

Chapter 2

Functional Dissection of Mitotic Regulators Through Gene Targeting in Human Somatic Cells

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Abstract

With the human genome fully sequenced (1, 2), biologists continue to face the challenging task of evaluating the function of each of the ~25,000 genes contained within it. Gene targeting in human cells provides a powerful and unique experimental tool in this regard (3–8). Although somewhat more involved than RNAi or pharmacological approaches, somatic cell gene targeting is a precise technique that avoids both incomplete knockdown and off-target effects, but is still much quicker than analogous manipulations in the mouse. Moreover, immortal knockout cell lines provide excellent platforms for both complementation analysis and biochemical purification of multiprotein complexes in native form. Here we present a detailed gene-targeting protocol that was recently applied to the mitotic regulator Polo-like kinase 1 (Plk1) (9).

Key words: Gene targeting, rAAV, human somatic cells, mitosis, Plk1.

1. Introduction

Over the past several years, the methods used to generate knock-out or knockin mutations in human somatic cells have greatly improved in efficiency (3–8). The current method utilizes the high recombination potential of adeno-associated virus (AAV) vectors, requires less than 6 months for homozygous mutation of both alleles in a diploid cell (10–12), and can be divided into several general stages. The first step is to design and assemble a targeting vector containing 5' and 3' arms that are homologous to the locus of interest. If desired, gene inactivation can be made conditional, simply by placing *loxP* sites on either side of a coding

exon of interest. This allows the exon to be rapidly and irreversibly deleted at the desired time, via expression of Cre recombinase. This approach permits the study of genes that are essential for cell proliferation, as expected for many mitotic regulators. The second step is to cotransfect the targeting vector with helper plasmids into HEK293 cells, in order to generate infectious AAV particles. The third step is to infect the cell line of interest with these viruses and subsequently screen for clones that have undergone correct locus-specific recombination. The final step is to verify gene disruption by PCR and Southern blotting assays, and finally, to carry out the procedure a second time to obtain homozygous mutant clones.

Multiple human cell types are amenable to gene targeting with AAV vectors, including both transformed and non-transformed cell lines and primary cells isolated from patients (7, 9, 13). Here we use telomerase-immortalized human retinal pigment epithelial cells (hTERT-RPE) to investigate Plk1 function in a nontransformed, nontumorigenic setting (9). Unlike established cancer cell lines, hTERT-RPE cells tolerate severe (>90%) depletion of Plk1 via RNAi without effect (14). In contrast, we find that homozygous deletion of the *PLK1* locus fully abrogates its function and recapitulates all known mitotic functions of this kinase (9). Furthermore, these *PLK1*^{Δ/Δ} cells could be reconstituted with a variant form of Plk1 that is uniquely susceptible to bulky ATP analogues, enabling chemical genetic dissection of Plk1's roles in late mitosis and cytokinesis (9).

2. Materials

2.1. Cell Culture

1. HEK293 cells (ATCC CRL-1573)
2. Telomerase-immortalized human retinal pigment epithelial cells (hTERT-RPE; ATCC CRL-4000)
3. Medium suitable for propagation of HEK293 cells: D-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Omega Scientific) and 0.1 mg/ml penicillin-streptomycin (Gemini Bio-Products)
4. Medium suitable for propagation of hTERT-RPE cells: D-MEM/F:12 medium with 15 mM HEPES, 2.5 mM L-glutamine, 2.4 g/L sodium bicarbonate (Invitrogen), supplemented with 10% FBS and 0.1 mg/ml penicillin-streptomycin
5. Hank's balanced salt solution (HBSS; Invitrogen)
6. 0.05% Trypsin-EDTA (Invitrogen)

7. Tissue culture-treated 96-, 48-, 24-, 12-, and 6-well plates (Corning)
8. Tissue culture treated T-25 and T-75 flasks (Corning)
9. G418 (Gemini Bio-Products)
10. Presterilized 8-port manifolds (CLPdirect)
11. Repeater Plus pipetter with sterile 50-ml Combitips (Eppendorf) or multichannel P200 and filter tips (Rainin)
12. 100 ml sterile basins (Fisher)

**2.2. Production of
rAAV Particles and
Genomic DNA
Extraction**

1. pNY, Jallepalli lab (9)
2. QuikChange II XL site-directed mutagenesis kit (Stratagene)
3. AAV Helper-free system (Stratagene, 240071)
4. Lipofectamine Transfection reagent/Plus reagent (Invitrogen)
5. OptiMEM I medium (Invitrogen)
6. Disposable cell scrapers (Fisher)
7. Deep-well blocks (BD Biosciences)
8. Wizard SV 96 genomic DNA purification system (Promega, A2371)
9. Beckman-Coulter Allegra 25R with deep-well block rotor (S5700) or Vac-Man 96 Vacuum Manifold (Promega)
10. Multichannel P200 and P1000 pipettors and filter tips (Rainin)
11. QIAampDNA Blood Mini Kit (Qiagen, 51106)

