting sequence does not already begin with a G, append a G as well to facilitate transcription from the U6 promoter. Oligo 1 for *AURKA* is

CACC**G**ATTCTGGAATATGCACCACT

c. Oligo 2 is the reverse complement of the targeting sequence, with the prefix AAAC. If you added a G to your target sequence, also add the complementary C as a suffix. Oligo 2 for *AURKA* is

AAAC**C**AGTGGTGCATATTCCAGAAT

(2) Anneal and phosphorylate oligos
1 μL Oligo 1 (100 μM stock)
1 μL Oligo 2 (100 μM stock)
1 μL T4 DNA ligase buffer (NEB)
6.5 μL ddH_2O
0.5 μL T4 PNK (NEB M0201S)
Incubate at 37°C for 30 min, then anneal in a thermocycler at 95°C for 5 min, then ramp down to 25°C at 5°C/min or slower.

(3) Dilute the resulting oligo reaction 1:200 in ddH_2O

(4) Digest and dephosphorylate the pLenti-sgRNA/pKM808 plasmid with *Bsm*BI, also marketed as Esp3I (Fisher Scientific FERFD0454) for 30 min at 37°C
5 μg of DNA
6 μL of FastDigest Buffer
3 μL FastAP
3 μL FastDigest Esp3I
ddH_2O to 60 μL

(5) Gel purify the digested plasmid. The stuffer fragment of ~2 kb can be discarded, and the larger fragment retained

(6) Ligate the diluted oligos and cut vector at room temperature for 10 min:
50 ng digested plasmid from step 5
1 μL diluted oligos from step 3
5 μL Quick Ligase Buffer (NEB)
1 μL Quick Ligase (NEB M2200S)
ddH_2O to 11 μL

(7) Transform into recombination-deficient bacteria. We use homemade Mach1 cells, propagated from ThermoFisher C862003

(8) Pick colonies into liquid culture, miniprep and sequence with a primer in the U6 promoter, e.g., LKO: GACTATCATATGCTTACCGT

To generate large numbers of sgRNA-expressing plasmids in parallel, these reactions can be performed in 96-well format.

2.3 GENERATION OF INDUCIBLE KNOCKOUT CELL LINES

2.3.1 Overview

We generate inducible knockout cell lines in two steps. First, we generate a clonal cell line in which the doxycycline-inducible Cas9 is stably integrated by transposition and confirm robust expression of the Cas9 upon induction. Second, we derive knockouts of specific genes from this clonal Cas9 cell line by viral transduction using the sgRNA-expressing plasmids generated in Section 2.2. It is possible to generate clonal cell lines carrying a given sgRNA at this stage. However, following induction and Cas9 cutting, NHEJ will generate a different mutation in each allele in each cell, such that the analyzed cells will not be clones.

Tight control of Cas9 induction is critical for the generation and propagation of these cell lines, as even low-level and/or transient expression of Cas9 will generate irreversible mutations resulting in a severe fitness defect if the gene of interest is essential. Thus, spurious Cas9 activation can rapidly select against cells competent to generate knockouts. In the system we describe here, Cas9 is tightly controlled by the TRE3G system. However, tetracycline in fetal bovine serum (FBS) can activate tetracycline/doxycycline-inducible promoters. Therefore, as a precaution, we culture and freeze all cell lines in FBS confirmed to be tetracycline free (Gemini #100–800 or Fisher #SH3007003T). In general, we maintain all cell lines in DMEM +10% tetracycline-free FBS, penicillin/streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2.

2.3.2 Considerations for choice of parental cell line

Cell lines expressing inducible Cas9 of the following backgrounds are available from the Cheeseman laboratory: HeLa, hTERT-RPE1, DLD-1, and U2OS. A protocol for the generation of cell lines in additional backgrounds is provided in Section 2.3.3. The cell line in which knockouts will be generated should be chosen according to the researcher's specific goals. When selecting a cell line, it is important to consider four characteristics:

(1) *Ploidy*

Our strategy relies on the introduction of disruptive indels at all alleles in a given cell to completely abrogate functional protein production. The more copies of the gene of interest exist within a cell, the more likely it is that at least one will repair with an indel that is no longer a substrate for the Cas9–sgRNA complex (i.e., is terminally repaired), but permissive for the production of functional protein (e.g., retains the reading frame). Thus, starting with a cell line with a minimal number of copies of the gene of interest increases the frequency at which nulls can be achieved. At the furthest end of the spectrum, haploid human cell lines require only a single lesion to generate a null (Elling & Penninger, 2014). However, on the other end of the spectrum, we have generated knockouts with high efficiency in highly aneuploid HeLa cell lines (McKinley & Cheeseman, 2017; McKinley et al., 2015). Selecting guide sequences that target an important protein domain increases the probability that all lesions will disrupt protein production, as described in Section 2.2.2.

