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Chapter 17

Applying CRISPR-Cas9 Genome Editing to Study Genes Involved in Peroxisome Biogenesis or Peroxisomal Functions

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Abstract

The development and application of the CRISPR-Cas9 technology for genome editing of mammalian cells have opened up a wealth of possibilities for genetically modifying and manipulating human cells, and use in functional studies or therapeutic approaches.

Here we describe the approach that we have been using successfully to generate multiple human cell lines with targeted (partial) gene deletions, i.e., knockout cells, or human cells with modified genomic nucleotide sequences, i.e., knock-in cells, in genes encoding known or putative proteins involved in peroxisome biogenesis or peroxisomal functions.

Key words CRISPR-Cas9, Gene editing, PEX genes, Gene deletion, Gene disruption, Gene knockout

1 Introduction

Primary skin fibroblasts of patients have long been the primary choice and option to unravel and study the roles of genes or the consequences of genetic defects in human peroxisome biogenesis and/or functioning [1, 2]. Biochemical, metabolic, and cell biological characterization of patient cells combined with identification of the underlying genetic defect, via genetic complementation, or a candidate gene approach and Sanger sequencing, has resolved the biochemical and genetic makeup for human peroxisome biogenesis and functioning, and identified the underlying genetic causes of a large number of peroxisomal diseases [2–5]. Whereas such studies in patient cells are still crucial to determine the functional consequences of patient-specific genetic variants [6, 7], other approaches that do not require patient cells have been used to study functional aspects and properties of different peroxisomal proteins. These include overexpression of the

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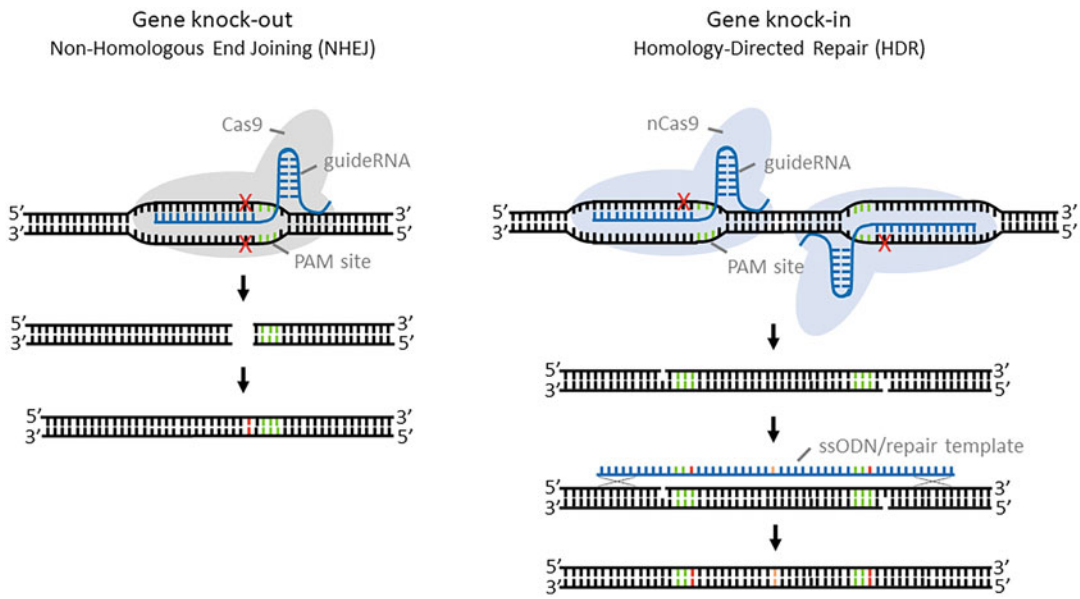

Fig. 1 Principle of CRISPR-Cas9 editing. The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 technology enables site-specific genomic editing in virtually every cell and can be used to generate gene knockouts or gene knock-ins