**2.3. PCR Screening
for Knockouts**

1. Thin-wall 96-well plates (Simport) and sealing films (Fisher)
2. Tissue culture-grade water (Sigma)
3. DMSO (Sigma)
4. 10X PCR buffer: 166 mM ammonium sulfate, 670 mM Tris-HCl, pH 8.8, 67 mM MgCl₂, 100 mM beta-mercaptoethanol
5. 10 mM dNTPs (USB)
6. Primers (Integrated DNA Technologies)
7. Platinum Taq polymerase (Invitrogen)
8. Taq Extender (Stratagene)
9. Mineral oil (Sigma)
10. Bio-Rad Sub-Cell Model 96/192 gel system
11. Multichannel P10, P20, P200 pipettors and filter tips (Rainin)

**2.4. FLP-Mediated
Excision of the Neo
Cassette**

1. Fugene 6 (Roche)
2. pCAGGS-FLPe (Gene Bridges)
3. Puromycin (Gemini Bio-Products)
4. Purified adenovirus expressing Cre recombinase (Vector Development Laboratory Baylor College of Medicine)

3. Methods

The following method can be used to generate a conditional knockout of any desired locus in human somatic cells. The targeting strategy should be planned in its entirety before starting work (Fig. 2.1).

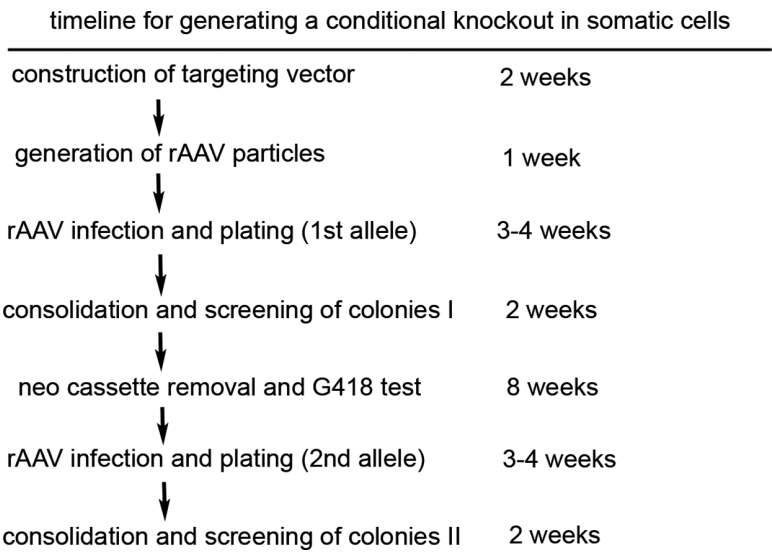


Fig. 2.1. Timeline for generating a conditional knockout in human somatic cells.

3.1. Characterization of the Genetic Locus and Primer Design

1. Choose the exon that you wish to conditionally delete. This exon will need to be flanked by tandemly oriented *loxP* sites, generating a so-called “floxed” allele. Use *Ensembl* to verify that length of this exon in basepairs is not a multiple of three (<http://www.ensembl.org/index.html>). This will ensure that the open reading frame undergoes a frameshift and premature termination after the exon is deleted.
2. Using RepeatMasker (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>), examine a 4-kb region centered on this exon for repetitive DNA content.
3. Choose 5’ and 3’ homology arms of about 1.0–1.5 kb in length, taking care to maximize the unique sequence content in each arm (see Fig. 2.2A and Notes 1 and 2).
4. Design oligonucleotides for amplifying and cloning the homology arms into the shuttle vector pNY, which contains a central *FRT*-neoR-*FRT*-*loxP* cassette (see Fig. 2.2A, primer pairs F1/R1 and F2/R2; see Notes 3 and 4).
5. Design several oligonucleotides for PCR screening and sequencing across both homology arms. The candidate

