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CRISPR Cas9 Genome Editing in Human Cell Lines with DONOR Vector Made by Gibson Assembly

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Abstract

CRISPR Cas9 genome editing allows researchers to modify genesin a multitude of ways including to obtain deletions, epitope-tagged loci, and knock-in mutations. Within six years of its initial application, CRISPR Cas9 genome editing has become widely employed, but disadvantages to this method, such as low modification efficiencies and off-target effects,need careful consideration. Obtaining custom donor vectors can also be expensive and time consuming. This chapter details strategies to overcome barriers to CRISPR Cas9 genome editing as well as recent developments in employing this technique.

Keywords

CRISPR Cas9; Custom Donor Vector; Gibson Assembly

1. Introduction

CRISPR Cas9 and related mechanisms are endogenous adaptive immune responses found widely in bacteria and archaea to fend off invading DNAs, such as those from viruses [1–8]. Short segments of invading DNA are inserted into a specialized bacterial chromosomal locus containing repetitive DNA elements, the clustered regularly interspaced short palindromic repeats (CRISPR) [9–15]. The resulting transcribed RNA is processed into several short RNAs (crRNAs) that are incorporated into a riboprotein complex [11–15].The riboprotein complex then targets the foreign nucleic acids in a sequence specific manner due to the presence of the crRNAs. CRISPR Associated Proteins (Cas proteins) Include those involved in transcription, RNA processing and formation of the riboprotein complex. Researchers ingeniously co-opted this bacterial mechanism to devise heterologous systems to edit the genome of nearly any organism at will [16–22].

Endogenous CRISPR Cas9 system from bacteria

Approximately 50% of bacteria and 90% of archaea harbor an adaptive CRISPR system to mount an immunological response against phage (viruses) and other mobile genetic elements [23–25]. So far, at least 6 subtypes of CRISPR systems have been identified. First

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discovered by Ishino and colleagues in1987, many variations of these systems have been identified and characterized [26]. At the forefront of this field are exciting, emerging issues. Chief among these, is the ability of some bacterial phage to counter the CRISPR immune response by expressing anti-CRISPR proteins that prevent DNA binding and Cas3 activity [27–29]. Additional functional roles for CRISPR in bacteria include promoting genome evolution, bacterial virulence and DNA repair [30–33].

Different types of CRISPR

Numerous distinctive CRISPR/Cas systems have been discovered that show incredible diversity regarding genomic locus architecture and gene composition. This diversity in the CRISPR/Cas system helps prokaryotes accurately identify and neutralize invading bacteriophages. The most recent approach to classification of CRISPR/Cas systems includes two main subclassifications based on their effector modules (Fig. 1) [34, 35].

Class 1 systems include approximately 90% of the CRISPR-Cas loci of prokaryotes and employs multi-subunit effector complexes: types I, III, and the rare type IV. Type I systems contain the Cas-3 nuclease helicase; type III systems possess the signature gene Cas10.Type IV systems include an uncharacterized large subunit Csf1 (Fig. 1) [34, 35].

Class 2 systems comprise ~10% of the CRISPR-Cas loci and contain single-subunit effector complexes, including types II, V, and VI. The type II system has a signature Cas9 nuclease. Type V systems consist of either Cas12a, Cas12b or Cas12C, which are like Cas9. Type VI systems possess either Cas13a, Cas13b or Cas13c (Fig.1). Apparently, the Class 2 systems convey more potential in gene editing and enhancing genetic assessment by using Cas9, Cas12a, Cas13a, and Cas13b [36].

The CRISPR Cas9 system remains the foremost and most extensively used for genetic engineering. In this system, the Cas9 protein is involved in processing the transcript into the mature crRNA [36]. Processing requires binding of a short trans activating RNA (tracrRNA), which is complementary to the repeat sequence found in type II CRISPR systems. After processing the transcript, the Cas9 protein remains bound to the crRNA and the tracrRNA, ultimately leading the complex to cleave a target sequence.

Heterologous CRISPR Cas9 system basics

CRISPR Cas9 genome editing using heterologous systems was pioneered by researchers including Dr. Doudna (UC Berkeley), Dr. Charpentier (currently at Max Planck), Dr. Church (Harvard), and Dr. Zhang (Broad Institute) [16, 19–22, 37]. In a breakthrough study, Drs. Doudna and Charpentier were the first scientists to show that a chimeric dual-tracrRNA:crRNA containing the sequence of a desired target could be engineered to direct the Cas9 nuclease to produce double strand breaks in a sequence specific manner [16]. This ground-breaking study end Ed with the statement "…*could offer considerable potential for gene targeting and genome editing applications* [37]." In 2013, the employment of CRISPR Cas9 technology to genome edit in human cells (by Dr. Zhang and colleagues) using heterologous systems became a reality [19]. These systems utilized chimeric guide RNAs to target a desired sequence, and an engineered human codon–optimized *S. pyogenes Cas9* (hSpCas9) in HEK 293 FT cells [19]. Nuclear localization sequences were fused to hSpCas9

to ensure DNA access [19]. CRISPR heterologous systems are continually being refined to increase efficiency and reduce off-target effects in a vast array of biological systems from *C. Elegans* to humans[9].

2. Materials

2.1 PCR to amplify Gibson Assembly Components for Donor Vector Construction

1. Phusion DNA Polymerase kit for PCR (Thermo-Fisher cat: F530S)
   a. Phusion DNA Polymerase (2 U/μL)
   b. 5X Phusion HF Buffer
   c. DMSO
   d. 50 mM $MgCl_2$ solution
2. 25 mM stock dNTP (prepared from Invitrogen cat: 10297–117)
3. Ultra-Pure DNase/RNase-Free Distilled Water (Invitrogen cat: 10977015)
4. Primers obtain from Sigma (custom oligos on Table 1.)
5. Template: CMV5-*FOXO3* prepared by miniprep kit (Qiagen cat: 27106)

2.2 Cloning Enzymes

1. *Dpn*I (NEB R0176S, Ipswich, MA)
2. *Dra*III (NEB cat: R3510S, Ipswich, MA)
3. *Bsm*I (NEB R0134S, Ipswich, MA)
4. CIP (Calf Intestine Phosphatase, NEB cat: M0290S, Ipswich, MA)
5. Gibson Assembly Kit (NEB cat: E2611S, Ipswich, MA)

2.3 Column Purification of DNA

1. DNA Purification Kit (Qiagen cat: 28104)
2. DNA Maxi Kit (12163)

2.4 Transformation of Bacteria for Cloning

1. 5-alpha competent *E. coli* (NEB cat: C2988J, Ipswich, MA)

2.5 Propagation of Human Cell Lines

1. MEM (Minimal Essential Media, Fisher cat: 10010cv)
2. DMEM (Dulbecco’s Modified Eagle Medium, Fisher cat: mt10013cv)
3. RPMI (Roswell Park Memorial Institute 1640, Fisher cat: 10040cv)
4. Penicillin/streptomycin (Fisher cat: 30002ci)
5. Fetal Bovine Serum (FBS, Fisher cat: 26140079)