To create gene knockouts, a single-guide RNA, composed of 20 nucleotides matching the DNA sequence 5′ of a so-called protospacer adjacent motif (PAM) site and a sequence interacting with a Cas9 nuclease, is generated to direct the Cas9 to a specific genomic location, i.e., the gene to be edited. The Cas9 nuclease generates double-stranded DNA breaks, which are then repaired via the non-homologous end joining (NHEJ) DNA repair pathway. This repair is error-prone and results in deletions and/or insertions that thus can disrupt gene function

To create gene knock-ins, two single-guide RNAs are generated that each direct a modified nCas9, displaying nickase activity, to specific genomic locations 5′ and 3′ of the site in the gene to be edited. The nCas9 nickases generate single DNA strand breaks creating 5′ overhang DNA sequences, which stimulate precise DNA repair via homology-directed repair (HDR). Co-transfection of the guide RNAs with a single-stranded oligodeoxynucleotides (ssODN)/repair template, which codes for a single-nucleotide change (indicated as orange in figure) or includes a larger non-homologous DNA insert (e.g. encoding a small reporter; not shown) and contains two homology arms matching the DNA sequences 5′ and 3′ of the genomic site of insertion, results in homologous recombination introducing the desired nucleotide change or insertion in the genomic sequence. To prevent re-cutting by the nCas9 nickases, the PAM sites are modified via the ssODN/repair template (indicated in red)

encoding cDNAs (via plasmids, viral vectors, or genomic integration, e.g., using the Flp-In technology) or knockdown of gene expression by means of RNA interference in human cell lines [8].

The development of genome editing tools, including in particular the easy accessible CRISPR-Cas9 technology (Fig. 1), which allow to genetically and precisely modify human cells at the genomic level has revolutionized the field of molecular biology and now offers ample and increasing opportunities to study a wide variety of functional aspects in human cells. While initially used primarily for ex vivo and academic purposes, the technical developments;

accuracy; increasing efficiency; minor, if any, adverse effects; and biological safety of the technology have made the technology a promising option for therapeutic approaches targeted at correcting disease-causing genetic defects [9–11]. Modifications and improvements of the initial technology, which was mainly used to create targeted genomic deletions, have resulted in the development of many different applications, including knock-in approaches, e.g., to introduce or correct specific genetic variants [9, 11, 12] or insertion of reporter constructs [13], promotor activation approaches [14], whole genome CRISPR screens [15], etc. Further refinements of the technology, creating more specificity and reducing the risk of off-target events and thus making the technique better suited for therapeutic approaches, include base-editing and prime-editing [9, 10].

Here we describe a basic, straightforward protocol that we have been using successfully to introduce a targeted disruption of one or multiple selected genes encoding peroxisomal proteins, or to introduce small DNA fragments or a patient-specific single-nucleotide variant in one of the genes and in a variety of cells.

2 Materials

2.1 Plasmids (Available with MTA via www.addgene.org)

1. pSpCas9(BB)-2A-GFP (pX458) (Addgene plasmid ID: 48138) [12].
2. pSpCas9(BB)-2A-Puro (pX459) (Addgene plasmid ID: 48139) [12].
3. pSpCas9n(BB)-2A-GFP (pX461) (Addgene plasmid ID: 48140) [12].

2.2 Reagents

1. Fast digest buffer (10×), Thermo Scientific, B64.
2. FastDigest *Bpt*I, Thermo Scientific, FD1014.
3. T4 ligase, Roche, 10716359001.
4. ATP (10 mM).
5. QIAprep Spin Midiprep kit, Qiagen, cat. number 12143.
6. jetPRIME® DNA and siRNA transfection, VWR, 101000046.
7. Puromycin 10 mg/mL, Sigma, P9620.
8. Phire Animal Tissue Direct PCR kit, Thermo Scientific, F140WH.
9. Plasmid DNA isolation kit.
10. Standard setup for agarose gel DNA analysis.

2.3 PCR and Sequence Primers

1. LKO1_5_primer, 5′- GACTATCATATGCTTACCGT -3′ (sequence primer for pX458/459/461).
2. Forward and reverse gene-specific primers extended with M13fw and/or M13rev primer sequence (see below).
3. M13fw primer: 5′-TGTAAAACGACGGCCAGT-3′.
4. M13Rev primer: 5′-CAGGAAACAGCTATGAC-3′.