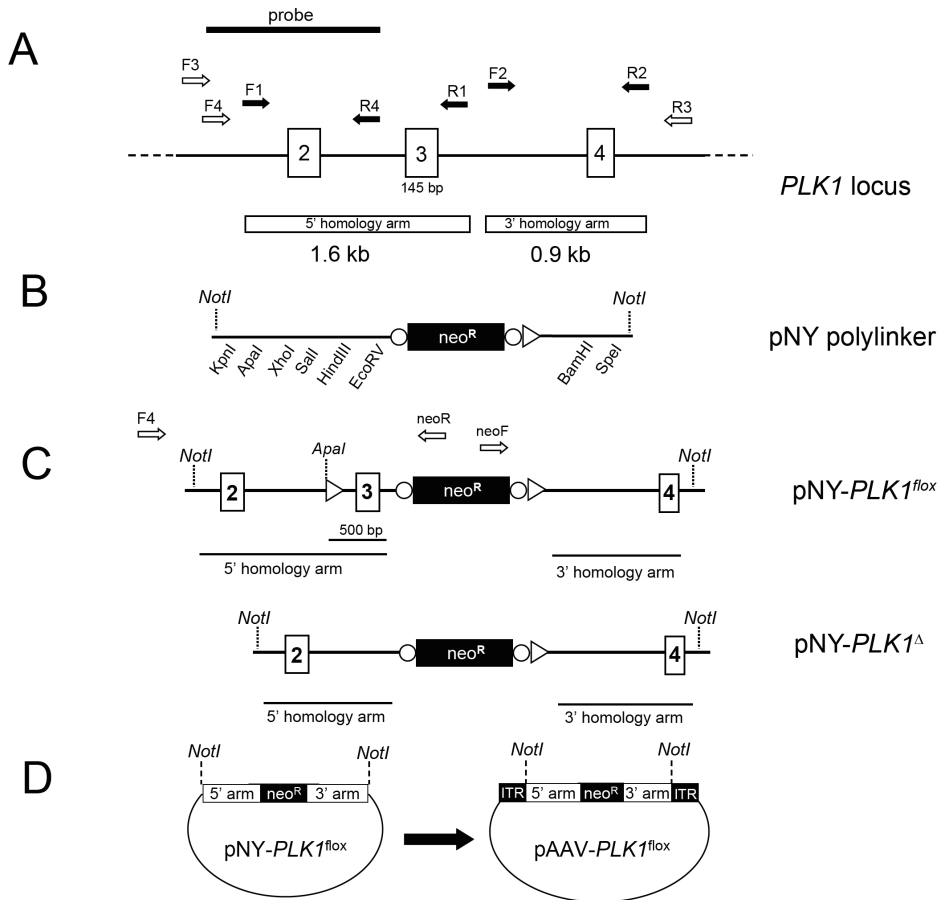


Fig. 2.2. Strategy for targeting exon 3 of *PLK1*. **(A)** Schematic of the 5' end of the *PLK1* locus. Primers used to amplify the homology arms for cloning are denoted by black arrows, and those used for PCR screening are denoted by white arrows. **(B)** Map of the pNY polylinker. *FRT* sites are shown as white circles and the *loxP* site is shown as a triangle. Unique restriction sites for cloning the 5' and 3' homology arms are also indicated. **(C)** Structure of the pNY-*PLK1*^{lox} and pNY-*PLK1*^Δ shuttle plasmids. **(D)** Transfer of the *NotI* fragment from pNY-*PLK1*^{lox} to pAAV generates the final construct (pAAV-*PLK1*^{lox}) used for virus production. ITR, AAV-specific inverted tandem repeats.

screening primers should correspond to unique genomic sequences about 100–500 bp outside the region delimited by the 5' and 3' homology arms (see Fig. 2.2A screening primers F3, F4, and R3).

6. Harvest wild-type RPE-hTERT cells from a T-25 flask and prepare genomic DNA (gDNA) using the QIAamp DNA Blood Mini Kit. Determine the concentration of your DNA by measuring the OD 260 of your sample in a spectrophotometer.
7. Perform test PCR reactions using the conditions specified in Section 3.6 with the candidate screening primers and locus-specific primers (F3/R1, F4/R1, F2/R3; see Fig. 2.2A),

using the gDNA prepared in step 6 as the template. Prepare 10-fold serial dilutions of your gDNA to identify those primers which have optimal sensitivity and specificity (*see Note 5*).

3.2. Construction of the Targeting Vector

1. Using a high-fidelity thermostable polymerase (e.g., Pfu-Turbo, Stratagene), amplify 5' and 3' homology arms from human gDNA (e.g., purified from the cell line of interest) or a human genomic BAC (bacterial artificial chromosome) clone identified by BLAST searching and obtained commercially (Invitrogen or BACPAC Resources).
2. Subclone homology arms into pNY and verify by sequencing (*see Fig. 2.2B*).
3. Introduce a *loxP* site into the appropriate homology arm of the targeting vector by site-directed mutagenesis (QuikChange II XL kit, Stratagene) or linker ligation. This *loxP* site should be in the same orientation as the existing *loxP* site in pNY. The introduced *loxP* site should also be marked with a novel restriction endonuclease site to facilitate downstream analyses. Verify the presence, orientation, and integrity of the inserted elements by sequencing and restriction digestion (*see Note 6*).
4. Restriction digest pNY containing the homology arms with NotI and gel-purify the insert (5' arm-[*FRT*-neoR-*FRT*-*loxP* cassette]-3' arm).
5. Restriction digest the recipient vector pAAV-*lacZ* with NotI. Treat this reaction with 1 μ l of Calf Intestinal Phosphatase (CIP) and leave reaction at 37°C for an additional 15 min. Extract the DNA with phenol:chloroform, ethanol precipitate, and resuspend in 20 μ l water. Gel-purify the NotI digested pAAV vector backbone (*see Note 7*).
6. Ligate the NotI digested insert from step 4 into the pAAV backbone from step 5 (*see Fig. 2.2D*).
7. Transform the ligations and screen the resulting colonies for recombinant AAV plasmids that contain your homology arms (*see Note 8*).
8. Prepare transfection-grade plasmid DNA using standard methods (silica-based kits or isopycnic centrifugation on cesium chloride gradients).