(2) *Division rate*

Upon introduction of indels, functional protein will no longer be generated, but protein that has already been produced will remain in the cell and continue to perform functions. To observe the null phenotype, this existing protein must be depleted. The rate at which this protein is depleted after the generation of indels depends both on the intrinsic stability of the protein of interest, and the rate at which it is depleted through cell division (for example, see McKinley et al., 2015, fig. S2). For very

stable proteins, depletion may only be feasible over the course of many cell divisions that dilute the existing protein. Thus, achieving robust protein depletion requires a cell line that proliferates fast enough to achieve this dilution in a reasonable time frame. Selecting appropriately proliferative cell lines is less of a challenge for mitosis researchers, who are inclined by the nature of their research to focus on cell lines that proliferate efficiently. Nonetheless, the contribution of cycling rate to depletion efficiency remains an important factor to bear in mind (also see Section 2.4).

(3) *p53 status*

The tumor suppressor p53 induces exit from the cell cycle in response to diverse cell cycle defects including DNA damage and failures of cytokinesis and centriole duplication (Bazzi & Anderson, 2014; Fong et al., 2016; Ganem et al., 2014; Lambrus et al., 2016; Meitinger et al., 2016). Thus, the phenotypes associated with knockouts of diverse cell cycle genes differ between cell lines in which p53 is functional, and cell lines in which p53 is mutant or suppressed (McKinley & Cheeseman, 2017).

(4) *Cell line handling (specific to the strategies described here)*

The strategy described in Section 2.3.3 is designed for adherent cell lines and involves introduction of the inducible Cas9 by transfection. However, these protocols can be modified for cells in suspension, and Cas9 can be introduced virally with modifications for transfection-resistant cell lines. In addition, we have found it valuable to generate clonal cell lines harboring Cas9 to select for strong expression. If polyclonal cell lines are used for cell lines that are refractory to single cell sorting, variability in Cas9 expression may cause additional heterogeneity in the knockout cells.

2.3.3 Protocol for introduction of inducible Cas9 into parental cell lines

To introduce the inducible Cas9, we cotransfect a plasmid containing the inducible Cas9, the reverse tetracycline-controlled transactivator (rtTA) and a neomycin-resistance marker in a transposon (plasmid HP138-neo) with a transposase (plasmid HP137).

Day 0: Plate cells of interest in six-well plate to achieve ~80% confluency the following day.
Day 1: Cotransfect 2 μg HP138-neo with 1 μg HP137 in six-well plate (we use Lipofectamine 2000; Life Tech 11668019).
Day 2: Passage to 15-cm plate.
Day 3: Select with G418/Geneticin (Life Technologies 11811023) at a concentration determined by the literature or determined by testing a range of drug concentrations for lethality.

After selection is complete, isolate single cells and expand clones. Screen the clones for robust Cas9 induction by Western blot after inducing cells with 1 μg/mL doxycycline hyclate (Sigma) for 48 h. We blot with the 7A9-3A3 antibody (Abcam ab191468).

2.3.4 Protocol for the introduction of sgRNA into cell lines

We integrate sgRNA-expressing DNA into the Cas9-expressing cell line by lentiviral transduction (Wang et al., 2015). This process occurs in two steps: first, the sgRNA-expressing plasmid is cotransfected with plasmids encoding components necessary to produce lentiviral particles into a packaging cell line, usually 293T cells. These cells then shed virus into the medium, which is harvested and used to infect the Cas9-expressing cell line (Fig. 5). The sgRNA-expressing plasmids carry drug resistance markers, allowing for selection of transduced cells with antibiotics.

Appropriate institutional biosafety precautions for lentiviral work should be followed for all steps involving the production or handling of virus.

1) Production of virus

Day 0: Plate 750,000 293T cells per well in complete medium in a six-well plate, one well per sgRNA.

Day 1: Cotransfect sgRNA plasmid and lentiviral helper plasmids. For each sgRNA:

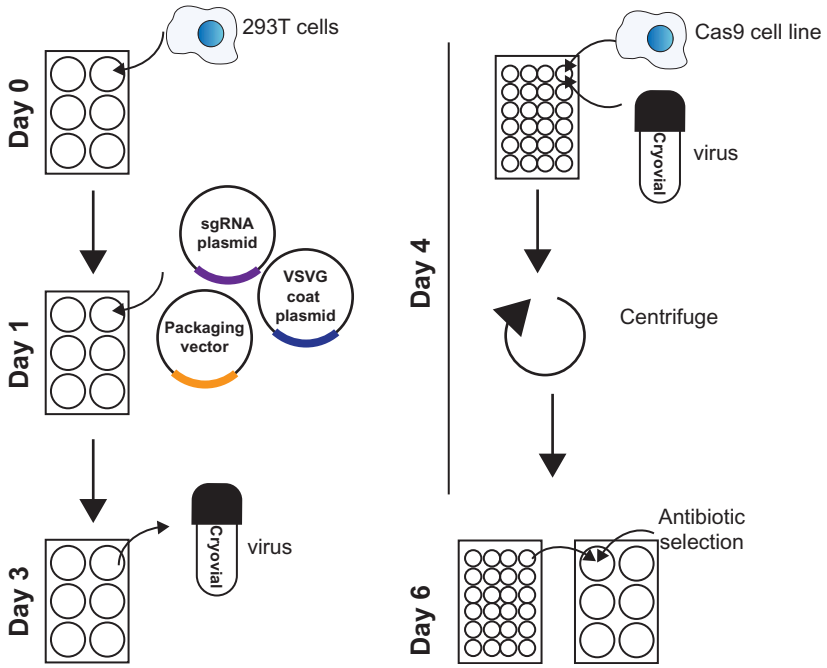

FIG. 5

Schematic of the protocol for the generation of sgRNA-expressing lentivirus (*left panels*) and its transduction into inducible Cas9-expressing cell lines (*right panels*).

