STAR Protocols

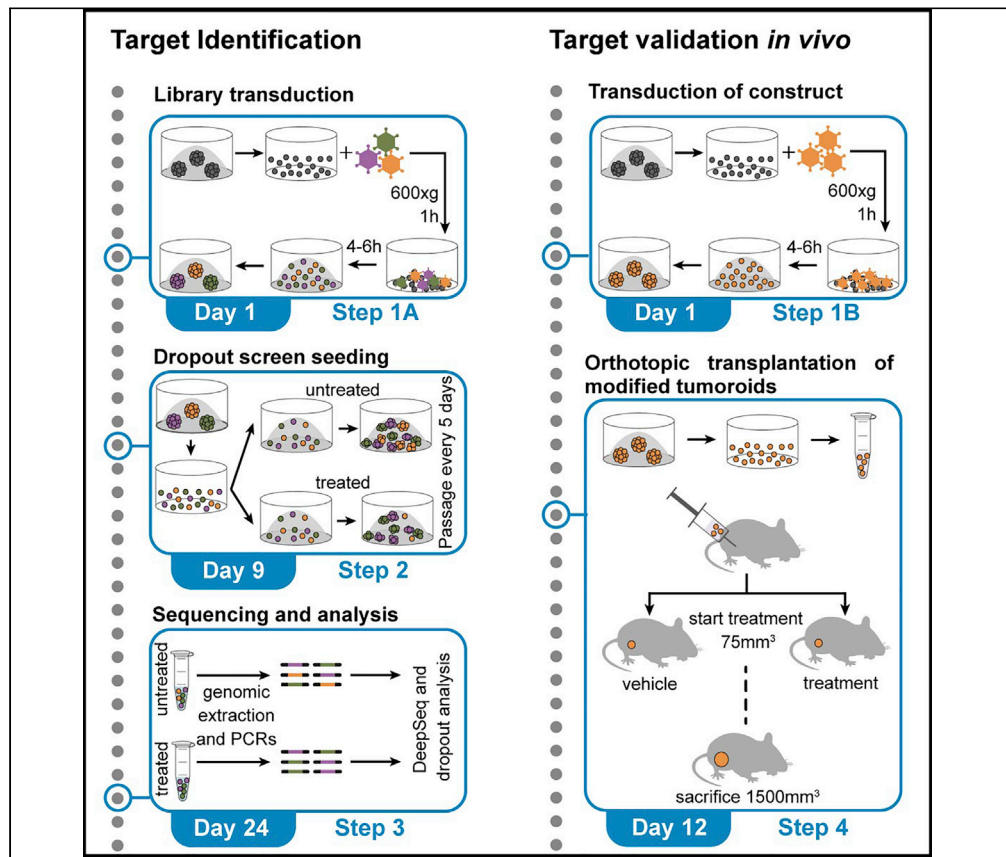**Protocol**

Functional genetic dropout screens and *in vivo* validation of candidate therapeutic targets using mouse mammary tumoroids

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Highlights

Functional genetic dropout screens using BRCA1/2-deficient mouse mammary tumoroids

Screen analysis and identification of candidate drug targets

In vitro genetic modification and orthotopic transplantation of tumoroids

Rapid and straightforward testing of candidate drug targets *in vivo*

We have previously developed 3D tumoroids derived from genetically engineered mouse models (GEMMs) of BRCA1/2-deficient breast cancer. Here, we describe how to genetically modify tumoroids, use them for functional genetic dropout screens, and carry out orthotopic transplantation of modified tumoroids. The purpose of this protocol is to screen for therapeutic targets and allow rapid and straightforward *in vivo* validation of the candidate targets.

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Functional genetic dropout screens and *in vivo* validation of candidate therapeutic targets using mouse mammary tumoroids

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SUMMARY

We have previously developed 3D tumoroids derived from genetically engineered mouse models (GEMMs) of BRCA1/2-deficient breast cancer. Here, we describe how to genetically modify tumoroids, use them for functional genetic dropout screens, and carry out orthotopic transplantation of modified tumoroids. The purpose of this protocol is to screen for therapeutic targets and allow rapid and straightforward *in vivo* validation of the candidate targets.
For complete details on the use and execution of this protocol, please refer to Paes Dias et al. (2021b).

BEFORE YOU BEGIN

Poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi) are a promising new therapeutic approach for the treatment of cancers with homologous recombination deficiency such as BRCA1/2-mutated cancers. Despite the success of this approach, drug resistance remains a clinical hurdle (Paes Dias et al., 2021a).