3.3. Generation of rAAV Particles

1. Thaw early-passage HEK293 cells in D-MEM supplemented with 10% FBS and 0.1 mg/ml penicillin-streptomycin (complete D-MEM). Grow cells at 37°C in a tissue culture incubator until 80–90% confluent.
2. Split HEK293 cells into two T-75 flasks and grow at 37°C in a tissue culture incubator until they are at 50–70% confluence.

3. Transfect each flask with 3 μg each of your targeting vector, pHELPER, and pAAV-RC (9 μg total) using Lipofectamine reagent and Plus reagent as follows. Mix DNAs in one sterile 1.5 ml tube with 750 μl OptiMEM I reduced serum medium and 36 μl of Plus reagent. In a second sterile 1.5 ml tube, mix 54 μl of Lipofectamine reagent with 750 μl OptiMEM I reduced serum medium. After 15 min at room temperature, drip the contents of the first tube into the second tube, and incubate for 30 min at room temperature to form DNA/lipid complexes. Wash the HEK293 cells twice with Hank's balanced salt solution (HBSS) and add 7.5 ml OptiMEM and incubate at 37°C for 15 min. Drip this DNA/lipid mixture onto your HEK 293 cells and incubate for 4 h at 37°C. Replace the medium with 15 ml of complete D-MEM and return the cells to the 37°C incubator.
4. Harvest the virus particles three days post-transfection. Begin by transferring the medium from each T-75 to a sterile 50 ml conical tube (*see Note 9*).
5. Add 5 ml of complete D-MEM to each flask, scrape off the remaining cells using a disposable cell scraper, and transfer each cell suspension to the 50 ml conical tube.
6. Set up a dry ice/methanol bath. Freeze the cell suspension in the bath. Transfer the tubes to a 37°C water bath and thaw. When the cell suspension is almost fully thawed, vortex the tubes at maximum speed for 1 min. Repeat this freeze/thaw cycle two more times (*see Note 10*).
7. Spin out the cell debris at 10,000 \times g for 30 min at 4°C. Decontaminate the outside of each tube with 70% ethanol and place within a sterile tissue culture hood.
8. Carefully transfer the supernatant fraction to a new 50 ml conical tube. This is your working stock of infectious rAAV particles. Aliquot in 5–10 ml fractions and store at –80°C until ready to use.

3.4. Infection of Target Cells with rAAV Particles and Selection for Stable Integrants

1. Thaw an early-passage stock of your target cells of interest (hTERT-RPE cells in the example given here). Passage cells 1–2 days prior to infection so that they are ~30% confluent in a T-75 flask on the day of infection (*see Note 11*).
2. Wash the cells twice with 5 ml of HBSS. Add 6 ml of D-MEM/F:12 supplemented with 10% FBS and 0.1 mg/ml penicillin-streptomycin (complete D-MEM/F:12) + 6 ml of your rAAV preparation. Incubate at 37°C in a tissue culture incubator for 4 h.
3. Bring the volume up to 15 ml with complete D-MEM/F:12. Allow infection to continue for 48 h at 37°C in a tissue culture incubator.

4. Plate the rAAV-infected cells at sufficient dilution into 15×96 well plates to obtain no more than one clone per well. To do this, remove the medium from the flask and trypsinize the cells. Transfer the trypsinized cell suspension into a tissue culture vessel containing 300 ml of complete D-MEM/F:12+ 0.4 mg/ml G418. Mix well by gentle inversion or pipetting up and down and pour the cell suspension into a sterile basin. Dispense 200 μ l/well into 15×96 -well plates using a repeat pipetter with an 8-port manifold attachment or a multichannel pipette.
5. Wrap the stack of 96-well plates in plastic wrap and incubate at 37°C in a tissue culture incubator for 2–3 weeks until colonies form (*see* **Note 12**).