2.4 Transformation of E. coli

1. Competent *Escherichia coli* Stbl3 cells.
2. LB agar plates +50 μg/mL ampicillin.
3. Standard setup for *Escherichia coli* cell transformation.

2.5 Mammalian Cells

1. Hek293, HeLa, HepG2, or other cell line of your choice.
2. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM), supplemented with 25 mM HEPES buffer, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 200 ng/mL fungizone.
3. Phosphate-buffered saline (PBS).
4. Trypsin-EDTA (0.25%), phenol red solution (Gibco, 25300062).
5. Cell culture plates, 96 well.

2.6 Equipment and Antibodies

1. Sony SH800 Cell Sorter (or equivalent).
2. Software: (a) Guide RNA design software resources (http://www.zlab.bio/guide-design-resources); (b) Benchling analysis software (http://www.benchling.com).
3. Standard setup for immunoblot analysis.
4. Anti-ACAA1 antibody, Sigma-Aldrich, HPA007244.
5. Anti-ACOX1 antibody, Abcam, ab184032.

3 Methods

For the generation of (partial) gene deletions (knockouts), the introduction of DNA fragments, or the introduction or correction of (missense) variants in cell lines, we have adapted and partly modified the protocol published by Ran et al. [12], which also should be cited when using this protocol. Using this approach, we have succeeded to generate cell lines with up to three different gene knockouts.

3.1 Design of Guide RNAs

3.1.1 General

1. There are different online software tools available to assist in identifying suitable protospacer adjacent motif (PAM) sites and design optimal guide RNAs for your gene of interest (for current listing, see www.zlab.bio/guide-design-resources). We mostly use the software from Benchling (www.benchling.com).

2. Open the DNA sequence file of the gene of interest in a software application that allows you to identify PAM sites and design guide RNA sequences.
3. Identify potential PAM sites with consensus sequence 5′-NGG-3′ in the coding region (*see* **Note 1**).
4. Select a guide RNA-encoding sequence of 20-nucleotide length matching the DNA sequence located 5′ of the PAM site(s) of choice according to the following considerations:
   (a) To assure the generation of cells with a functional gene knockout, it is recommended to use PAM site(s) as close as possible to the 5′ end of the coding region of the gene.
   (b) Ensure that no potential in-frame start codon (ATG) is present 3′ of the PAM site to exclude the production of an N-terminally truncated protein with residual activity.
   (c) Because the first exon of a gene is often GC-rich, which can complicate PCR amplification used to validate cells by Sanger sequencing (see below), we often opt for PAM sites in the second exon.
   (d) In case multiple transcripts are known to be generated from the gene of interest due to alternative splicing, one should select PAM site(s), which are present in all isoforms of the gene to create complete functional knockout cells. Alternatively, one can choose PAM site(s) that are only present in selected transcripts to disrupt only the expression of these.
5. To avoid off-target activity by Cas9, the target sequence to which the guide RNA-encoding sequence is designed ideally should be unique throughout the genome. Most available online software tools, such as the Benchling software, provide information of potential off-target activity of guide RNA-encoding sequences. When no unique sequence can be found, it is recommended to select those for which potential off-target activity is not expected to interfere with your phenotype. Even then, it is highly recommended to exclude off-target activity via PCR and Sanger sequencing of the predicted genomic areas (as described in Subheading 3.4).
6. We recommend to design guide RNA-encoding sequences targeting DNA sequences 5′ of at least two different PAM sites of the same gene, in order to generate different functional gene knockout cell lines due to different disruptions of the same gene. This will allow to discriminate phenotypic features due to the desired gene knockout from (possible) off-target events.

7. When working with cell lines such as HeLa, Hek293, and HepG2, for which the genome sequence is available (https://cancer.sanger.ac.uk/cosmic), it is highly recommended to verify that there is no silence nucleotide change in the target sequence selected for the guide RNA-encoding sequence as this may affect the efficiency. Alternatively, one may consider to Sanger sequence the selected gene region in the cell line of choice.
8. Although not very common, there are human genes with overlapping, non-coding regions, such as promoter or regulatory regions [16]. Make sure when designing guide RNA-encoding sequences that they do not target such non-coding regions.