Tumoroids can be easily genetically modified and, compared to 2D, screens in 3D tumoroids have the advantage of using cells that retain crucial features of the original tumor (e.g., morphology, drug response, 3D structure) and that require a small culturing surface area (1 million tumoroid cells can fit in a few wells while 1 million 2D cells require one or more 15 cm dishes). The protocol below describes the specific steps to carry out functional genetic dropout screens using PARPi-resistant mouse mammary tumoroids to identify new therapeutic targets to improve response to PARPi and revert resistance, as in Paes Dias et al. (2021b). Additionally, this protocol describes how tumoroids can be used to validate the identified candidate targets *in vivo*.

The steps below focus on the use of tumoroids to study PARPi response, however a similar protocol can be used to identify drug targets to sensitize to other therapeutic interventions. While this protocol assumes the use of previously established tumoroid lines (Annunziato et al., 2019; Duarte et al., 2018), one could also derive new tumoroid lines with different characteristics (e.g., GEMMs with different genotype or treated with other inhibitors) as previously described in Duarte et al., (2018).

Virus preparation and concentration

⏱ Timing: 4 days

1. Culture HEK293FT cells in IMDM media supplemented with 10% FBS and 50 units/mL penicillin/ streptomycin.
2. Produce lentiviral stocks, pseudotyped with the VSV-G envelope, by transient transfection of HEK293FT cells as described before (Follenzi et al., 2000).
   a. Seed 7–8 million cells in one 15 cm dish.
   b. The next day (approx. 24 h), transfect cells with the library/construct of interest.
   c. The following day, refresh medium (16 mL).
   d. Collect viral supernatant 48 h post transient transfection using a 0.22 μm filter and syringe to avoid collection of cells into the supernatant.
3. Concentrate viral supernatant using ultracentrifugation.
   a. Upon collection of viral supernatants, ultracentrifuge for 2 h at 70,000 × *g* at 7°C in ultracentrifuge specific tubes.
   b. Remove supernatant by inverting the tube once and let it dry while inverted on a tissue for 2 min. The viral pellet will stay attached to the tube.
   c. Resuspend the viral pellet corresponding to one 15 cm plate in 200 μL PBS.

   Note: Per construct, we suggest collecting supernatant from two 15 cm dishes. For sgRNA/ shRNA libraries, the number of plates may increase according to the size of the screen (complexity/coverage).

   Optional: Instead of ultracentrifugation, one can use commercial protein concentration filter tubes (e.g., MilliporeSigma UFC905024).

4. Calculate lentiviral titer. This can be done by using a qPCR lentivirus titration kit for small sample volumes, such as the kit from Applied Biological Materials https://www.abmgood.com/ qpcr-lentivirus-titration-titer-kit.html. Ideal virus titer should vary from 0.2×10^9 to 1×10^9 units/mL.

   Optional: Lentiviral titer can also be calculated by limiting dilution and colony counting.

Culture and passage of tumoroid lines

   ⏱ Timing: 1 h

5. Spin down tumoroids at 300 × *g* for 5 min at 4°C. If tumoroids have been dissociated and are single cells (please see dissociation of tumoroids into single cells), centrifugation should be done at 1,000 *g*.

   ⚠ CRITICAL: Tumoroids tend to stick to plastic surfaces. Always coat tubes and pipettes with media before use by inverting the tube or pipetting medium up and down. Coating can also be done with bovine serum albumin (BSA).

6. Resuspend pellet in Cultrex Reduced Growth Factor Basement Membrane Extract (BME) Type 2 mixed 1:1 with cold mouse mammary gland tumoroid complete medium (ADDF6+) and seed in 40 μL droplets per well, on 24-well suspension plates. Troubleshooting 1. For regular culture, we usually seed three to six droplets/wells for each line.

   ⚠ CRITICAL: It is very important to maintain BME at 4°C as it solidifies when at temperatures superior to 8°C. If this happens, freezing BME at −20°C followed by thawing at 4°C is required to revert to liquid form.