1. To 50 µL of Serum-Free Medium (DMEM + Pen/Strep + Glutamine, no FBS), Add
   1.2 µg of your pLenti-sgRNA plasmid
   1 µg pCMV-dR8.2 dVPR (Addgene 8455)
   0.3 µg pCMV-VSVG (Addgene 8454)
   6 µL Xtremegene-9 (Roche 06365787001)
2. Finger tap to mix and incubate 15 min.
3. Pipet dropwise onto 293T cells.

Day 2: Gently remove medium from the plate and replace with 1.5 mL fresh complete medium.

Day 3: Gently harvest the medium from the plate into cryovials and freeze at −80°C overnight. This will kill any 293T cells inadvertently harvested, as this medium lacks cryoprotectant. It is also possible to remove contaminating 293T cells by filtering through a 0.45-µm filter. This virus can be stored long-term at −80°C and freeze-thawed three times.

2) Transduction of Cas9 cell line by spinoculation

Day 1: Trypsinize inducible Cas9 cells, resuspend in medium, and collect in a falcon tube. Dilute the cell suspension to ∼833,000 cells/mL.

1. Add 10 µg/mL polybrene (Millipore TR-1003-G) to the inducible Cas9 cell suspension
2. Add 600 µL of this suspension to a well of a 24-well plate (one well per sgRNA).
3. Add 400 µL of virus harvested in step 1.
4. Spin the plate at 1200 × *g* in a swinging bucket centrifuge for 45 min at 37°C.

Day 2: Remove and dispose of medium containing virus and replace with 1 mL of fresh complete medium.

Day 3: Passage cells to a 6-well plate (1 well of the 24-well plate transfers to 1 well of the 6-well plate). To select for infected cells, add complete medium with puromycin (for pLenti-sgRNA plasmids) or blasticidin (for pKM808 plasmids). In parallel, plate a well of uninfected cells into the same antibiotic medium, to validate the efficiency and rate of drug selection.

The concentrations of these drugs that we used for HeLa, U2OS, hTERT-Rpe1, and DLD1 cells are outlined in Table 1. For other cell lines, the appropriate concentrations can often be determined from the literature, or by testing a range of the drug for lethality.

Day 4+: Monitor cells and passage to larger plates as they reach confluency. We recommend maintaining these cell lines in the appropriate selection drug for 4 days–1 week, or until uninfected control cells have been completely killed. Subsequently we maintain these cell lines in the absence of drug without noticeable depletion of sgRNA-harboring cells.

Table 1 Recommended Concentrations of Antibiotics for Cas9-Expressing Cell Lines

Cell Line	Puromycin Concentration	Blasticidin Concentration
HeLa + iCas9 (cTT20.11 from Cheeseman laboratory)	0.5 μg/mL	2 μg/mL
hTERT-Rpe1 + iCas9 (cTT33.1 from Cheeseman laboratory)	5 μg/mL	10 μg/mL
DLD1-osTir1 + iCas9 (cTT25.8 from Cheeseman laboratory)	N/A, cell line already puromycin resistant	N/A, cell line already puromycin resistant
U2OS + iCas9 (cKM257.1 from Cheeseman laboratory)	2 μg/mL	10 μg/mL

2.3.5 Generation of multiple knockouts

It is often desirable to eliminate multiple targets simultaneously in one cell to perform epistasis analyses, including the disruption of redundant pathways. Introducing sgRNAs targeting different genes allows for the efficient disruption of multiple targets. One strategy for this is to introduce each sgRNA in a separate virus. For this, one sgRNA can be cloned into pLenti-sgRNA and another sgRNA into pKM808 and the cell line selected with both blasticidin and puromycin (see McKinley & Cheeseman, 2017). Alternatively, the researcher can array multiple copies of the sequence containing the U6 promoter, targeting sequence and chimeric scaffold on a single construct, and introduce the arrayed sequences through viral transduction or transposition.

2.4 CONSIDERATIONS FOR ANALYSIS OF THE INDUCIBLE KNOCKOUTS

(1) *Knockout frequency* The knockout process is initiated in these cell lines by the addition of 1 μg/mL doxycycline to the medium to induce the expression of Cas9. Following induction of the knockout, each cell will harbor different lesions in the target gene. In some cells, at least one allele will repair the break in a way that can no longer be cut by Cas9 but retains protein function. Thus, only a subset of cells generated in this manner will exhibit the null phenotype. The cells in the population will give rise to progeny that share their specific collection of modified alleles, such that, for nonmotile cell types, such as the HeLa cells used to generate cTT20.11, contiguous clones of phenotypically wild-type or null cells can be observed by immunofluorescence (Fig. 6).