3.5. Consolidation of Colonies and gDNA Preparation

1. Two weeks after plating, inspect the 96-well plates for colonies using an inverted bright field microscope (*see* **Note 13**).
2. To consolidate colonies for PCR screening, carefully aspirate the media from the plates using an 8-port manifold attached to a vacuum line. Apply 50 μ l of 0.05% Trypsin-EDTA using a P200 multichannel pipette and incubate plates for 10 min at 37°C in a tissue culture incubator.
3. Using an inverted brightfield microscope, verify that the colonies are fully detached from the well. Using a multichannel P200 pipette, disaggregate the cells and transfer 40 μ l from each well to a deep-well block. We regularly pool 5–10 plates per deep-well block in order to reduce the number of PCR reactions required for the initial screen. However, single clones can also be analyzed if desired (*see* **Notes 14 and 15**).
4. Using the P200 multichannel pipette or a repeat pipetter with a 50 ml Combitip and 8-port manifold, refeed the 96-well plates with 190 μ l of D-MEM/F:12 complete medium per well. Cover the plates in plastic wrap to minimize evaporation and return to the 37°C tissue culture incubator.
5. To prepare gDNA, add a sufficient quantity of HBSS to each well of the deep-well block to bring it to a final volume of 300 μ l. Purify gDNA using the Wizard SV 96 genomic DNA purification kit (Promega). It is recommended that the eluted gDNA be used immediately for PCR screening, but if this is not feasible, it can be stored at –20°C (*see* **Notes 16 and 17**).

3.6. PCR Screen

1. All PCR reactions are done in thin-walled 96-well plates. The reaction conditions per reaction are as follows:

dd H ₂ O	6.3 μ l
10 \times PCR buffer	1.25 μ l
10 mM dNTPs	1.5 μ l
DMSO	0.75 μ l
primerF (350 ng/ μ l)	0.25 μ l
primerR (350 ng/ μ l)	0.25 μ l
platinum Taq polymerase	0.125 μ l
Taq extender	0.125 μ l
genomic DNA	2.0 μ l

Prepare a master mix (100 reactions per plate) and dispense 10.5 μ l to each well. Add 2 μ l of gDNA to each reaction using a P10 multichannel pipette and filter tips. Overlay each well with a drop of mineral oil and cover the plate with a piece of sealing film (*see* **Note 18**).

- Place the plate in a thermal cycler and cycle as follows: 94°C \times 30 s (1 cycle); 94°C \times 15 s, 63°C \times 30 s, 70°C \times 2 min (4 cycles); 94°C \times 15 s, 60°C \times 30 s, 70°C \times 2 min (4 cycles); 94°C \times 15 s, 57°C \times 30 s, 70°C \times 2 min (40 cycles; *see* **Note 19**).
- After completion of PCR reactions, remove the sealing film and add 12.5 μ l of 2 \times DNA loading dye to each reaction.
- Using a P20 multichannel pipette, load 12.5 μ l of each reaction onto a 0.8% agarose gel containing ethidium bromide cast with 104 wells (multichannel compatible). Run the gel at 150 volts for 30–45 min and photograph on a UV light box.
- Identify the wells giving rise to correct PCR products (*see* **Fig. 2.3B**, white arrowhead).
- Gel-purify positive PCR products from step 4 above and digest with the appropriate restriction enzyme (chosen during the design stage in **Section 3.2**) to assess where recombination occurred relative to the exogenously introduced *loxP* site (*see* **Fig. 2.3C** and **Note 20**).
- Go back to your stack of 96-well plates and trypsinize those wells comprising each PCR-positive pool with 50 μ l of 0.05% Trypsin-EDTA. Transfer each well to a new well of a 96-well tissue culture plate and add 150 μ l of complete D-MEM/F:12. Return the plate to the 37°C tissue culture incubator and grow the cells until they reach confluence.
- Prepare gDNA using the SV96 genomic DNA purification kit and rescreen individual clones by PCR. These clones are your targeted heterozygotes (i.e., cells with a *floxneo*/+ genotype; *see* **Note 21**).
- Expand your heterozygote clones to sufficient quantities and prepare gDNA using the Blood Mini Kit. Use this