3.1.2 Introduction of Small DNA Sequences, Including Single-Nucleotide Variants

1. For the genomic introduction of small DNA sequences or single-nucleotide changes (knock-in), a mutated form of Cas9 is used, which displays nickase activity (*see* Fig. 1 for principle). In contrast to Cas9 nucleases that cleave both strands of DNA substrates, the nickase Cas9 only cuts one DNA strand, introducing DNA nicks, which induce precise DNA repair by homologous recombination, but not NHEJ. To promote the exchange of a genomic DNA sequence by an external DNA "repair" template (containing the desired nucleotide change or insert) via homologous recombination, two guide RNA-encoding sequences are designed, one targeting a PAM site located 5′ and one targeting a PAM site located 3′ of the genomic site of insertion. These PAM sequences should be as close as possible to the genomic site of insertion and located in opposing DNA strands so that the nickase Cas9 will create 5′ overhang sequences.
2. To target and align the single-stranded oligodeoxynucleotides (ssODN)/repair template to the correct genomic location, it should contain 5′ and 3′ nucleotide sequences of ~50 nucleotides (homology arms) matching the DNA sequences 5′ and 3′ of the genomic site of insertion.
3. Modify the PAM sequences used for the targeting of the guide RNAs in the ssODN/repair template to prevent (re-) cutting of the ssODN/repair template after genomic integration. Make sure that the modification of the PAM sequence does not result in a different amino acid after translation.
4. Although this method allows to introduce single-nucleotide changes, there have been alternative approaches developed, including in particular base- and prime editing, which can introduce specific single-nucleotide changes with higher specificity and efficiency [9, 10].

3.2 Cloning of gRNA-Encoding Sequences in pSPCas9 Plasmids

1. Order reverse complementary oligonucleotides matching the guide RNA-encoding sequences.
2. Mix 0.1 nmol of each of the two complementary oligonucleotides dissolved in 1× FastDigest buffer, incubate for 5 min at 95 °C, and let it cool down slowly to room temperature to anneal.
3. For the restriction and ligation reaction, combine 0.2 pmol of annealed oligonucleotides, 100 ng pX458, pX459, or pX461 plasmid, 1× FastDigest buffer, 0.5 mM ATP, FastDigest *Bpi*I, and T4 ligase.
4. Incubate for 5 min at 37 °C to digest the plasmids followed by 1 h incubating at 21 °C to ligate the annealed oligonucleotides into the *Bpi*I sites of the plasmids.
5. Transform the ligation mixtures into Stbl3-competent *Escherichia coli*, and select for colonies containing plasmids on ampicillin 50 μg/mL LB agar plates (overnight at 37 °C).
6. Extract and purify the plasmids from individual colonies using your plasmid DNA isolation method of choice.
7. Verify the sequence of the guide RNA-encoding inserts of the plasmids by Sanger sequencing using the LKO1_5 primer.

3.3 Transfection of pCRISPR-Cas9 Plasmids into Human Cells

Which cell line to use for your experiments may depend on which functional studies you wish to perform (*see* **Note 2**). Virtually, all human cell lines contain peroxisomes, and there are different established cell lines one can use to study peroxisome biogenesis or peroxisomal functions. Most commonly, we use HeLa, Hek293, or HepG2 cell lines.

1. Culture the cell line of your choice in a T75 flask until sub-confluent. Remove medium, and wash with PBS.
2. Detach the cells by incubating with a minimum amount of EDTA/Trypsin solution at room temperature.
3. Add DMEM to dilute the trypsin solution at least 3 times after cells have been detached (to inactivate the trypsin).
4. Plate the cells in a 6-well plate (60–70% confluent), and incubate overnight at 37 °C in a 5% CO_2 incubator.
5. On the next day, after cells are attached, refresh the medium with 2 mL of prewarmed DMEM.
6. Transfect the cells as indicated below using your favorite method. We use jetPRIME DNA Transfection according to the manufacturer's protocol:
   (a) For (partial) gene deletions (knock-out): Use 2 μg of pX458 or pX459 containing the guide RNA-encoding insert (*see* **Note 3**).