As a result of this cell-to-cell variation, inducible knockouts generated in this manner are not ideal tools for population-level analyses, which will average the null and wild-type states. In contrast, knockouts generated in this manner are

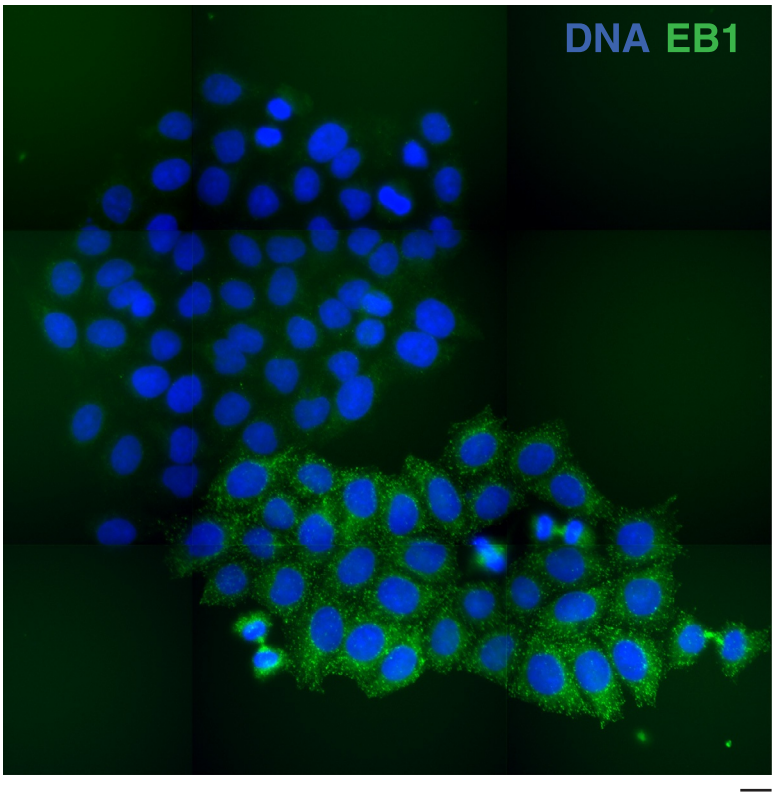

FIG. 6

Immunofluorescence image of cells stained for DNA (*blue*) and end-binding protein 1, EB1 (*green*). These cells have been induced to knock out the *MAPRE1* gene, which encodes EB1. In the *top left corner* is a clone of cells in which EB1 expression is not detectable (apparently null). In the *bottom right corner* is a clone of cells in which EB1 expression persists despite induction of the knockout. This can arise when the founder cell of the clone repairs at least one allele in a manner that maintains the production of functional protein. The image was assembled by stitching together multiple adjacent fields of view. Scale bar, 15 μm.

Image reproduced with permission from McKinley, K.L., & Cheeseman, I.M. (2017). Large-scale analysis of CRISPR/Cas9 cell-cycle knockouts reveals the diversity of p53-dependent responses to cell-cycle defects. Developmental Cell 40, *405-420.e402.*

excellent tools for single cell analyses, such as immunofluorescence studies, as phenotypically wild-type cells provide a robust internal control.

(2) *Length of knockout induction and the use of tools for inducible protein degradation or mistargeting*

The time it takes for potential phenotypes to appear after knockout induction will depend on the stability of the protein (see Section 2.3.2). In our cell

cycle gene knockout study, we have often observed strong phenotypes for most targets after 3–4 days of induction of cell division knockouts in HeLa cells and 5–6 days of induction in hTERT-Rpe1 cells. It is important to note that, over this window, cells will transit through partially depleted states (McKinley et al., 2015), and thus that the phenotype observed at the endpoint may represent a combination of the null phenotype as well as errors accumulated over the course of the depletion.

These limitations arise due to the strategy of directly targeting the DNA, such that protein produced before lesions are generated persists in the system until it is depleted through a combination of its intrinsic stability and dilution through cell division. To circumvent these problems, existing protein function can be disrupted directly by using tags to either (A) target the protein for degradation or (B) target the protein to a cellular compartment where it can no longer execute its function.

A. The protein can be inducibly targeted for degradation, for example by using an auxin-inducible degron (AID) (Holland, Fachinetti, Han, & Cleveland, 2012; Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009). The AID strategy is discussed in detail by Holland and colleagues (see Chapter "Applying the auxin-inducible degradation system for rapid protein depletion in mammalian cells" by Lambrus et al.). In brief, the protein of interest is fused to an AID derived from plants and expressed in cells that also express a plant F-box subunit, Tir1. Addition of derivatives of the plant hormone, auxin, targets the protein for degradation by SCF complexes containing Tir1, resulting in rapid protein elimination, in some cases in less than an hour (Guo et al., 2017; Hoffmann et al., 2016).

B. Alternatively, protein function may be disrupted by mistargeting the protein, known as a knocksideways strategy (Robinson, Sahlender, & Foster, 2010). Briefly, one component of an inducible dimerization system, such as FKBP, is appended to the protein of interest. The complementary component, such as FRB, is fused to a protein that resides in a cellular compartment that is distinct from the physiological localization of the protein of interest. For example, a kinetochore protein can be tagged with FKBP and a mitochondrial protein tagged with FRB. Upon addition of rapamycin, the kinetochore protein will be sequestered at the mitochondria.