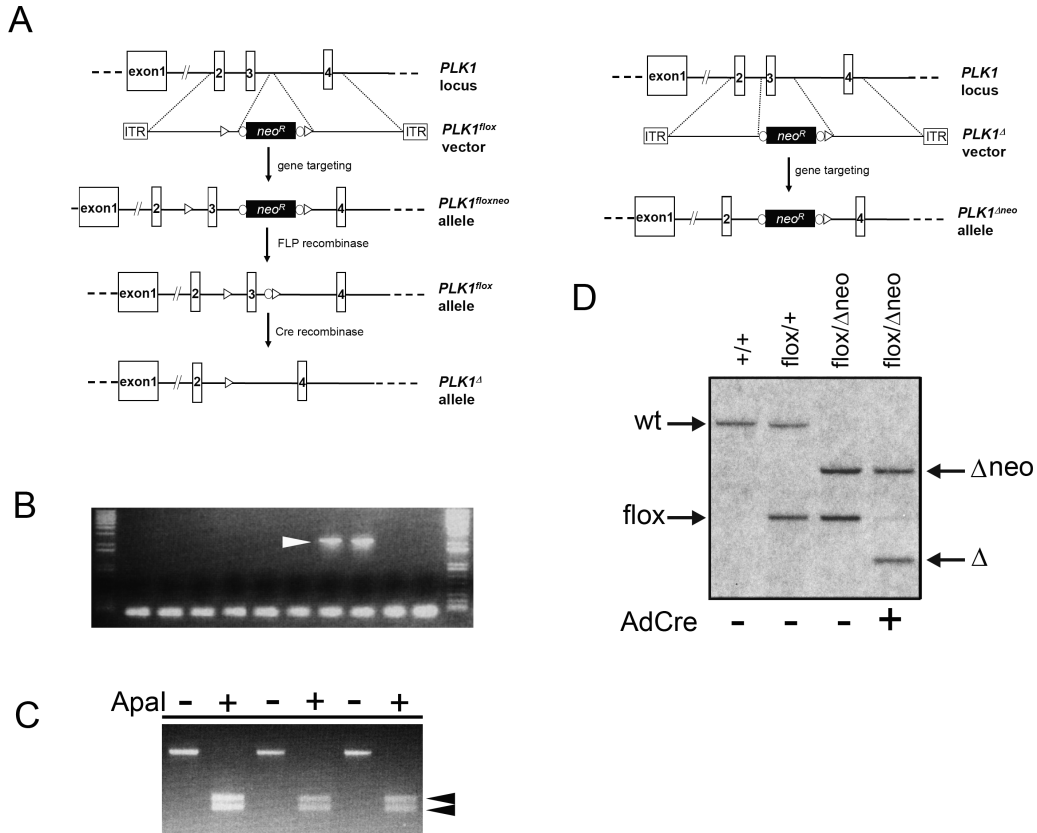


Fig. 2.3. Generation of *PLK1* conditional-knockout cells. **(A)** Structure of *PLK1* alleles generated after gene targeting, removal of the neomycin-resistance cassette, and deletion of exon 3. In this instance, the first allele of *PLK1* was targeted with a conditional-null vector (*left panel*), and the second allele was targeted with a constitutive-null vector (*right panel*). **(B)** Example of a positive “hit” from a genomic PCR screen using F4 and neoR primers. The expected product of 2266 bp is marked by a white arrowhead. **(C)** Apal restriction digests demonstrate that recombination between the 5' homology arm and the chromosomal locus took place upstream of the loxP site between exons 2 and 3; in other words, a favorable crossover occurred. Fragments of 1107 bp and 1159 bp are highlighted by black arrowheads. **(D)** Verification of genotypes by Southern blotting. Genomic DNAs were digested with BamHI and SacI and hybridized with a [³²P]-labeled probe (see Fig. 2.2A). Wild-type (4.8 kb), *flox* (2.1 kb), *Δneo* (3.2 kb), and *Δ* (1.6 kb) alleles are indicated with black arrows. Note that conversion of the *PLK1^{flox}* allele to a *PLK1^Δ* allele requires infection with an adenovirus expressing Cre recombinase (AdCre).

gDNA for additional PCR and Southern blot analyses to verify correct recombination at the locus (15).

- Expand heterozygote clones for long-term cryopreservation.

3.7. Removing the Neomycin Cassette by FLP Recombinase and Testing the Functionality of the “Floxed” Allele

- Expand heterozygously targeted (*floxneo/+*) cells to 60–80% confluence in a T-25 flask (see Note 22).
- Transfect heterozygously targeted cells with the pCAGGS-FLPe plasmid as follows. In a sterile 1.5 ml tube combine 9 μl Fugene with 500 μl of OptiMEM I and incubate at room temperature for 5 min. Add 3 μg of pCAGGS-FLPe,

mix, and incubate at room temperature for 15 min. Wash the target cells twice with HBSS and replace with 2.5 ml of complete D-MEM/F:12. Drip the DNA/Fugene mix onto the cells and return the flask to the 37°C incubator (*see Note 23*).

3. Trypsinize the cells 24 h post-transfection, and plate into a T-75 containing 3 µg/ml puromycin. Maintain the puromycin selection for 48 h, changing the medium after 24 h to remove the bulk of dead cells (*see Note 24*).
4. After selection, replace the medium with complete D-MEM/F:12 medium lacking puromycin and allow the cells to recover at 37°C in a tissue culture incubator for several days until they reach about 80% confluence.
5. Trypsinize the cells and prepare dilutions of the cell suspensions so that you can plate the cells using a multichannel pipette into 2–4 96-well plates at a density of 0.5 cells/well and 2.5 cells/well.
6. Wrap the plates with plastic wrap and allow them to grow for 2–3 weeks at 37°C in a tissue culture incubator until colonies form.
7. Check the plates for colonies using a brightfield microscope and identify wells that contain single colonies. Trypsinize these wells and transfer 48–96 clones to a new 96-well plate. Add complete medium and expand cells to confluence. These are your candidate *flox*/+ cells.
8. Prepare gDNA from the entire 96-well plate as in **Section 3.5**.
9. Set up a PCR screen using a *neo*-specific primer (*neo*R; **Fig. 2.2C**) and a locus-specific primer to identify clones that have lost the *neo* cassette due to FLP-mediated excision (*see Note 25* and **Fig. 2.3A**).
10. Expand putative *flox*/+ clones to sufficient quantities and prepare gDNA using the Blood Mini Kit. Verify the *flox*/+ genotype by PCR and Southern blotting (*see Fig. 2.3D*).
11. Prior to the 2nd allele targeting, plate 10⁶ *flox*/+ cells into complete D-MEM/F:12 medium + 0.4 mg/ml G418 in a T-75 and allow cells to grow for 2 weeks. Check for complete G418 sensitivity by scoring for any colony growth (*see Note 26*).
12. Seed *flox*/+ cells in a T-25 at a confluency of ~10%. The following day, when the cells reach ~20% confluence, infect with adenoviruses expressing Cre recombinase (AdCre (*see Section 2.4*)) at an MOI of 100–200.
13. Remove the AdCre-containing medium 24 h after infection and replace with complete D-MEM/F:12 medium.