6. Trypsin (0.25% Fisher: 25053ci)

2.6 Transfection of Human Cell Lines

1. Scepter Cell Counter (Millipore, Burlington, MA); can also use hemocytometer
2. Nucleofector V solution (Lonza, Cologne, Germany Kit V cat: VCA-1003)
3. *FOXO3* guide RNA plasmid (Sigma: HSL0001339461)
4. *FOXO3* guide RNA plasmid (Sigma: HSR0001339464)
5. CRISPR Cas9D10A-GFP Nickase vector (Sigma cat: CAS9D10AGFPP)

2.7 Selection of neomycin resistant clones

1. G418 (Corning cat: 30–234-CR)

2.8 Isolating Neomycin Resistant Clones

1. Vaseline (Amazon: original solution, autoclaved in a glass beaker)
2. Cloning cylinders (Fisher: 0955221, autoclaved)
3. 1XPBS (Fisher cat: 21–031-cv)

2.9 Western Blot Analysis

1. Cell scraper (Fisher cat: 7200365)
2. 2X Sample Buffer
   a. 125 mM Tris-HCL at pH 6.8
   b. 2% sodium dodecyl sulfate (SDS)
   c. 10% 2-mercaptoethanol
   d. 20% glycerol
   e. 0.05% bromophenol blue
   f. 8 M urea
   g. prepared with ultra-pure distilled water
3. Polyacrylamide gel Mini-PROTEAN TGX 4–20, 10W (Bio Rad cat: 4561093E)
4. Protein Ladder (Bio Rad: 1610374)
5. Polyacrylamide Gel Running Buffer
   a. 25 mM Tris
   b. 190 mM glycine
   c. 0 .1% S D S
6. Transfer Buffer
   a. 25 mM Tris

   b. 190 mM glycine
   c. 10% methanol
7. PVDF (polyvinylidene fluoride) membrane, (Fisher cat: ipvh00010)
8. TBST
   a. 20 mM Tris pH 7.5
   b. 150 mM NaCl
   c. 0.1% Tween 20
9. Carnation Non-fat Dry Milk(Amazon)
10. FOXO3 antibody (Cell Signaling, Danvers, MA, Cat: 75D8)
11. GAPDH (Santa Cruz Biotechnology, Dallas, TX, cat: G-9)
12. Goat-anti-rabbit secondary antibody for FOXO3 blots (Fisher/Pierce cat: 31460)
13. Goat-anti mouse secondary antibody for GAPDH blots (Fisher/Pierce cat: 31432)
14. Super Signal West Dura Extended Duration Substrate luminol (Fisher/Pierce: 37075)

3. Methods

Protocol to disrupt *FOXO3* using CRISPR Cas9 with a Custom Donor Vector

The protocol highlighted in this chapter was published (in less detail) in Open Access BMC Molecular Biology; parts of this protocol are reproduced with permission from the initial journal [9]. This method employs a modified Cas9 nickase (Cas9D10A) purchased from Sigma: CRISPR Cas9D10A-GFP Nickase (catalog: CAS9D10AGFPP, Sigma, St. Louis, MO). This mutant nuclease is from *Streptococcus pyogenes* and will only nick one strand of DNA. Cas9D10A retains the HNH domain activity (to cleave the target strand) and lacks the RuvC activity (that would normally cleave the non-target strand).

Two guide RNAs were employed to target *FOXO3*

Guide RNAs contain a sequence resembling the repeat sequence in the CRISPR Cas 9 system plus a sequence to target the gene of interest [37]. The chimera guide RNA then takes the place of the endogenous crRNAs in binding Cas9 and targeting the nuclease to the specified sequence [37]. Guide RNAs were purchased from Sigma to nick the *FOXO3* genomic locus (on chromosome 6) 41 bases apart on opposite strands.

Sequences: We used two *FOXO3*-targetting gRNAs from Sigma: HSL0001339461, 5’-CTTACTGAAGGTGACAGGCTGG-3’ (chromosomal position: 108663888–108663909) and HSR0001339464, 5-’CACGGCTGACTGATATGGCAGG-3’ (chromosomal position 108663933–108663954). Researchers can also readily devise custom guide RNAs using recommendations by Zhang *et al* (among others) to target virtually any gene [38, 39].

Construction of a donor vector to select for *FOXO3* disruption mutants

To select for *FOXO3* disruption mutants, we prepared a donor vector containing the neomycin resistance gene via Gibson assembly using the commonly employed mammalian expression vector pcDNA3 as the backbone [9]. First, PCR was used to construct two "arms" containing *FOXO3* chromosomal sequences. Then, a two-step Gibson assembly cloning procedure was performed to insert the *FOXO3* arms on either side of the *NPTII* gene (encoding neomycin resistance) found in the pcDNA3 vector; see Fig.2(permission granted from BMC Molecular for reproduction) [9]. The donor vector had *FOXO3* sequences of enough length to allow for homology directed repair in order to disrupt the chromosomal *FOXO3* gene. One *FOXO3* arm (termed Arm 1) in the donor vector included 418 bases (chromosomal position: 108663463–108663880) and the other *FOXO3* arm (termed Arm 2) had 750 bases (chromosomal position 108663921–108664670); see Fig.2. Homology directed repair of the CRISPR Cas9D10A-induced nicks produced a truncated *FOXO3* gene in several human cell lines examined [9].

Gibson assembly protocol to obtain Custom Donor Vector

Gibson assembly was developed by Daniel G. Gibson in 2009 [40]. The method joins together fragments containing identical sequences of DNA (typically ranging from 20–40 nucleotides long) on their ends. The first step of Gibson assembly involved 5' to 3' exonuclease activity that Produced 3' overhangs, which could then anneal to another fragment with a compatible overhang. By using PCR primers containing vector sequences, the pcDNA3 vector and *FOXO3* fragment had3' overhangs that couldanneal. After annealing, a DNA polymerase extended from the 3' ends to fill in missing bases. DNA ligase sealed the phosphodiester backbone [40]. In this manner two fragments with "overlapping," sequences could be joined. The amount of fragment overlap depends (in part) on the insert and vector sizes. In this protocol, an overlap of 20–30 bases Was enough for efficient cloning. For larger inserts, 30–40 bases might be needed for efficient cloning. A guide to Gibson assembly can be found at: (https://www.biocat.com/bc/files/Gibson_Guide_V2_101417_web_version_8.5_x_11_FINAL.pdf). We used a basic Gibson assembly reaction with three steps described above (NEB Gibson Assembly Kit E2611S); all took place at 50 degrees Celsius in the span of one hour. Specifics of the reactions including preparing the PCR products and vector are detailed below:

PCR reactionto amplify *FOXO3* Arm 1to be inserted into pcDNA3 vector at *Dra*III site

- **i.** 20 microliters of 5 x Phusion HF Buffer (Thermo-Fisher cat: F530S)
- **ii.** 8 microliters 25 mM dNTP
- **iii.** 2.5microliters of 50 micromolar forward primer (***FOXO3*ARM 1 F)**
- **iv.** 2.5 microliters of 50 micromolar reverse primer (***FOXO3*ARM 1 R)**
- **v.** 1 microliter of plasmid DNA template (200ng/microliter concentration contained *FOXO3* gene)
- **vi.** 3 microliters of DMSO
- **vii.** 1 microliter of Phusion polymerase (Thermo-Fisher cat: F530S)

viii. 59.5 microliters of water

ix. 2.5 microliters of 50 mM $MgCl_2$

Cycling parameters: 94 degrees Celsius for 10 minutes (initial heat step done only once); then, 94 degrees Celsius for 1minute, 56 degrees Celsius for 1 minute, and 72 degrees Celsius 3 minutes (three steps for forty cycles). One last extension of 72 degrees Celsius for 20 minutes was followed by a hold at 4 degrees Celsius until reactions were collected.