For either of these systems to generate a null phenotype, all copies of the protein of interest in the cell must be fused to the tag so that it can be degraded or mistargeted. To achieve this, either all alleles of the endogenous gene must be modified to append the tag (Lambrus et al., 2015; McKinley et al., 2015) (see Section 3), or endogenous protein must be eliminated and replaced with a transgene expressing the protein fused to the tag. For the latter strategy, robust elimination of the endogenous protein can be achieved with the inducible knockout system in combination with a CRISPR-resistant transgene fused to the tag, as described in Section 2.6. The DLD-1-osTIR1 cell line, cTT25.8

(Table 1) (McKinley & Cheeseman, 2017), carries both the plant F-box subunit, Tir1, and the inducible Cas9 and is ideal for such analyses using the AID system. Ultimately, we recommend that the inducible knockouts and degron or knocksideways systems be employed in combination as they have different advantages and limitations. The inducible knockout system can be scaled efficiently to rapidly generate cell lines to analyze many different targets but lacks the high temporal resolution of the degron and knocksideways systems. In contrast, degron or knocksideways-tagged cell lines are more laborious to generate, but are valuable to refine the null phenotype and to determine the consequences of protein elimination at specific points in the cell cycle.

2.5 GENERATION OF STABLE KNOCKOUTS

Inducible knockout cell lines are ideal tools for acute gene disruption to facilitate the analysis of essential genes. However, if no phenotypic abnormalities are observed following induction of the knockout and the sgRNAs used have been validated to abolish protein production, it may be possible to maintain null cell lines. In this case, following knockout induction, single cells may be isolated and expanded to generate clonal cell lines in which all cells harbor the same lesions in their alleles. Stable knockouts can also be generated by transient transfection of a plasmid constitutively expressing both Cas9 and sgRNA such as pX330, described in Section 3.3. The precise mutations can be defined by PCR amplification of the targeted region, TOPO cloning, and Sanger sequencing.

2.6 RESTORING AND MODIFYING PROTEIN FUNCTION WITH CRISPR-RESISTANT TRANSGENES

CRISPR/Cas9 genome engineering offers several powerful strategies for structure–function analyses and the analyses of disease-associated mutations. Defined mutations can be directly introduced into the endogenous gene of interest using CRISPR/Cas9 genome editing. However, this strategy does not allow for analysis of mutant proteins that cannot support viability, as homozygous mutant cells will not be recovered. To analyze the functions of nonviable mutants, CRISPR-resistant transgenes carrying a mutation of interest can be introduced into the relevant inducible knockout. In this way, the researcher can analyze the acute phenotypes associated with the mutant in the absence of the endogenous protein. CRISPR-resistant transgenes can also be used to perform rescue experiments as controls for off-target effects as described in Section 2.2.2, or to facilitate rapid protein degradation or mistargeting as described in Section 2.4.

(1) Generation of a CRISPR-resistant wild-type transgene

The cDNA encoding the protein of interest can be isolated by PCR from bulk cDNA, purchased (for example, from the Mammalian Gene Collection,

MGC, available from Dharmacon) or synthesized. Note that the cDNA will harbor a sequence complementary to the targeting sequence of the sgRNA. Left intact, this sequence will allow the cDNA to be cut by the Cas9–sgRNA complex in the same manner as the endogenous sequence. However, since Cas9 critically relies on the PAM sequence, mutating the PAM sequence in the cDNA will render it resistant to Cas9 cutting. Thus, the most straightforward strategy to render the transgene resistant to cutting is to make synonymous mutations in the PAM sequence, such that Cas9 cutting will be disrupted, but the protein sequence remains intact. If it is not possible to mutate the PAM (e.g., there are no alternative codons), synonymous mutations can be introduced to disrupt complementarity with the targeting sequence.

We recommend cloning the CRISPR-resistant transgene into a vector with appropriate tags (e.g., fluorescent proteins to monitor protein localization) and a drug selection cassette that is not already represented in the relevant knockout cell line.

(2) Introduction of the CRISPR-resistant wild-type transgene into the inducible knockout cell line

The relevant transgene can be stably integrated into the genome of the relevant inducible knockout cell line by viral transduction (for example, see Shah et al., 2004) or transposition. Following integration, we recommend isolating clonal cell lines to ensure homogenous expression of the transgene within the population. Multiple clonal cell lines, each with a different expression level, can be analyzed to determine the appropriate expression level.

(3) Validation of transgene function

Following the generation of cell lines harboring the wild-type transgene, the knockout can be induced with doxycycline and phenotypes analyzed by single-cell analyses at the appropriate time point as described in Section 2.4.

Expression of this CRISPR-resistant but otherwise wild-type cDNA should be sufficient to restore the wild-type phenotype to knockout cells. Failure to rescue the knockout may indicate that the phenotype is off-target (see Section 2.2.2), may be due to interference from any appended tags, or may indicate that the knockout disrupts isoforms that are not represented by the cDNA. Distinguishing these possibilities with the wild-type transgene is a critical precursor to the analysis of mutant cDNAs.