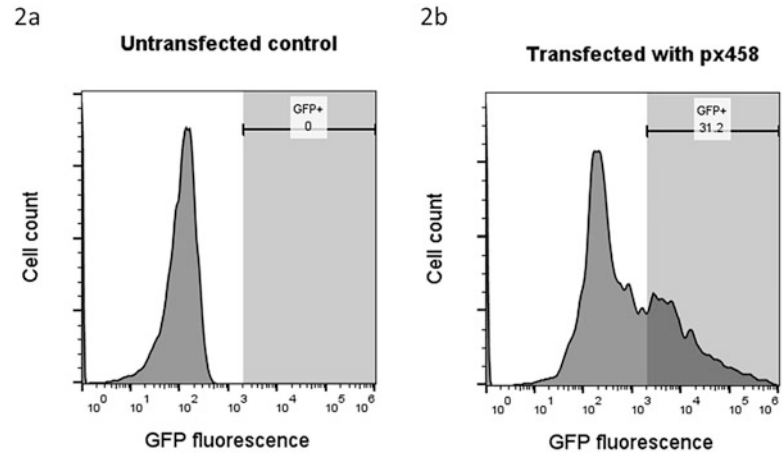

Fig. 2 FACS sorting of cells transfected with pX458 plasmid. The design of the pX458 plasmid (pSPCas9(BB)-2A-GFP; Addgene #48138) [12] ensures the simultaneous expression of a single-guide RNA directed against a PAM site of the gene of interest under transcriptional control of the U6 promoter and of the *S. pyogenes* Cas9 protein coupled with (E)GFP protein, separated by a T2A peptide and under transcriptional control of the CBh promoter. Cells transfected with the plasmid will express the guide RNA, the Cas9 protein, and the (E)GFP protein, which displays green fluorescence that can be used for sorting. (**a**) Fluorescence-activated cell sorting (FACS) analysis of untransfected HeLa cells; (**b**) fluorescence-activated cell sorting (FACS) analysis of cells transfected with pX458. The population of GFP-positive cells is indicated by the gray area. 31.2% of GFP-positive cells were identified after cells were transfected with pX458 plasmid

(b) For insertion or nucleotide changes (knock-in): Combine 0.75 μg of each pX461 plasmid containing the guide RNA-encoding insert with 0.02 nmol ssODN/repair template.

7. Incubate transfected cells at 37 °C in a 5% CO_2 incubator.
8. 48–72 h after transfection, repeat **steps 2–3** to detach the cells. Collect the cells in a 15 mL reaction tube in 0.5–1 mL of DMEM.
9. Vortex the cell suspension, and use FACS sorting to separate cells that express green fluorescent protein (GFP) from cells that do not express GFP. You may use non-transfected cells as a control for non-fluorescent cells (Fig. 2). We use an SH800 Sony sorter with a 488 nm laser for excitation and a 525/50 nm channel for emission. Note: this only applies for cells transfected with pX458 or pX461, in which GFP expression is coupled to the expression of Cas9. (For cells transfected with pX459, *see* **Note 3**).

10. From GFP-positive cells, select cells with low-to-average fluorescent intensity because high intensities correlate with high expression levels of Cas9, which may lead to more frequent off-target events.
11. To generate isogenic "clonal" cell lines, sort the GFP-positive cells as one cell per well into 96-well plates containing 200 μL of prewarmed DMEM. Because on average only 10–20% of the sorted single cells will survive and grow, fill at least two different 96-well plates per guide RNA-encoded sequence containing plasmid.
12. Incubate the sorted cell plates at 37 °C in a 5% CO_2 incubator, and refresh the medium once a week until individual colonies are formed. Only one colony should be observed per well.
13. When the cell populations per well are almost confluent, repeat **steps 2–3** to detach the cells, and transfer half of the cell suspension to a 48-well plate, and use the other half for screening by PCR and Sanger sequence analysis (see below).
14. Incubate the 48-well plates at 37 °C in a 5% CO_2 incubator, and further expand only those cells that after screening by PCR and Sanger sequence analysis are found to have the expected/suitable genomic DNA changes.
15. When the cell cultures have been sufficiently expanded, they can be used for experiments and/or stored via cryopreservation for later use, using standard protocols. For cell lines with cell-doubling times <36 h, it takes 4–6 weeks to expand from single cell to T-175 flasks.