After the PCR Reactions were complete

1. Added 2 microliters of *Dpn*I (NEBR0176S, Ipswich, MA) to *FOXO3* Arm1 PCR reaction and digested at 37 degrees Celsius for two hours to remove the plasmid DNA template.
2. These digested PCR reactions were column purified using Qiagen PCR purification system (Hilden, Germany Cat No./ID: 28104) and eluted with sterile 30 microliters of water.
3. Purified *FOXO3* Arm 1 PCR product was quantified with a Nanodrop spectrophotometer.

Preparing pcDNA3 vector for Gibson Assembly

1. Cut pcDNA3 vector with *DraIII* for two hours at 37 degrees Celsius
   i. 1 microgram of pcDNA3
   ii. 4 microliters of Cut Smart buffer (10x, NEB Ipswich, MA)
   iii. 3 microliters of *DraIII* enzyme (NEB cat: R3510S, Ipswich, MA)
   iv. 27 microliters of water for final volume of 40 microliters
2. CIP (NEB cat: M0290S, Ipswich, MA) was added to the above restriction digest and samples were incubated at 37 degrees Celsius for one additional hour.
3. The *Dra*III-cut (and phosphatased) vector was purified using Qiagen PCR purification system (Hilden, Germany Cat No./ID: 28104) and eluted with sterile 30 microliters of water.
4. The purified vector was quantified with a Nanodrop spectrophotometer.

Gibson Assembly reaction to add *FOXO3* Arm1 into the pcDNA3 vector at *Dra*III site

i. 10 microliters of NEB Gibson Assembly Master Mix (E2611SNEB, Ipswich, MA)

ii. 2 microliters (10 nanograms) of prepared *Dra*III-cut pcDNA3 vector (see above for preparation details)

iii. 8 microliters of *FOXO3* Arm1 insert prepared as described above (80 nanograms)

Gibson assembly reactions were incubated for one hour at 50 degrees Celsius. 5 microliters of the Gibson assembly reaction were added to 50 microliters of 5-alpha competent *E. coli*

(NEB, Ipswich, MA cat: C2988J) and were incubated for 30 minutes on ice. Then samples were heat shocked at 42 degrees Celsius for 30 seconds followed by placement on ice for 5 minutes. After this, 250 microliters of SOC recovery media were added and samples were incubated for one hour at 37 degrees Celsius. Finally, dilutions of cells (1:10, 1:100, 1:1000) were plated onto LB plates with ampicillin (100 micrograms per mL) and incubated overnight at 37 degrees Celsius. Hundreds of colonies were obtained and confirmed by restriction digest analysis and Sanger sequencing.

Adding second *FOXO3* Arm to CRISPR Cas9 Donor vector

1. PCR was performed to amplify *FOXO3* Arm2 exactly as described above (for *FOXO3* Arm 1), except using Arm 2 primers described in Table 1. The PCR product was cut with *Dpn*I (NEB R0176S) to remove template and column purified as described above.
2. The pcDNA3 vector that contained *FOXO3* Arm 1 was prepared for Gibson Assembly in order to add *FOXO3* Arm 2. Vector pcDNA3 *FOXO1* Arm 1 was cleaved with *BsmI* for two hours at 65 degrees Celsius:
    i. 1 microgram of pcDNA3 *FOXO3* Arm 1 vector
    ii. 4 microliters of Cut Smart buffer (10x NEB, Ipswich, MA)
    iii. 3 microliters of *Bsm*I enzyme (NEB R0134S, Ipswich, MA)
    iv. 27 microliters of water for final volume of 40 microliters
3. CIP (NEB M0290S, Ipswich, MA) was added to the above restriction digest and samples were incubated at 37 degrees Celsius for one additional hour.
4. The *Bsm*I-cut (and phosphatased) vector was purified using Qiagen PCR purification system (Hilden, Germany Cat No./ID: 28104) and eluted with sterile 30 microliters of water.
5. Purified vector was quantified with a Nanodrop spectrophotometer.

The Gibson assembly reaction to add *FOXO3* Arm 2 (to obtain final donor vector) and transformation of 5-alpha cells were carried out as described above for construction of the plasmid containing Arm 1 [9]. After confirmation of the desired construct by restriction digest analysis and Sanger sequencing, plasmids were prepared for transfection of cell lines by maxiprep (Qiagen Maxi Kit: 12163) according to manufacturer's instructions.

Transient transfection of human cell lines to disrupt the *FOXO3* gene with a neomycin resistance cassette

We disrupted the *FOXO3* gene in several human cell lines and compared mutation efficiencies. Glioblastoma cell line U87MG was grown in MEM (Minimal Essential Media Fisher cat: 10010cv). Human embryonic kidney cells (HEK 293) were grown in DMEM (Dulbecco's Modified Eagle Medium, Fisher cat: mt10013cv). BT549 basal breast cancer cells were grown in RPMI 1640 (Roswell Park Memorial Institute 1640, Fisher cat: 10040cv). All cell lines were grown with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin (Fisher cat: 30002ci).

1. Cells were plated three days before the Transfection in media that lacked antibiotic.
2. On transfection day, 70% confluent cells were treated with 0.25% trypsin solution (Fisher cat: 25053ci) for 3 minutes at 37 degrees Celsius.
3. Cells were then mixed 1:10 with fresh media (lacking antibiotic) and were counted using a Scepter Cell Counter (Millipore, Burlington, MA).
4. One million cells were put into a 15 mL screw cap tubes (Fisher cat: 14–959–70c) and centrifuged at 100 x g for 10 minutes (Eppendorf 5810R benchtop centrifuge) to pellet the cells.
5. Cells were prepared for electroporation
    i. Resuspended one million cells in 100 microliters of Nucleofector V solution (Lonza, Cologne, Germany Kit V cat: VCA-1003).
    ii. Added 250 nanograms of first guide RNA plasmid to nick *FOXO3* chromosome: HSL0001339461(5'-CTTACTGAAGGTGACAGGCTGG-3' chromosomal position: 108663888–108663909)
    iii. Added 250 nanograms of second guide RNA plasmid to nick *FOXO3* chromosome: HSR0001339464 (5'-CACGGCTGACTGATATGGCAGG-3' chromosomal position 108663933–108663954).
    iv. 250 nanograms of the vector enabling Cas9D10A expression (catalog: CAS9D10 AGFPP, Sigma, St. Louis, MO)
    v. 1.0 microgram of *FOXO3* donor vector (prepared as described above)
    vi. Program P-20 for was utilized for U87MG and BT549 and program X-001 was used for HEK 293) to electroporate with Lonza Nucleofector machine (Lonza, Cologne, Germany). Pre-warm the media for recovery ahead of time. Another useful practice is to have the recovery media ready in the plastic dropper included in the kit before electroporation in order to add it to the cells right away for recovery. Recovery media lacked antibiotic.
    vii. Cells recovered for two days in a 10 cm dish at 37 degrees Celsius and 5% CO_2.