(4) Generation and analysis of a CRISPR-resistant mutant transgene

Once a functional CRISPR-resistant cDNA with relevant tags has been defined, mutations of interest for the particular study can be introduced into the CRISPR-resistant cDNA. The mutant transgene (with equivalent tags) can then be introduced into knockout cells as in step 2. The phenotype can be compared between knockout cells without a transgene, with the wild-type transgene, and with the mutant transgene.

3 INSERTION OF TAGS INTO ENDOGENOUS LOCI TO REPORT ON AND PERTURB GENE FUNCTION

3.1 OVERVIEW

Cas9 cleavage at a specific site can be used to facilitate integration of sequences nearby (Yang et al., 2013). In this section, we will describe strategies to introduce sequences encoding tags to report on and modify protein function (Fig. 7). For this, sgRNAs are designed to generate DSBs as close as possible to the desired insertion site, within ~100 bp. Plasmids encoding Cas9 and the sgRNA are cotransfected into the cells of interest with a plasmid harboring a repair template. The repair template contains the tag sequence to be introduced, flanked on both sides by sequences homologous to the targeted region of the gene. Targeted cells are selected with antibiotics and/or fluorescence-activated cell sorting (FACS).

3.2 CONSIDERATIONS FOR ENDOGENOUS TAGGING

There are two primary considerations for generating cell lines in which endogenous genes are tagged:

(1) *Position of the tag: at the N- or C-terminus of the protein*

The position of the tag may affect protein function in ways that are challenging to predict; thus, an inert position of the tag must be determined empirically. From a technical standpoint, C-terminal tags are more efficient to introduce than N-terminal tags, as an antibiotic selection marker driven by a synthetic promoter can be introduced downstream to allow enrichment of cells that have recombined in the template DNA. In contrast, selection markers are discouraged for N-terminal tagging due to disruption of the endogenous promoter. However, cells that have been tagged at the N-terminus can be enriched by FACS if the tag encodes a fluorescent protein.

(2) *Copies of the tag: whether the cells are homozygous or heterozygous for the tagged allele*

Depending on the application, it may be sufficient for the tagged protein to be expressed from only one allele. However, it may be necessary for all protein to contain the tag (for example, if the tag is to be used for loss-of-function studies, such as with the AID as described in Section 2.4). In this case, we recommend using a diploid or near-diploid cell line (such as hTERT-Rpe1 or DLD-1), instead of an aneuploid cell line, so that only two alleles need to be modified. In this case, there are two potential strategies to ensure that all protein in the cell contains the tag:

(i) Selecting clones in which both alleles are repaired with the template (homozygotes)

Homozygosity can be achieved following introduction of a single tag by simply screening through clones by PCR to identify homozygotes. Alternatively, homozygotes can be enriched by introducing two

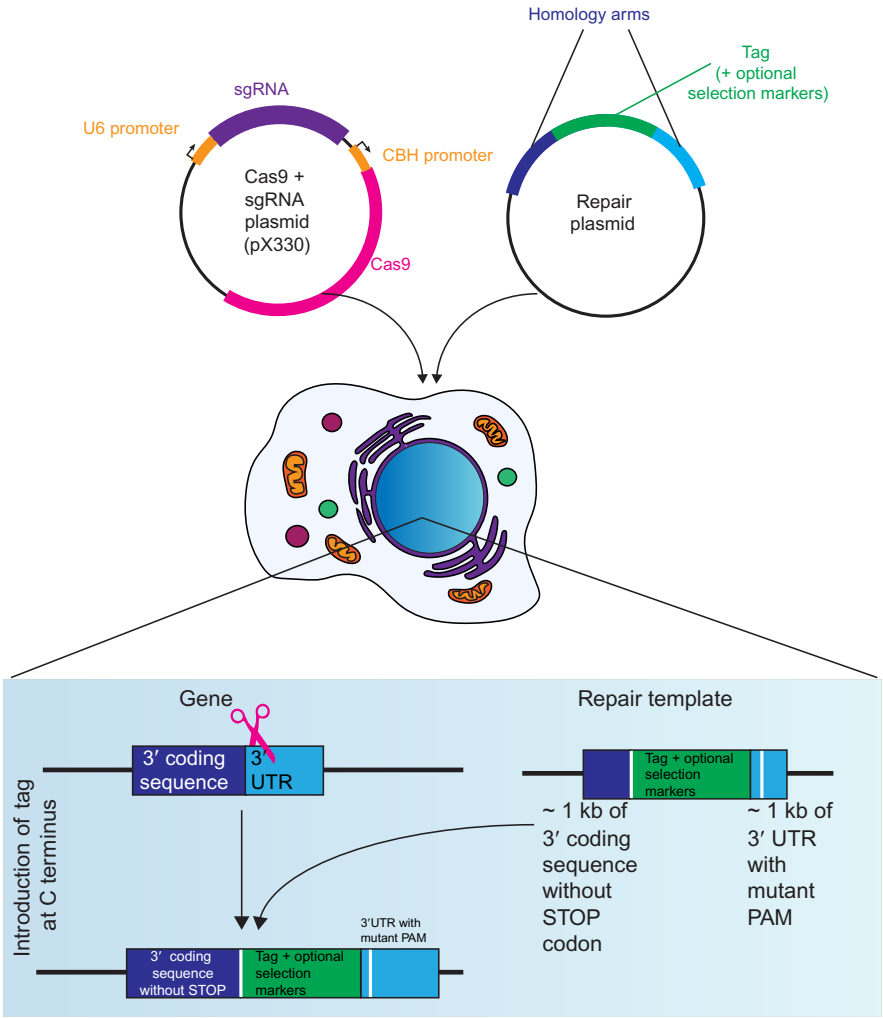

FIG. 7

Schematic of the strategy used to modify a gene of interest to introduce a sequence tag at the C-terminus. A plasmid expressing Cas9 and an sgRNA targeting the 3′ UTR is cotransfected with a plasmid harboring a repair template.

distinct repair templates, encoding either different antibiotic resistance cassettes (if using a C-terminal tag) or different fluorophores and selecting for double-positive cells.