14. Harvest the cells 48 h after infection and prepare gDNA using the Blood Mini Kit for Southern blotting to confirm the functionality of the *loxP* site (i.e., deletion of floxed sequences; see **Fig. 2.3A**, *PLK1*^Δ allele).

3.8. Targeting the Second Allele

1. Thaw and expand early-passage *flox/+* cells that have passed the G418 sensitivity test (described in **Section 3.7, step 11**) to a T-75 flask, such that they are ~30% confluent on the day of rAAV infection.
2. Thaw your rAAV virus preparation made in **Section 3.3**.
3. Infect *flox/+* cells with rAAV particles as in **Section 3.4** above (see **Notes 27–29**).
4. After 2–3 weeks, check for G418-resistant colonies using an inverted brightfield microscope.
5. Consolidate colonies and prepare gDNA as in **Section 3.5**.
6. PCR screen colonies and identify individual PCR positive wells as in **Section 3.6**.
7. To identify floxed homozygotes (*floxneo/flox*), perform a secondary PCR screen on the clones that scored positively in the first PCR screen, but in this case use locus-specific primers that span the *loxP* site, rather than a *neo*-specific PCR primer. Gel-purify PCR products and digest with the restriction enzyme used to mark the *loxP* site. The PCR product of a bi-allelic mutant (*floxneo/flox*) should cut completely, whereas the PCR product of a monoallelic (*floxneo/+*) mutant will cut only partially (less than 50%, due to random reannealing of the Watson and Crick strands during PCR).
8. Expand candidate *floxneo/flox* clones to verify their genotype by Southern blotting.
9. Expand candidate *floxneo/flox* clones for cryopreservation.
10. Generate *flox/flox* cells by removal of the neomycin cassette as in **Section 3.7**.
11. Expand early-passage *flox/flox* cells to 20–30% confluence and infect with AdCre.
12. 24 h after infection remove the AdCre containing medium and replace with complete D-MEM/F:12 medium.
13. Harvest cell pellets at 24 h intervals for several days post AdCre infection.
14. Prepare gDNA using the Blood Mini Kit to verify homozygous deletion of the targeted exon by Southern blotting (see **Fig. 2.3D**).

4. Notes



1. Where possible, minimize the repetitive DNA content of each homology arm to maximize the efficiency of locus-specific integration.
2. The packaging limit of AAV is 4.7 kb. Targeting vectors containing sequences larger than this will not package well, resulting in extremely low titers of transducing virus.
3. The NetPrimer program is invaluable in generating effective screening primers (<http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html>).
4. The pNY vector is optimized for conditional knockouts, as marker removal is controlled by the Flp/FRT recombination system, whereas conditional exon removal is driven by Cre/lox recombination.
5. We stress the importance of testing the screening primers in order to identify those that give a strong and specific PCR product from very low levels of DNA (typically about 30 copies/reaction). For these tests, each screening primer is paired with a locus-specific primer that gives a PCR product of roughly the same size as that we wish to detect in the actual screen (2.5–3.5 kb). It may be helpful to vary the concentration of DMSO while testing your screening primers to determine optimal amplification conditions.
6. To avoid interference with splicing, we recommend placing the *loxP* site at least 100 bp upstream of the 5' end or downstream of the 3' end of the targeted exon. At the same time, the distance between the *loxP* site and the *neomycin*-resistance cassette should be minimized in order to reduce the chances of a nonproductive crossover between these two elements.
7. The direct juxtaposition of two inverted tandem repeats (ITRs) results in plasmid instability. For this reason we use pAAV-*lacZ* as the source of the pAAV vector backbone.
8. The 5' and 3' ITRs in pAAV contain *SmaI* sites that can be used to confirm successful transfer of the *NotI* fragment from pNY to pAAV. The orientation of the insert does not seem to affect gene targeting efficiency as both the (+) and (–) strands are packaged.
9. Transfection of HEK293 cells with AAV producer plasmids may cause a cytopathic effect, but this is not strictly correlated with high-titer virus production.
10. Freezing the cells should take about 10 min in the dry ice/methanol bath. Remove the cells from the 37°C bath

when nearly all of the suspension is thawed. In our hands this takes about 13 min.