Selecting *FOXO3* disruption clones using neomycin

Selecting clones takes weeks (and possibly longer if the mutation is deleterious to cell growth). After recovery for 2 days in media lacking antibiotic, transformed cells were treated with trypsin (Fisher cat: 25053ci) for three minutes at 37 degrees Celsius. After this, cell suspensions were mixed with 10 mL of fresh media (containing 5% penicillin/streptomycin solution: Corning: 30–002-CL) and passed through a cell strainer (BIOLOGIX 100 micrometer diameter cell strainer cat: 15–1100) eliminating clumps to better isolate

single clones. Ten plates with ~100,000 cells were prepared from strained cells. Neomycin was added the next day to select for single clones: G418 (Corning 30–234-CR: 0.5 mg/mL final concentration for U87MG and BT549 and 1.5 mg/mL G418 for HEK 293 cells) [9]. Samples were incubated for one month at 37 degrees Celsius and 5% CO_2. Approximately 2–10 clones were isolated from each 10 cm plate. Single clones were purified using cloning cylinders.

Isolation of single clones from 10 cm dishes

i. Wash 10 cm plate (with clones on it) twice with 2 mL sterile 1XPBS (Fisher cat: 21–031-cv). Important: make sure that all PBS is carefully removed from the plate. Residual PBS canmake cloning cylinders move around.

ii. Cloning cylinders (Fisher: 0955221) were placed onto the 10 cm plate (directly over individual colonies) using sterile forceps and autoclaved Vaseline (to make the cylinder stick to the plate).

iii. 200 microliters of 0.25% trypsin were placed into the cloning cylinder and incubated at room temperature for 5 minutes.

iv. After this, gently pipette up and down ten times and remove the 200 microliters with cells. Place cells into fresh media in a well of a 6-well plate. Allow cells to grow for roughly a week.

Confirming mutants by western blot analysis

After cloned cells recovered for about a week, they were treated with 0.25% trypsin (Fisher cat: 25053ci) and re-plated at a 1:2 dilution to propagate and collect protein lysates for western blot Analysis as described previously [9]. Total protein was obtained from cells by rinsing out the 6 well plate wells with1mL1XPBS followed by direct lysis in 2x sample buffer (125 mM Tris-HCL at pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20 % glycerol, 0.05% bromophenol blue, 8 M urea). To do this, 2x sample buffer was added to each well and scraped [9]. The lysate was collected from each well, placed into a 1.5 ml microcentrifuge tube and heated for 10 minutes at 90°C in a dry-bath heat block [9]. Protein lysates were separated by sodium dodecyl sulfate – polyacrylamide gel (SDS-PAGE) electrophoresis at 100V for 1 hour. The protein was then transferred onto a polyvinylidene fluoride (PVDF) membrane for an hour and 30 minutes then blocked in a 5% milk solution (Carnation powdered milk, 1X Tris-buffered saline with Tween 20 (TBST)) for an hour. The membrane was incubated with FOXO3 (Cell Signaling, Danvers, MA, Cat: 75D8), or GAPDH (Santa Cruz Biotechnology, Dall as, TX, cat: G-9) antibodies overnight at 4° C. After primary antibody incubation, blots were washed for 20 minutes with TBST in 5-minute intervals [9]. After the first set of washes, blots were incubated with secondary antibody (goat-anti-rabbit for FOXO3 blots: Fisher/Pierce 31460 and goat-anti mouse for GAPDH blots: Fisher/Pierce cat: 31432) for one hour at room temperature with gentle rocking. After secondary antibody incubation, the membrane was washed again for 20 minutes in 5-minute intervals. Blots were developed with Super Signal West Dura Extended Duration Substrate luminol solution for 5 minutes (Pierce: PI-37075) [9]. A BioRad ChemDoc XRS+ molecular imager was used to detect light emitted from protein-containing

complexes. Western blot SCN files from the (BioRad ChemDoc XRS+) were analyzed using NIH Image J. Disruption of the *FOXO3* gene as described in this protocol led to a much smaller protein (349 amino acids versus 673), [9]. Therefore, western blot analysis allowed us to quickly screen hundreds of putative mutants (that were cloned as G418 positive) for production of a truncated FOXO3 protein.

The results of our mutagenesis reactions in several cell lines can be found in Table 2 [9]. One can see that mutation frequencies varied depending on the cell line. HEK 293 cells had the lowest mutation frequency of approximately 1.0% [9]. Of note, U87MG and BT549 cells had 5-fold higher mutation frequencies than HEK 293 cells [9]. One possible reason for this is that HEK 293 cells are immortalized and have fewer mutations than the cancer cell lines U87MG and BT549. DNA repair mechanisms such as non-homologous end joining may be more efficient in HEK 293 cells leading to fewer disruption mutants obtained via homology-directed repair of chromosomal lesions.

Homozygous disruption mutants of the *FOXO3* gene were also confirmed by PCR amplification of the disrupted region and Sanger sequencing. One million cells were removed from plates using trypsin and centrifuged at 700 x g for 5 minutes. Cells were re-suspended in 500 microliters of Buffer (10 mM Tris 7.4, 10 mM NaCl, 25 mM EDTA, 1% SDS). 25 microliters of Proteinase K (50 U per mL stock, catalog: 03115828001, Roche, Mannheim, Germany) Were added to each sample followed by an 18-hour incubation at 37°C [9]. Next, samples were extracted with an equal volume of phenol, followed by centrifugation at top speed in phase lock tubes (Quanta Biosciences, Beverley, MA, catalog: 2302820). DNA was precipitated from the supernatant as previously described by adding 3 microliters of 20 mg/mL glycogen, 50 microliters of sodium acetate and 1 mL of 100%ethanol (stored at −80°C over-night followed by 20 minutes of centrifugation at 17,000 x g) [9]. The DNA pellets were washed with 70% ethanol, dried and re-suspended in sterile water. PCR was performed to identify clones that had *NPTII* integration into the *FOXO3* locus using the primers in Table 3. The forward primer for PCR was approximately sixty bases upstream of the *FOXO3* fragment found in Arm 1 of the *FOXO3* donor vector [9]. Therefore, primer sequences employed for mutant detection by PCR were absent from the donor vector and were only found in chromosomal *FOXO3*. The reverse primer was part of the *NPTII* cassette used to disrupt *FOXO3* [9].PCR products (employed for detecting *FOXO3* disruption) were column purified using Qiagen PCR purification system (Hilden, Germany Cat No./ID: 28104) and eluted with 30 microliters of sterile water. Purified PCR products were quantified with a Nanodrop spectrophotometer and analyzed by Sanger sequencing using primer in Table 3.