7. Incubate 24-well plate at 37°C for 30 min and add 0.5 mL warm ADDF6+ medium per well. ADDF6+ medium must be pre-warmed at 37°C. Cold medium disrupts BME droplets.
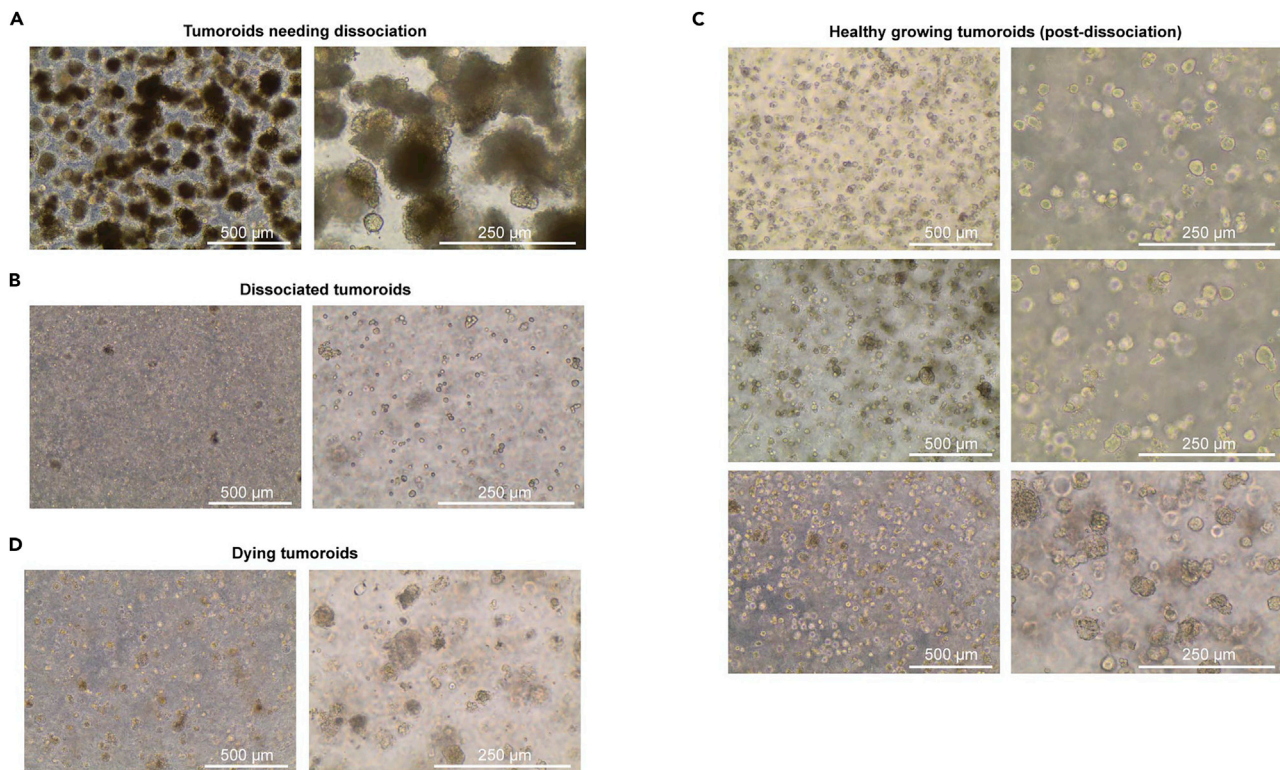
Figure 1. Tumoroid cultures

(A) Large tumoroids in need of dissociation in order to maintain proliferation. Medium color changes to yellow very fast (1–2 days), tumoroids form large dense aggregates that appear opaque.

(B) Tumoroids immediately after dissociation.

(C) Healthy growing tumoroids post-dissociation. After 3–5 days, the medium starts to change to yellow, tumoroids look visibly transparent and form smooth spheres.

(D) Dying tumoroids. Medium stays pink, cells are sparse, and do not form tumoroids. Images taken with 40× magnification, scale bar: 500 μm; images taken with 100× magnification, scale bar: 250 μm.

8. Culture tumoroids under standard conditions (37°C, 5% CO_2).
9. Passage tumoroids twice per week, at dilutions between 1:3 to 1:6.
   a. Carefully aspirate warm media from each well without disrupting the BME droplet.
   b. Place 2 mL cold ADDF3+ medium in 15 mL tube (on ice), take 1 mL using a P1,000 pipette and collect BME droplet(s) containing tumoroids.
   c. Resuspend tumoroids in the 2 mL.
   d. Spin down at 300 × g for 5 min at 4°C.

⚠ CRITICAL: Use of cold media to collect tumoroids is critical for BME to be dissolved.

Note: Tumoroids do not grow well if seeded too sparsely. It is preferable to seed organoids confluently when in doubt. In case they are too big, dissociate tumoroids to enable better growth (Figures 1A–1D). Depending on the tumoroid line, this may be required every 3–6 weeks.