11. It is important to use early-passage target cells for rAAV infection.
12. Without removing the plastic wrap, check the stacks 3–5 days after plating to make sure the media is not yellow and the G418 selection is working.
13. The number of colonies can vary from infection to infection. We often get anywhere from 300 to 1500 colonies per experiment. Of these, typically 1–10% represent locus-specific recombinants. We find that HCT116 cells form compact colonies, whereas hTERT-RPE colonies are looser in structure because of the greater motility of this cell type.
14. The number of plates that you pool will depend on the total number of G418 colonies obtained in the experiment. If you have ~1 colony in every well, you will want to pool a maximum of 5 plates. However, if you have many fewer colonies, you may want to pool 10 plates. If you consolidate more than 5 plates in a deep-well block, be sure to adjust the volume of cells taken from each well such that the total volume in each well of the deep-well block is 300 μ l (e.g., for 10 plates, take 30 μ l from each well). Be sure to label each individual plate with a unique identifier so that you can accurately deconvolve your PCR positives from the pool stages.
15. The deep-well block can be stored at -20°C for a day or two or used immediately for gDNA preparation. We usually freeze the cells in the deep-well block for several hours prior to gDNA extraction to help with the lysis. If storing, cover the deep-well block with a piece of sealing film. Keep in mind that your candidate targeted heterozygotes (*floxneo/+* cells) will be confluent in several days and so it is critical to complete the PCR screening to identify positive clones in this time frame.
16. This protocol is optimized for a centrifuge outfitted with a swinging-bucket rotor that can accommodate deep-well blocks. Alternatively, one can use the Vac-Man 96 Vacuum Manifold with comparable results.
17. After preparing gDNA, we recommend setting up the PCR screen immediately as the gDNA may not be very stable and is susceptible to degradation with freeze/thaw cycles.
18. Using mineral oil avoids potential problems with reaction evaporation and cross-contamination.
19. Extension time may be varied according to the expected size of the PCR product (1 min per kb).
20. If you are only able to successfully PCR screen across the homology arm that does not contain the *loxP* site (i.e., you

cannot determine a favorable crossover), you will have to expand PCR positive clones and try to rescreen the *loxP*-containing homology arm using higher purity DNA prepared by the DNA Blood Mini Kit.

21. You should expect to identify a single PCR positive clone from each pool you deconvolve. If your initial plates contain multiple clones per well, a subsequent round of subcloning by limiting dilution will be necessary to reach clonality (and confirm heterozygosity) prior to the removal of the neomycin cassette.
22. We recommend expanding at least two independently derived *floxneo*/+ clones for FLP mediated excision of the *neo* cassette in order to eliminate clonal bottlenecks in downstream analyses.
23. It is helpful to transfect an extra flask of *floxneo*/+ cells with a GFP-expressing plasmid, which provides both an indication of transfection efficiency and a negative control for puromycin selection. For HCT116 cells we transfect pCAAGS-FLPe using the Lipofectamine Plus protocol as described in **Section 3.3**, as this gives a higher transfection efficiency than Fugene in this cell type.
24. We find that the puromycin selection is more effective for HCT116 cells than RPE cells. Because of this, we typically screen more FLP-transfected RPE clones (96) than HCT116 clones (48) in order to ensure recovery of *neo*-excised derivatives.
25. It can be helpful to perform a control PCR using locus-specific primers to confirm that your gDNA is present and of sufficient quality for screening.
26. Occasionally one may detect the presence of a small fraction of G418-resistant cells in a putatively *neo*-excised clone, presumably through low-level contamination by the parental clone or a *neo*-positive sibling. In our experience, such contamination can always be cured by an additional round of limiting dilution.
27. The amount of virus used in the second round of infection can be adjusted depending on the number of colonies obtained from the first round of infection. However, application of large amounts of virus (in excess of 6 ml per T-75) may also increase the risk of multiple integration events.
28. In general, homology arms do not have to be made from DNA that is isogenic to the target cell line. However, in rare cases where substantial polymorphisms exist, it is possible that these sequence differences may result in preferential and recurrent integration into one allele. In this unusual situation, we have found that reconstructing targeting vectors based on the specific polymorphisms present in the second (untargeted) allele can overcome this allele bias and

- generate a homozygous knockout cell line (M.-E.T. and P.V.J., unpublished data).
29. It is also possible to target the second allele with a modified construct in which the targeted exon is deleted outright, rather than flanked by *loxP* sites (*see* **Fig. 2.3A**, right panel). Although this requires some additional cloning steps, it eliminates the need to consider where crossovers occur relative to the targeted exon. This can be beneficial in instances where the distribution of crossovers obtained with the first-allele targeting construct is found to be biased towards nonproductive integrants that failed to incorporate both *loxP* sites.

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