4. Notes

4.1 Streamlining Mutation Isolation by CRISPR Cas9 Mutagenesis

We initially tried to examine pools of neomycin resistant cells directly after CRISPR Cas9 mutagenesis but found little *FOXO3* disruption. We concluded that the efficiency of the mutagenesis was not high enough to examine pools of mutagenized cells. Therefore, isolation of clones was required to obtain a sizeable pool of mutants to investigate. To accomplish this, we selected for disruption of the *FOXO3* gene using neomycin resistance

followed by western blot analysis and sequencing. Using three tiers of Screening helped to quickly isolate the desired mutants. Upon clone isolation, one can see from Table 1. that our mutation frequencies were only 1–5% when seeking disruption alleles for *FOXO3*.

4.2 Overcoming Off-target Effects for our Mutagenesis

One major issue with CRISPR Cas9 genome engineering is off-target effects [41]. Our protocol took numerous approaches to decrease off-target effects from CRISPR Cas9 mutagenesis.

- **i.** First, we employed transient expression of the Cas9D10A nuclease from a mammalian expression vector. This decreased the time that the Nuclease was expressed in the cells, reducing the likelihood of multiple mutation events and greatly limiting the possibility of off-target mutations. Others have addressed this issue by employing an inducible form of Cas9 nuclease [42–44].
- **ii.** We utilized two guide RNAs to direct the Cas9D10A nickase in order to introduce a recombinogenic (staggered) double strand break in the chromosomal locus of *FOXO3*. In this case, both strands needed to be targeted separately by the Cas9D10A nickase. This approach has been shown to increase specificity by 100-fold or more in other systems [45].

4.3 Looking into the Cutting Edge: Novel CRISPR Systems for Genome Editing

Efforts are continually being made to make CRISPR Cas9 genome editing more precise and efficient. CRISPR/Cas-based DNA and RNA editing tools have revolutionized biological research and extended the capability to manipulate genomes. CRISPR Cas9, which belongs to the class 2 type II CRISPR, is the most extensively used high-throughput tool for genome editing.

Initially, CRISPRCas9 was a valuable tool for targeted DNA cleavage, but with the development of programmable RCas9, sequence-specific sites on RNA can now be recognized and cleaved for studying RNA regulation [46]. In RCas9, specific PAM-presenting oligonucleotides (PAMmers) target sites on RNA and avoids sites on genomic DNA, which stimulates the cleavage of RNA in a site-specific manner. CRISPR Cas13 is another powerful tool for precise RNA editing. Cas13a (previously known as C2c2) belongs to class 2 type VI CRISPR and is a single-effector RNA-guided RNase, which can be programmed for cleavage of specific sites of mRNA transcripts, mediated by the Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains [47]. The CRISPR Cas13 system is more efficient than RNA interference and can be used in mammalian and plant cells to knockdown specific mRNA transcripts [48].

REPAIR (RNA editing for programmable A to I replacement) technology further advances RNA editing. This novel technique employs Cas13 orthologs, dCas13b, which are catalytically inactive, and can be used to convert a single A base to I (functionally equivalent to G) with the help of ADAR2 (adenosine deaminase acting on RNA type 2). With REPAIR, it is possible to target coding and noncoding RNA, and it has higher specificity. However, REPAIR can allow only single base A to I editing, which is a major drawback [49, 50]. To

expand the editing capability to other nucleotide bases, Abudayyeh and his group proposed a programmable RESCUE system (RNA Editing for Specific C to U Exchange), which is capable of a highly specific and precise C to U conversion in RNA. As an advantage, RESCUE can target amino acids encoding signals for posttranscriptional modifications including phosphorylation, glycosylation, and methylation [50]. REPAIR and RESCUE RNA editing tools have revolutionized the capability of CRISPR and expanded the ability to treat potential genetic diseases.

Another advance has come from Sternberg and his group who developed a new fully programmable CRISPR/Cas model based on the bacterial Tn7-like transposons. In this system, highly specific DNA insertion in the genome is guided by the RNA complex known as Cascade (Type I CRISPR) without any double-strand breaks [51]. Transposon-based CRISPR/Cas models 26 provide a new approach for site-specific insertion of DNA, which could be leveraged in the production of biopharmaceuticals, gene therapies, improvement of crops, and future development of knock-in methods [51].

4.4 Recent Advances in reducing CRISPR Cas9 Off-Target Effects

Off-target effects remain the major drawback for utilizing CRISPR Cas9 as a genome editing tool, especially in the area of precision medicine. However, Wienert and colleagues, have created a method, the DISCOVER-Seq, for better identification of off-target sites [52]. This technology is built on the idea that after a cut is made on a DNA strand, the cell recruits DNA repair machinery as part of the body's natural repair process. Finding those DNA repair factors would mean finding the locations of the CRISPR off-target effects [52]. This testing has been completed on induced pluripotent stem cells and patient cells and has shown be more accurate than the initial CRISPR Cas9 system.

After Cas9 completes its job by cleaving the target DNA strand, continued activity of the protein can become a potential danger by producing non-target breaks in DNA strands. As a solution to this problem, current research is examining ways to make "off switches" to regulate the activity of Cas9 or similar proteins [53]. Much of the research on solving the problem of off-target effects began in 2012 at the University of Toronto where researchers attempted to find different ways to block the Cas9 enzyme and break its interaction with the guiding RNA strand [54–64]. A challenge was that Cas9 cuts the DNA with a specific pair of nuclease domains that must be disabled until needed. Amit Choudhary and colleagues focused on the interaction between Cas9 and a short DNA segment that Cas9 uses to begin cutting, the protospacer adjacent motif (PAM) sequence [65]. Luckily, Dr. Choudhary noted, that since this is a short sequence, "the recognition [does not] have to be very strong." His team conducted different assays to discover which compounds could effectively disrupt the Cas9-PAM binding interaction. The result was a pair of small molecules that could penetrate cells and also inhibit this binding interaction without exhibiting off-target toxic effects [65].

In 2019, Kocak and colleagues introduced a new way of increasing the precision and decreasing off-target effects of bacterial CRISPR Cas9 nucleases [66]. Researchers added hairpin secondary structures to the spacer region of single guide RNAs (hp-sgRNAs). It was discovered that these hp-sgRNAs added specificity when utilizing five different Cas12a or Cas9 protein variants [66]. The RNA secondary structure was created by elongating the

hairpin on the 5' end of the sgRNA to function as a steric barrier to R-loop formation. R-loop formation is critical to helping a trans-activator based on Cas9 from *Streptococcus pyogenes* (SpCas9) to function alongside the active nuclease [66]. Thus, by altering the strength of the RNA secondary structure, the R-loop could form at target sites, but not at off-target sites due to mispairing. This allowed for an increase in specificity by an average of 55-fold with five of the most commonly used Cas proteins in CRISPR [66].

Ultimately, much research remains to be done to increase the precision of CRISPR technologies. New future projects, such as introducing regulatory molecules along with the CRISPR technology, using the body's natural repair capabilities, or engineering new secondary structures, show promise in the field of cell biology and biomedicine [67–69].