Dissociation of tumoroids into single cells

⏱ Timing: 20–40 min

10. Collect tumoroids in one tube as described in step 9 and centrifuge at 300 × g for 5 min at 4°C.

11. Aspirate supernatant and resuspend pellet in TrypLE Express Enzyme. 1 mL of TrypLE can be used to dissociate tumoroids from 12 droplets. For bigger tumoroids, higher volume and/or longer incubation might be required (we do not advise longer than 30 min).
    a. Resuspend tumoroid pellet in TrypLE.
    b. Incubate at 37°C for 10 min. Resuspend pellet every 5 min using a P1000 pipette. A glass slide can be used to observe tumoroid disruption into single cells by microscope. Troubleshooting 2.

    ⚠ CRITICAL: Keep tube vertical and do not invert during incubation with TrypLE to avoid tumoroids sticking to the tube wall.

    c. Add 1 volume of ADDF3+ to neutralize TrypLE.
    d. Seed cells as described in steps 5 to 8. Dissociation of tumoroids into single cells will be used in transduction and selection of mouse mammary tumoroids (step 1), seeding of tumoroids for functional genetic dropout screen (steps 9, 12 and 13), and *In vivo* validation of target (step 18).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
IMDM	Gibco	Cat#21980-032
Advanced DMEM/F12	Gibco	Cat#12634-010
B-27 Supplement (50×)	Gibco	Cat#17504-044
HEPES Buffer Solution (1M)	Gibco	Cat#15630-056
N-acetylcysteine	Sigma-Aldrich	Cat#A9165
GlutaMAX (100×)	Gibco	Cat#35050-061
EGF Mouse Protein, Native	Thermo Fisher Scientific	Cat#53003-018
Penicillin-Streptomycin (5,000 U/mL)	Gibco	Cat#15070-063
TrypLE™ Express Enzyme (1×)	Gibco	Cat#12605010
Cultrex Reduced Growth Factor Basement Membrane Extract Type 2 (BME)	Trevigen	Cat#3533-001-02
Polybrene	Sigma-Aldrich	Cat#28728-55-4
Olaparib (AZD2281), PARP inhibitor	Syncom, Groningen, the Netherlands	CAS: 763113-22-0
Critical commercial assays		
qPCR Lentivirus Titer Kit	Applied Biological Materials	Cat#LV900
Gentra Puregene Cell Kit	QIAGEN	Cat#158745
Phusion™ High-Fidelity DNA Polymerase (2 U/μL)	Thermo Fisher Scientific	Cat#F-530L
MiniElute PCR Purification Kit	Qiagen	Cat#28004
DNA 7500 Kit	Agilent	Cat#5067-1506
Experimental models: Cell lines		
HEK293FT	ATCC	RRID:CVCL_6911
ORG-KB1P4N.1 (PARPi-naïve)	Duarte et al. (2018)	N/A
ORG-KB1P4R.1 (PARPi-resistant)	Duarte et al. (2018)	N/A
ORG-KB1PM7N.1 (PARPi-naïve)	Duarte et al. (2018)	N/A
ORG-KB1PM7R.1(PARPi-resistant)	Duarte et al. (2018)	N/A
ORG-KB2P17N.1 (PARPi-naïve)	Duarte et al. (2018)	N/A
ORG-KB2P17R.1 (PARPi-resistant)	Duarte et al. (2018)	N/A
ORG-KP.5	Duarte et al. (2018)	N/A
ORG-KPM.1	Duarte et al. (2018)	N/A
ORG-WB1P	Annunziato et al. (2019)	N/A
ORG-WB1P.Myc	Annunziato et al. (2019)	N/A