(ii) Repairing one allele with the template and knocking out the other allele (compound heterozygotes)

Compound heterozygotes can be achieved for N-terminal tags by targeting Cas9 to generate a DSB in the 5′ coding exon. Thus, one allele can be repaired with the template, and the other allele may acquire an indel that renders it null, as described in Section 2.

3.3 GENERATION OF sgRNA-EXPRESSING PLASMIDS

3.3.1 Considerations for selection of targeting sequences

The sgRNA should target within 100 bp of the insertion site. It can be in either a coding sequence, or in an untranslated region. For an N-terminal tag, either the 5′ UTR can be targeted, or the first coding exon can be targeted if it is desirable to knock out the other allele as described in Section 3.2. For a C-terminal tag, the 3′ UTR should be targeted. The targeting sequence should be selected to be (1) feasible (i.e., directly upstream of a PAM sequence) and (2) specific (i.e., with maximal number of mismatches to other sequences) as outlined in Section 2.2.2.

3.3.2 Cloning targeting sequences into transient vectors

To introduce sequences at specified sites, the Cas9, sgRNA, and repair template only need to be temporarily present within the cells to be targeted. Therefore, for this application we will introduce them by transient transfection. We will transfect two plasmids (Fig. 7): one that constitutively expresses both Cas9 and the sgRNA, and one that carries the repair sequence.

For transient expression of Cas9 and the sgRNA, we use the plasmid pX330 from Feng Zhang's laboratory (Addgene #42230). The cloning for pX330 uses the same overhangs as the pLenti-sgRNA/pKM808 plasmids used in Section 2.2.3. Therefore, the protocol for cloning is the same as in Section 2.2.3 with one important exception: in this case, the plasmid is cut with *Bbs*I, also marketed as Fast Digest *Bpi*I (Thermo Fisher 10569110). We recommend use of the Fast Digest *Bpi*I, as traditional formulations of *Bbs*I are poorly stable at −20°C.

As described in Section 2.2.2, the cleavage efficiency of sgRNAs is variable. Therefore, we recommend designing two sgRNAs per target. If tag insertion fails, cleavage efficiency can be validated using T7 endonuclease I or Surveyor assays.

3.4 GENERATION OF THE REPAIR TEMPLATE

The repair template is comprised of the sequence to be inserted, flanked on both sides by sequences with homology to the region to be targeted (here termed homology arms) (Figs. 7 and 8). We introduce this sequence on a plasmid, although linear DNA can also be used.

We will focus on the introduction of C-terminal tags (as in McKinley & Cheeseman, 2014; McKinley et al., 2015). The plasmid backbone for introduction of a fluorescent tag is pKM471 (deposited at Addgene) (Fig. 8), derived from pL452 (Liu, Jenkins, & Copeland, 2003). This plasmid introduces a C-terminal YPet