References

1. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, and Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173–1183 [PubMed: 23452860]
2. van Duijn E, Barbu IM,Barendregt A, Jore MM, Wiedenheft B, Lundgren M, Westra ER, Brouns SJ, Doudna JA, van der Oost J, and Heck AJ (2012) Native tandem and ion mobility mass spectrometry highlight structural and modular similarities in clustered-regularly-interspaced shot-palindromic-repeats (CRISPR)-associated protein complexes from Escherichia coli and Pseudomonas aeruginosa. Mol Cell Proteomics 11, 1430–1441 [PubMed: 22918228]
3. Haurwitz RE, Sternberg SH, and Doudna JA (2012) Csy4 relies on an unusual catalytic dyad to position and cleave CRISPR RNA. EMBO J 31, 2824–2832 [PubMed: 22522703]
4. Sternberg SH, Haurwitz RE, and Doudna JA (2012) Mechanism of substrate selection by a highly specific CRISPR endoribonuclease. RNA 18, 661–672 [PubMed: 22345129]
5. Sashital DG, Jinek M, and Doudna JA (2011) An RNA-induced conformational change required for CRISPR RNA cleavage by the endoribonuclease Cse3. Nat Struct Mol Biol 18, 680–687 [PubMed: 21572442]
6. Jore MM, Lundgren M, van Duijn E, Bultema JB, Westra ER, Waghmare SP, Wiedenheft B, Pul U, Wurm R, Wagner R, Beijer MR, Barendregt A, Zhou K, Snijders AP, Dickman MJ, Doudna JA, Boekema EJ, Heck AJ, van der Oost J, and Brouns SJ (2011) Structural basis for CRISPR RNA-guided DNA recognition by Cascade. Nat Struct Mol Biol 18, 529–536 [PubMed: 21460843]
7. Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, and Doudna JA (2010) Sequence-and structure-specific RNA processing by a CRISPR endonuclease. Science 329, 1355–1358 [PubMed: 20829488]
8. Wiedenheft B, Zhou K,Jinek M, Coyle SM, Ma W, and Doudna JA (2009) Structural basis for DNase activity of a conserved protein implicated in CRISPR-mediated genome defense. Structure 17, 904–912 [PubMed: 19523907]
9. Vazquez N, Sanchez L, Marks R, Martinez E, Fanniel V, Lopez A, Salinas A, Flores I, Hirschmann J, Gilkerson R, Schuenzel E, Dearth R, Halaby R, Innis-Whitehouse W, and Keniry M (2018) A protocol for custom CRISPR Cas9 donor vector construction to truncate genes in mammalian cells using pcDNA3backbone. BMC Mol Biol 19, 3 [PubMed: 29540148]
10. Lee JS, Kallehauge TB, Pedersen LE, and Kildegaard HF (2015) Site-specific integration in CHO cells mediated by CRISPR/Cas9 and homology-directed DNA repair pathway. Sci Rep 5, 8572 [PubMed: 25712033]
11. van Erp PB, Jackson RN, Carter J, Golden SM, Bailey S, and Wiedenheft B (2015) Mechanism of CRISPR-RNA guided recognition of DNA targets in Escherichia coli. Nucleic Acids Res 43, 8381–8391 [PubMed: 26243775]
12. Charpentier E (2015) CRISPR-Cas9: how research on a bacterial RNA-guided mechanism opened new perspectives in biotechnology and biomedicine. EMBO Mol Med 7, 363–365 [PubMed: 25796552]

13. Rollins MF, Schuman JT, Paulus K, Bukhari HS, and Wiedenheft B (2015) Mechanism of foreign DNA recognition by a CRISPR RNA-guided surveillance complex from Pseudomonas aeruginosa. Nucleic Acids Res 43, 2216–2222 [PubMed: 25662606]

14. Dyda F, and Hickman AB (2015) Mechanism of spacer integration links the CRISPR/Cas system to transposition as a form of mobile DNA. Mob DNA 6, 9 [PubMed: 27408625]

15. Moore R, Spinhirne A, Lai MJ, Preisser S, Li Y, Kang T, and Bleris L (2015) CRISPR-based self-cleaving mechanism for controllable gene delivery in human cells. Nucleic Acids Res 43, 1297–1303 [PubMed: 25527740]

16. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, and Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910–918 [PubMed: 23643243]

17. Zhang Q, Rho M, Tang H, Doak TG, and Ye Y (2013) CRISPR-Cas systems target a diverse collection of invasive mobile genetic elements in human microbiomes. Genome Biol 14, R40 [PubMed: 23628424]

18. Jiang W, Bikard D, Cox D, Zhang F, and Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31, 233–239 [PubMed: 23360965]

19. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, and Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 [PubMed: 23287718]

20. Tzur YB, Friedland AE, Nadarajan S, Church GM, Calarco JA, and Colaiacovo MP (2013) Heritable custom genomic modifications in Caenorhabditis elegans via a CRISPR-Cas9 system. Genetics 195, 1181–1185 [PubMed: 23979579]

21. Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, Church GM, and Calarco JA (2013) Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat Methods 10, 741–743 [PubMed: 23817069]

22. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, and Church GM (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res 41, 4336–4343 [PubMed: 23460208]

23. Horvath P, and Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327, 167–170 [PubMed: 20056882]

24. Horvath P, Coute-Monvoisin AC, Romero DA, Boyaval P, Fremaux C, and Barrangou R (2009) Comparative analysis of CRISPR loci in lactic acid bacteria genomes. Int J Food Microbiol 131, 62–70 [PubMed: 18635282]

25. Sorek R, Kunin V, and Hugenholtz P (2008) CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol 6, 181–186 [PubMed: 18157154]

26. Ishino Y, Shinagawa H, Makino K, Amemura M, and Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169, 5429–5433 [PubMed: 3316184]

27. Zhang H, Li Z, Daczkowski CM, Gabel C, Mesecar AD, and Chang L (2019) Structural Basis for the Inhibition of CRISPR-Cas12a by Anti-CRISPR Proteins. Cell Host Microbe 25, 815–826 e814 [PubMed: 31155345]

28. Wandera KG, Collins SP, Wimmer F, Marshall R, Noireaux V, and Beisel CL (2019) An enhanced assay to characterize anti-CRISPR proteins using a cell-free transcription-translation system. Methods

29. Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Mastel M, Borner K, Eils R, Grimm D, and Niopek D (2019) Cell-specific CRISPR-Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins. Nucleic Acids Res 47, e75 [PubMed: 30982889]

30. Babu M, Beloglazova N, Flick R, Graham C, Skarina T, Nocek B, Gagarinova A, Pogoutse O, Brown G, Binkowski A, Phanse S, Joachimiak A, Koonin EV, Savchenko A, Emili A, Greenblatt J, Edwards AM, and Yakunin AF (2011) A dual function of the CRISPR-Cas system in bacterial antivirus immunity and DNA repair. Mol Microbiol 79, 484–502 [PubMed: 21219465]