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: FVB/NRj, 6 weeks, female	Janvier Labs	N/A
Mouse: (FVB/N X 129/Ola)F1, 6 weeks, female	N/A	N/A
Mouse: NMRI nude (or other immunocompromised strain) , 6 weeks, female	Janvier Labs	N/A
Oligonucleotides		
PCR1 Fwd-5′ ACACTCTTTCCCTACACGACG CTCTTCCGATCTNNNNNNGGCTTTATATAT CTTGTGGAAAGGACG3′ NNNNNN represents a sequence of six-nucleotide barcodes (e.g., CGTGAT, ACATCG, GCCTAA, TGGTCA, CACTGT, ATTGGC, GATCTG, TCAAGT, CTGATC, AAGCTA, GTAGCC, TACAAG, etc)	N/A	N/A
PCR 1 Rev (pLKO.1 backbone library)- 5′ GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTGTGGATGAATACTGCCATTTGTCTC3′	N/A	N/A
PCR 1 Rev (pLentiguide backbone library)-5′ GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTTCTACTATTCTTTCCCCTGCACTGT3′	N/A	N/A
PCR 1 Rev (lentiCRISPRv2 backbone library)-5′G TGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTACTGACGGGCACCGGAGCCAATTCC3′	N/A	N/A
PCR2 Fwd-5′AATGATACGGCGACCACC GAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT3′	N/A	N/A
PCR2 Rev-5′CAAGCAGAAGACGGCATACGAGAT NNNNNNGTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT3′ NNNNNN represents a sequence of six-nucleotide barcodes (e.g., CGTGAT, ACATCG, GCCTAA, TGGTCA, CACTGT, ATTGGC, GATCTG, TCAAGT, CTGATC, AAGCTA, GTAGCC, TACAAG, etc)	N/A	N/A
Software and algorithms		
MAGeCK	Li et al. (2014)	N/A
Other		
24-well suspension plates	Greiner CELLSTAR	662102

MATERIALS AND EQUIPMENT

ADDF3+

Reagent	Final concentration	Amount
Advanced DMEM/F12 (1×)	1×	485 mL
Penicillin-streptomycin (5,000 U/mL)	50 units/mL	5 mL
HEPES (1M)	10 μM	5 mL
GlutaMAX (100×)	1×	5 mL
Total	n/a	500 mL

Store at 4°C for a maximum of 6 months

ADDF6+ (tumoroid complete medium)

Reagent	Final concentration	Amount
ADDF3+ (1×)	1×	490 mL
N-acetylcysteine (500 mM)	125 μM	125 μL
B-27 Supplement (50×)	1×	10 mL
EGF (100 μg/mL)	250 ng/mL	250 μL
Total	n/a	500 mL

Store at 4°C for a maximum of 6 months

Alternatives: For culturing tumoroids, other basement membrane matrices can be used such as Matrigel. For genomic extraction of screening samples, other methods or kits can be used such as QIAmp DNA Mini Kit (Qiagen). For measuring molarity after PCR2, an alternative is the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

STEP-BY-STEP METHOD DETAILS
Transduction and selection of mouse mammary tumoroids

⏱ Timing: 4 days

In this step viral transduction of tumoroids is carried out by spinoculation as previously described (Koo et al., 2012). The same step should be followed for the screen library transduction (graphical abstract step 1A, required for carrying out functional genetic dropout screens and target identification) as well as for transduction of single constructs (graphical abstract step 1B, required for single gene modification and target validation *in vivo*).

1. Dissociate tumoroids into single cells as described in preparation steps 10 and 11 and count.

    Note: We advise to transduce between 0.25 million to 1 million cells per well. We advise using a multiplicity of transduction (MOI) of 0.3–1 for library transduction (A) and a MOI of 50–100 for single constructs (B). Troubleshooting 3. For screens, the number of cells to be transduced should be at least three times the coverage to compensate for possible cell death with antibiotic selection. Example of library transduction: for one screen using a library with a complexity of 2,000 sgRNA/shRNAs and carried out with a coverage of 1,000×, 6 million cells should be transduced per replicate.

2. Pellet the required number of cells by centrifuging at 1,000 × *g* for 5 min, discard supernatant and resuspend in virus suspension (concentrated virus in PBS plus ADDF6+ medium, supplemented with 8 μg/mL polybrene). The total volume of virus suspension should be between 250 and 1000 μL; the volume of concentrated virus should not be more than the volume of ADDF6+ medium.

    ⚠ CRITICAL: Spinoculation is carried out in 24-well suspension plates.

3. Transfer cell suspensions to the 24-well suspension plate and centrifuge at 600 × *g* for 60 min at 25°C.
4. After centrifugation, single cells should be at the bottom of the well. Incubate the plate at 37°C for 4–6 h.
5. Resuspend single cells in the supernatant and transfer cell suspensions into tubes. Centrifuge at 1,000 × *g* for 5 min.
6. Discard the supernatant, resuspend pellet in BME-ADDF6+ mix and seed as described in preparation steps 6 to 8 (Figure 1B). Single cells transduced in one well can be seeded in one to three wells.
7. 24 h post transduction, add selection reagent (e.g., puromycin). Expand tumoroids without dissociation for the following step. For *in vivo* target validation (graphical abstract step 1B), target modification must be confirmed prior to experiment (e.g., western blot, PCR and/or sanger sequencing)

    Note: After transduction, dissociation of tumoroids using TrypLE should be avoided for the following few days (approx. 5 days) as tumoroids do not respond well to additional stress post transduction.