3.4 Screening of Clonal Cells

1. Spin the cells collected during Subheading 3.3, **step 13** for 5 min at 100–200 *g*.
2. Wash the cells once with 500 μL of PBS.
3. Lyse the cells, and use a small aliquot as template for PCR amplification of the edited gene region using a forward and reverse gene-specific primer extended with an M13fw or M13rev sequence (as an easy alternative, we use the Phire Animal Tissue Direct PCR kit).
4. Separate 5 μL of the PCR product on a 1% agarose-TBE gel to confirm specific PCR amplification. PCR products with a clearly different molecular weight indicate the presence of a larger deletion or insertion. We prefer to Sanger sequence only the cells that do not show an apparent change in molecular weight; these cells usually have small deletions or insertions.

5. Analyze the PCR fragments by Sanger sequencing using either the M13fw or the M13rev primer to identify the CRISPR-Cas9 introduced genetic changes. For gene knockouts, these changes can show a wide variability due to the error-prone nature of DNA repair by NHEJ, although some changes appear to occur more frequently.
6. For gene knockout cells, select those cell lines that are homozygous or compound heterozygous for out-of-frame variants (*see* **Notes 4** and **5**). For knock-in cells, select the cells with at least one correct DNA change; these may be homozygous or, more frequently, compound heterozygous for the correct change and out-of-frame changes.

3.5 Validation and Functional Studies

1. To confirm the actual gene knockout, the loss of protein should be confirmed in the gene knockout cell lines by immunoblot analysis using specific antibodies against the protein when available and when recognizing the protein in control cells (*see* **Note 6**).
2. Another important control experiment required to validate the knockout cell line is functional complementation of the cells by means of transfection with an expression plasmid containing the cDNA encoding the wild-type version of the protein disrupted in the cell line. This should reverse the mutant phenotype to wild type. This is an essential control when only clonal cell lines have been obtained based on gene editing of one PAM site. Moreover, this control will allow to discriminate effects due to off-target activities from effects due to the knockout of your gene.
3. When specific antibodies against the protein of interest are not available or not sensitive enough to detect the protein in control cells, functional studies can be performed to confirm that the cell line has the phenotype expected/predicted from the respective gene knockout. For validation of peroxisomal gene knockouts (often reflecting peroxisomal defects previously reported in patient cells), numerous metabolic, biochemical, and cell biological assays are available [1, 6]. Figure 3 provides an example for the validation of a *PEX1* (and *PEX7*) gene knockout in HeLa cells generated by the protocol described above.

4 Notes

1. The Cas9 protein from *Streptococcus pyogenes* encoded by the pX458, pX459, and pX461 plasmids only recognizes a PAM motif with the 5′-NGG-3′ consensus sequence. Cas9 proteins from other bacterial species have other PAM recognition sites and thus may be used for specific gene targeting using a