31. Bikard D, Hatoum-Aslan A, Mucida D, and Marraffini LA (2012) CRISPR interference can prevent natural transformation and virulence acquisition during in vivo bacterial infection. Cell Host Microbe 12, 177–186 [PubMed: 22901538]
32. Hatoum-Aslan A, and Marraffini LA (2014) Impact of CRISPR immunity on the emergence and virulence of bacterial pathogens. Curr Opin Microbiol 17, 82–90 [PubMed: 24581697]
33. Sampson TR, Saroj SD, Llewellyn AC, Tzeng YL, and Weiss DS (2013) A CRISPR/Cas system mediates bacterial innate immune evasion and virulence. Nature 497, 254–257 [PubMed: 23584588]
34. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, and Koonin EV (2015) An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol 13, 722–736 [PubMed: 26411297]
35. Koonin EV, Makarova KS, and Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol 37, 67–78 [PubMed: 28605718]
36. Tang Y, and Fu Y (2018) Class 2 CRISPR/Cas: an expanding biotechnology toolbox for and beyond genome editing. Cell Biosci 8, 59 [PubMed: 30459943]
37. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, and Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 [PubMed: 22745249]
38. Xie K, Zhang J, and Yang Y (2014) Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. Mol Plant 7, 923–926 [PubMed: 24482433]
39. Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, and Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31, 688–691 [PubMed: 23929339]
40. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, and Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6, 343–345 [PubMed: 19363495]
41. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, and Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31, 822–826 [PubMed: 23792628]
42. Dow LE, Fisher J, O'Rourke KP, Muley A, Kastenhuber ER, Livshits G, Tschaharganeh DF, Socci ND, and Lowe SW (2015) Inducible in vivo genome editing with CRISPR-Cas9. Nat Biotechnol 33, 390–394 [PubMed: 25690852]
43. Polstein LR, and Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11, 198–200 [PubMed: 25664691]
44. Zetsche B, Volz SE, and Zhang F (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. Nat Biotechnol 33, 139–142 [PubMed: 25643054]
45. Chiang TW, le Sage C, Larrieu D, Demir M, and Jackson SP (2016) CRISPR-Cas9 (D10A) nickase-based genotypic and phenotypic screening to enhance genome editing. Sci Rep 6, 24356 [PubMed: 27079678]
46. O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, and Doudna JA (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature 516, 263–266 [PubMed: 25274302]
47. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV, and Zhang F (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 353, aaf5573
48. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, and Zhang F (2017) RNA targeting with CRISPR-Cas13. Nature 550, 280–284 [PubMed: 28976959]
49. Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, and Zhang F (2017) RNA editing with CRISPR-Cas13. Science 358, 1019–1027 [PubMed: 29070703]

50. Abudayyeh OO, Gootenberg JS, Franklin B, Koob J, Kellner MJ, Ladha A, Joung J, Kirchgatterer P, Cox DBT, and Zhang F (2019) A cytosine deaminase for programmable single-base RNA editing. Science 365, 382–386 [PubMed: 31296651]
51. Klompe SE, Vo PLH, Halpin-Healy TS, and Sternberg SH (2019) Transposon-encoded CRISPR-Cas systems direct RNA-guided DNA integration. Nature 571, 219–225 [PubMed: 31189177]
52. Wienert B, Wyman SK, Richardson CD, Yeh CD, Akcakaya P, Porritt MJ, Morlock M, Vu JT, Kazane KR, Watry HL, Judge LM, Conklin BR, Maresca M, and Corn JE (2019) Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. Science 364, 286–289 [PubMed: 31000663]
53. Trasanidou D, Geros AS, Mohanraju P, Nieuwenweg AC, Nobrega FL, and Staals RHJ (2019) Keeping crispr in check: diverse mechanisms of phage-encoded anti-crisprs. FEMS Microbiol Lett 366
54. Bondy-Denomy J, Davidson AR, Doudna JA, Fineran PC, Maxwell KL, Moineau S, Peng X, Sontheimer EJ, and Wiedenheft B (2018) A Unified Resource for Tracking Anti-CRISPR Names. CRISPR J 1, 304–305 [PubMed: 31021273]
55. Bondy-Denomy J, Garcia B, Strum S, Du M, Rollins MF, Hidalgo-Reyes Y, Wiedenheft B, Maxwell KL, and Davidson AR (2015) Multiple mechanisms for CRISPR-Cas inhibitionby anti-CRISPR proteins. Nature 526, 136–139 [PubMed: 26416740]
56. Lee J, Mir A, Edraki A, Garcia B, Amrani N, Lou HE, Gainetdinov I, Pawluk A, Ibraheim R, Gao XD, Liu P, Davidson AR, Maxwell KL, and Sontheimer EJ (2018) Potent Cas9 Inhibition in Bacterial and Human Cells by AcrIIC4 and AcrIIC5 Anti-CRISPR Proteins. MBio **99**
57. Maxwell KL (2016) Phages Fight Back: Inactivation of the CRISPR-Cas Bacterial Immune System by Anti-CRISPR Proteins. PLoS Pathog 12, e1005282 [PubMed: 26741979]
58. Maxwell KL (2017) The Anti-CRISPR Story: A Battle for Survival. Mol Cell 68, 8–14 [PubMed: 28985512]
59. Maxwell KL, Garcia B, Bondy-Denomy J, Bona D, Hidalgo-Reyes Y, and Davidson AR (2016) The solution structure of an anti-CRISPR protein. Nat Commun 7, 13134 [PubMed: 27725669]
60. Pawluk A, Bondy-Denomy J, Cheung VH, Maxwell KL, and Davidson AR (2014) A new group of phage anti-CRISPR genes inhibits the type I-E CRISPR-Cas system of Pseudomonas aeruginosa. MBio 5, e00896 [PubMed: 24736222]
61. Pawluk A, Davidson AR, and Maxwell KL (2018) Anti-CRISPR: discovery, mechanism and function. Nat Rev Microbiol 16, 12–17 [PubMed: 29062071]
62. Pawluk A, Shah M, Mejdani M, Calmettes C, Moraes TF, Davidson AR, and Maxwell KL (2017) Disabling a Type I-E CRISPR-Cas Nuclease with a Bacteriophage-Encoded Anti-CRISPR Protein. MBio 8
63. Pawluk A, Staals RH, Taylor C, Watson BN, Saha S, Fineran PC, Maxwell KL, and Davidson AR (2016) Inactivation of CRISPR-Cas systems by anti-CRISPR proteins in diverse bacterial species. Nat Microbiol 1, 16085 [PubMed: 27573108]
64. Stanley SY, and Maxwell KL (2018) Phage-Encoded Anti-CRISPR Defenses. Annu Rev Genet 52, 445–464 [PubMed: 30208287]
65. Gangopadhyay SA, Cox KJ, Manna D, Lim D, Maji B, Zhou Q, and Choudhary A (2019) Precision Control of CRISPR-Cas9 Using Small Molecules and Light. Biochemistry 58, 234–244 [PubMed: 30640437]
66. Kocak DD, Josephs EA, Bhandarkar V, Adkar SS, Kwon JB, and Gersbach CA (2019) Increasing the specificity of CRISPR systems with engineered RNA secondary structures. Nat Biotechnol 37, 657–666 [PubMed: 30988504]
67. Raikwar SP, Kikkeri NS, Sakuru R, Saeed D, Zahoor H, Premkumar K, Mentor S, Thangavel R, Dubova I, Ahmed ME, Selvakumar GP, Kempuraj D, Zaheer S, Iyer SS, and Zaheer A (2019) Next Generation Precision Medicine: CRISPR-mediated Genome Editing for the Treatment of Neurodegenerative Disorders. J Neuroimmune Pharmaco/
68. Schermer B, and Benzing T (2019) [Genome Editing with CRISPR/Cas9: First Steps Towards a new Era in Medicine?]. Dtsch Med Wochenschr 144, 276–281 [PubMed: 30759475]
69. Vaughan A, and Yang IA (2019) CRISPR-Cas9 technology: A new direction for personalized medicine in respiratory disease? Respirology 24, 614–615 [PubMed: 31033105]