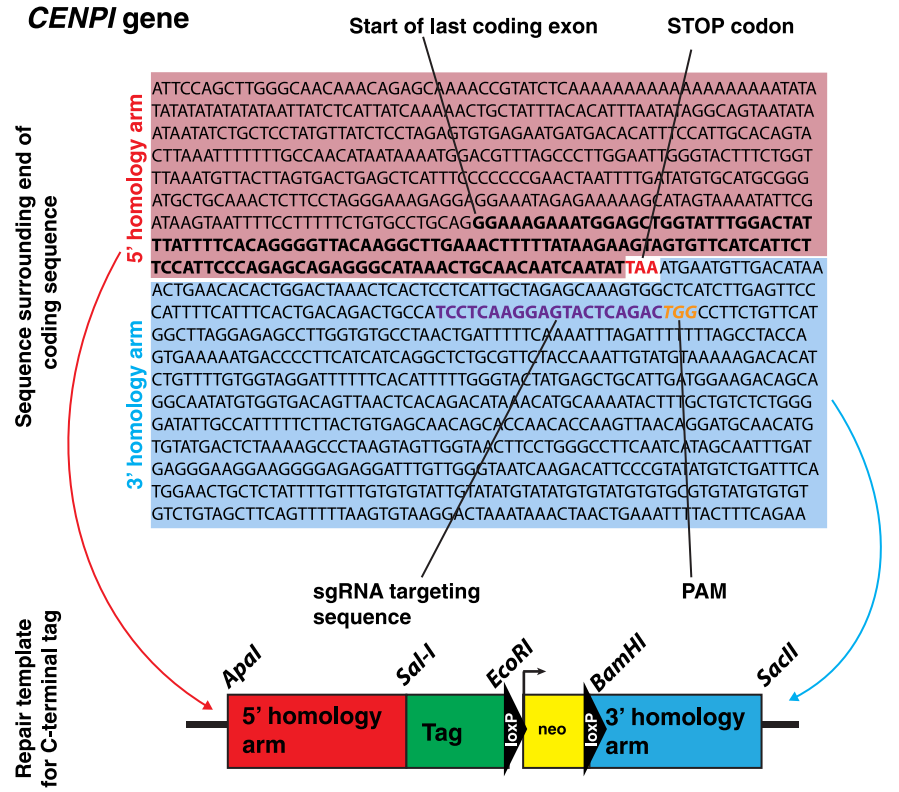

FIG. 8

Top: annotation of the 3′ coding sequence of the CENPI gene to demonstrate the corresponding the 5′ and 3′ homology arms used to introduce a C-terminal tag. *Bottom*: schematic of the repair template used for introducing tags to the C-terminus.

(YFP-variant) tag, followed by a neomycin resistance cassette flanked by loxP sites. Thus, cells repaired with the template can be selected using G418/Geneticin, and the resistance cassette can subsequently be excised if desired by transient transfection with a Cre recombinase. This vector can be modified for the introduction of N-terminal tags, different tags, and different resistance cassettes. For the introduction of a C-terminal auxin-inducible degron and eGFP (AID-eGFP), the equivalent plasmid is pKM502 (deposited at Addgene).

3.4.1 Design of homology arms

We use homology arms of ~500 bp–1 kb on each side of the tag, although other options with shorter arms are possible. The 5′ homology arm consists of the gene sequence 5′ of the STOP codon. The 3′ homology arm consists of the gene sequence 3′ of the STOP codon (Fig. 8).

Since the sgRNA targeting site was designed to cut in the 3′ UTR, it will be represented in the 3′ homology arm. Therefore, after the template is integrated into the genome, it will continue to be vulnerable to cutting by Cas9, which may generate indels in this region. To avoid this, the corresponding PAM site in the 3′ homology arm can be mutated, such that the repaired gene is resistant to additional Cas9 cutting.

3.4.2 Amplification of homology arms

The homology arms can either be commercially synthesized or amplified by PCR from genomic DNA. For commercial synthesis, the mutated PAM can be incorporated into the design. If amplifying from genomic DNA, the PAM mutation can be introduced subsequently by site-directed mutagenesis. For amplification of the homology arms by PCR from genomic DNA, we use either iProof (BioRad 1725301) or Bio-X-Act Short DNA polymerase (Bioline BIO-21065) according to the recipes below:

iProof

200 ng genomic DNA
10 μL 5 × GC Buffer or HF Buffer
2.5 μL DMSO
1 μL 10 mM dNTPs
1.5 μL each oligo (20 μM stock)
0.5 μL iProof
H_2O to 50 μL

Cycling conditions are according to the manufacturer's instructions, using 30 s/kb extension time.

Bio-X-Act

200 ng genomic DNA
5 μL 10 × Opti Buffer
3 μL DMSO
1.5 μL $MgCl_2$ (50 mM stock from manufacturer)
1.5 μL 10 mM dNTPs
1.5 μL each oligo (20 μM stock)
1 μL Bio-X-Act Short DNA polymerase
(optional: 10 μL HiSpec additive)
H_2O to 50 μL.

Cycling conditions are according to the manufacturer's instructions, using 1 min/kb extension time.

3.4.3 Cloning of homology arms

Homology arms can be introduced into the repair template plasmid by restriction enzyme or Gibson cloning. The relevant restriction enzyme sites are shown in Fig. 8.

3.5 GENERATION OF KNOCK-IN CELL LINES

Knock-in cell lines are generated by cotransfection of the pX330 plasmid generated in Section 3.3 and the repair template plasmid generated in Section 3.4 as follows:

Day 0: Plate cell line of interest in one well of a six-well plate for ~80% confluency the next day.
Day 1: Transfect 1–2.5 μg each of the pX330 plasmid and repair template plasmid. We use Lipofectamine 2000 (Thermo Fisher 11668027) according to the manufacturer's instructions.
Day 2: Passage the transfected well to a 15-cm plate.
Day 3: Select with G418/Geneticin (Life Technologies 11811023) at a concentration determined by the literature or determined by testing a range of drug concentrations for lethality.

Once selection is complete, clonal cell lines can be isolated and correct targeting confirmed based on protein localization and/or PCR.

4 CONCLUSIONS AND OUTLOOK

CRISPR/Cas9 technology provides a powerful tool for specific and robust gene editing in human cells. In this chapter, we have outlined strategies for generating inducible knockout human cell lines for studies of acute gene disruption, and knock-in human cell lines to report on endogenous protein behavior. In addition to the reverse genetic tools presented here, CRISPR/Cas9 gene editing tools facilitate forward genetic approaches such as genome-wide screens (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014) for the discovery of novel players or synthetic interactions (for example, see Fong et al., 2016; Lambrus et al., 2016; Meitinger et al., 2016). Together, these tools will facilitate further cell biological analyses of the molecular mechanisms of cell division.

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