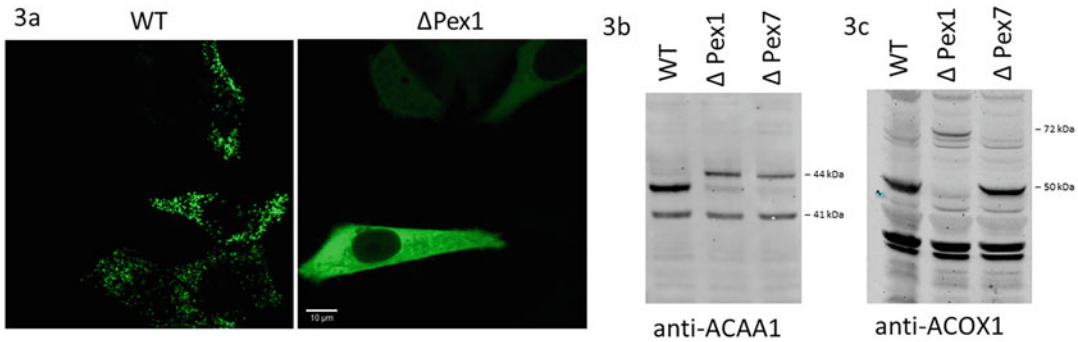

Fig. 3 Functional characterization of HeLa cells with a functional gene knockout for *PEX1* and *PEX7*. The function and consequences of a defect of many peroxisomal proteins are known from studies in patient cells or model organisms. A complete absence of one of the PEX proteins in most cases results in a peroxisome biogenesis defect [1]. (**a**) In HeLa cells with a functional *PEX1* knockout introduced by CRISPR-Cas9 editing following the protocol described in this chapter, peroxisomal matrix proteins or, as shown here, a GFP-PTS1 peroxisomal reporter protein is no longer imported into peroxisomes but mislocalized to the cytosol as visualized using fluorescence microscopy. The peroxisomal import defect in the PEX1-deficient cells is also evident from the lack of processing of the peroxisomal matrix proteins thiolase (**b**) and acyl-CoA oxidase 1 (**c**). Both proteins are synthesized as larger proteins (44 kDa and 72 kDa, respectively), which only after import in peroxisomes become processed (41 kDa and 50 kDa, respectively) but in the cytosol remain unprocessed [17]. In PEX7-deficient HeLa cells, also generated via CRISPR-Cas9 editing, only the peroxisomal import of thiolase, but not Acyl-CoA oxidase, is defective. Shown are immunoblot results prepared with lysates of the cells and incubated with specific antibodies against thiolase (anti-ACAA1) or acyl-CoA oxidase (anti-ACOX1). The unprocessed and processed proteins are indicated. Bar, 10 μm

different PAM site. In some cases, we have used SaCas9 (from *Staphylococcus aureus*), which recognizes PAM sites with the 5′-NNGRR(N)-3′ consensus sequence (available as pX601-mCherry; Addgene plasmid ID: 84039) [11].

2. Most peroxisomal genes are expressed in all cell lines, although their total and relative expression levels may vary markedly between different cell lines. Some peroxisomal genes are only expressed in some cells or tissues, however, such as the AGXT and BAAT genes that are exclusively expressed in hepatic cells and thus cannot be studied in most cell lines. We strongly suggest to first confirm that your gene of interest is expressed in the selected cell line before trying to knock it out.
3. When using pX459 instead of pX458, add 0.25–0.5 μg/mL puromycin 24 h after transfection, followed by culturing for up to 1 week at 37 °C in a 5% CO_2 incubator until most cells have died in the untransfected control. After this, let the cells recover for 1 week in DMEM without puromycin followed by plating one cell per well using a cell sorter to generate clonal cell lines. Note that these transfected cells will not be fluorescent during FACS sorting.

4. Most cancer(−like) cell lines may contain more than two alleles of the gene due to genomic rearrangements and genomic instability. In case CRISPR/Cas9 editing resulted in different genetic changes in more than two alleles, it might be difficult to analyze the sequencing results. In these cases, decomposition software might be helpful (http://shinyapps.datacurators.nl/tide/).
5. Be aware that cells with (small) in-frame deletions or insertions may still allow synthesis of proteins with residual function and thus may not represent functional knockout cells.
6. Out-of-frame mutations often lead to nonsense-mediated mRNA decay. If specific antibodies are not available, you may confirm the loss of the mRNA with RT-PCR. RT-PCR sequencing can also be used to confirm the absence of exon skipping.

Disclaimer

The authors wish to state that they have no commercial interest in nor are in any way connected to any of the companies or suppliers mentioned in this chapter. The reagents, kits, equipment, or software mentioned in this chapter are the ones that we have used in our laboratory for our experiments, but similar reagents, kits, equipment, or software from other suppliers are expected to work equally well.

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