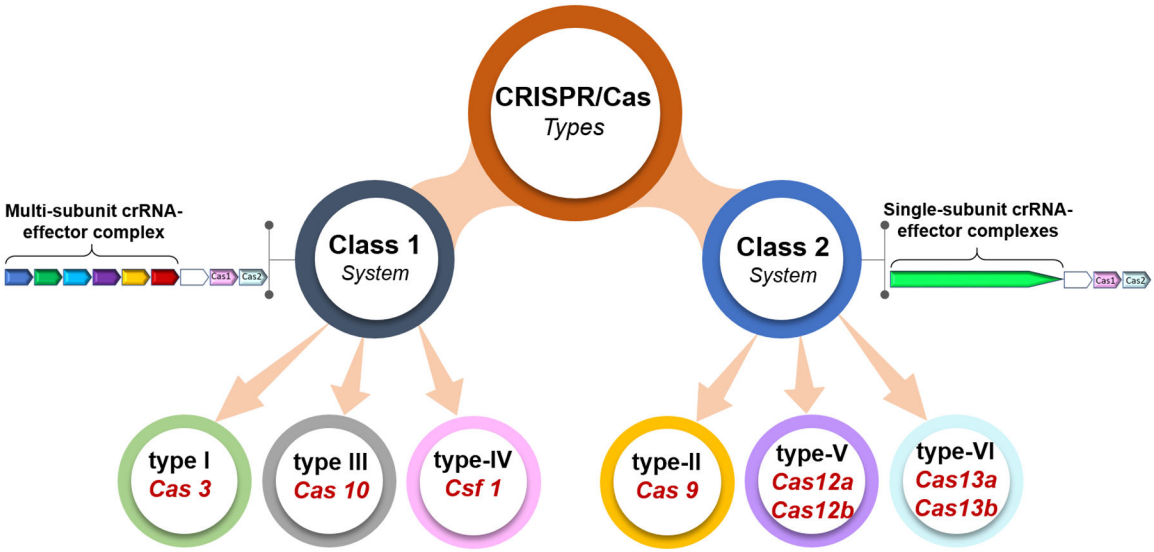

Fig. 1. Classification of CRISPR Systems.

CRISPR Class 1 and 2 systems are depicted.

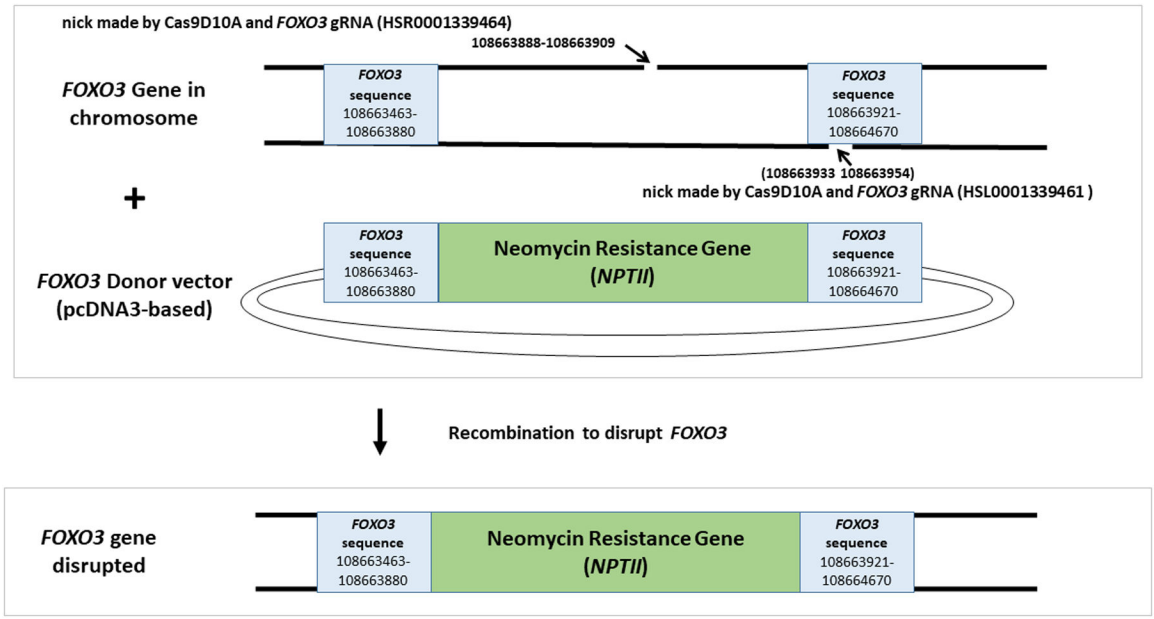

Fig. 2. Schematic of *FOXO3* gene disruption with neomycin resistance cassette.(*NPTII*).

Guide RNAs were employed to nick *FOXO3* gene in mammalian cell lines. The lesions were resolved by homology directed repair with a donor vector that contained a neomycin resistance gene (*NPTII*) flanked by *FOXO3* sequences (that were proximal to the CRISPR Cas9-generated nicked strands of *FOXO3* in the chromosome).

Table 1:

PCR primers utilized to amplify two separate FOXO*3* gene fragments (Arm 1 and Arm 2) for donor vector Gibson assembly reactions

Primer	Sequence*
FOXO3 ARM 1 F	5-GTGCTTTACGGCACCTCGACCCCCCGGCACAACCTGTCACTGC-3’
FOXO3 ARM 1 R	5’-CCGTCTATCAGGGCGATGGCCGCTGTAGAGCATGGGCGAGAG-3’
FOXO3 ARM 2 F	5- CAAATAAAGCATTTTTTTCACTCGGTGGAACTGCCACGGCTG-3’
FOXO3 ARM 2 R	5-GAGTTTGGACAAACCACAACTAGGTCCAAGTCGCTGGGGAAC-3’

*Nucleotides in underlined blue correspond to sequences in pcDNA3 vector. Sequences in black were to amplify the indicated *FOXO3* arm [9].

Table 2.

CRISPR Cas9 mutation frequencies in three mammalian cell lines

Cell Line	G418-resistant isolates screened by western blot analysis	Number of Homozygous Truncation Mutant	Homozygous Mutation Frequency
U87MG Trial 1	77	4*	5%
U87MG Trial 2	50	3*	6%
BT549	56	5	9%
HEK293	77	1	1.3%

* Table originally published in Vazquez *et al* and reproduced with permission [9].

Table 3.

Primers utilized to detect and sequence *FOXO3* gene disruption

Primer Name	Sequence
*FOXO3*F (For detection of disruption)	5’- GTGCTTCAGGATCGCTTCA −3’
Neo cassette R (For detection of disruption)	5’ -TGCATGCTTTGCATACTTCTG-3’
FOXO3 seq. (For sequencing disruption mutants)	5’- CTCGGTTTTGGACCATTCTG −3’

*Table originally published in Vazquez *et al* and reproduced with permission [9].