Seeding of tumoroids for functional genetic dropout screen

⏱ Timing: 15–21 days

This step follows screen library transduction as defined in transduction and selection of mouse mammary tumoroids (graphical abstract step 1A) and describes how to seed tumoroids for a functional genetic dropout screen and how to keep them in culture throughout the assay. Screens using tumoroids should be carried out for 2–3 weeks, depending on the cycling time of the tumoroid line and drug used (for screens in the absence of drug treatment to study direct lethality, 2 weeks should suffice; in the presence of olaparib, three weeks is advised). For functional genetic dropout screens using drug treatment as negative selection, we suggest a coverage of 500× to 1,000× due to increased variability across replicates.

Here, we use the PARPi olaparib as treatment. Olaparib concentration should be titrated in order to find a drug concentration where resistant cells remain alive (IC_{50}–IC_{100}) while responsive cells do not survive (IC_0–IC_{20}). Competition assays where both resistant and responsive lines are cultured together under drug selection conditions can help identify optimal drug concentration in a screen setting. The effect of frequent dissociation on tumoroid proliferation and drug sensitivity should be accounted for.

8. After transduction of tumoroids with the screen library, make sure there are enough cells for the different conditions and replicates. Expansion of tumoroids for 1 to 3 passages is usually required.

   Note: Example of number of cells required before seeding the screen: for one screen using a library with a complexity of 2,000 sgRNA/shRNAs, carried out with a coverage of 1,000× and with two different treatment conditions (untreated and treated), one should make sure to have at least 6 million cells per replicate (2 million for untreated condition, 2 million for treated condition and 2 million for Day 0 control (T0)).

9. Dissociate tumoroids into single cells as described in preparation steps 10 and 11 and count cells.
10. Collect cells equivalent to coverage for a Day 0 control (T0) and freeze pellet.
11. Seed tumoroids for screen.
    a. Pellet cells by spinning down at 1,000 × g for 5 min.
    b. Seed and culture tumoroids as described in preparation steps 6 to 8. The same number of single cells should be present in each 40 μL BME-ADDF6+ droplets. Untreated and olaparib treated tumoroids should be seeded at equal densities.
    c. Add olaparib to the medium on the same day of seeding.

    Optional: Olaparib treatment can start at later time points if required (e.g., to look for effects in formed tumoroids, treatment combinations), however this can increase the drug concentration required to sensitize tumoroids.

12. Every 5 days, dissociate tumoroids into single cells and re-seed as in step 11. Olaparib treatment should be maintained across the entirety of the screen.
13. At the end of the screen, collect all tumoroids, dissociate into single cells, count (maintain screen coverage) and pellet. Take along T0 pellets collected in step 10 and proceed to genomic extraction (step 25). Optionally, screen pellets can also be frozen before proceeding to step 14.

   △ CRITICAL: Dissociation of tumoroids before genomic extraction (as well as for RNA and protein extraction) is critical for optimal lysis.

   Note: The number of cells per matrix droplet depends on the tumoroid line. The number of cells seeded should allow tumoroids to grow for 5 days without getting overconfluent. We suggest between 50,000 and 200,000 cells per well. Example of seeding conditions: for one screen using a library with a complexity of 2,000 sgRNA/shRNAs and carried out with a coverage of 1,000×, 2 million cells should be seeded per replicate per condition (50,000 cells/well results into forty wells and corresponds to two 24-well plates), 2 million cells should be collected at T0 and 2 million cells should be collected at the end of the screen per replicate per condition.

Sequencing and analysis of screen

⏱ Timing: 3–4 weeks

In this step, genomic DNA extraction, library preparation (two-step PCR (Xu et al., 2015)) and sequencing, and screen analysis are outlined.

14. Proceed to genomic extraction of all samples, including the T0 controls, using the Gentra Pure-gene Cell Kit according to the manufacturer's protocol: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/gentra-puregene-kits/. The protocol is optimized for genomic extraction of 1–2 million or 10–20 million cells. Depending on the size of the screen, different approaches may be followed.

⚠ CRITICAL: Resuspend/vortex cell pellet in the leftover medium supernatant before proceeding to cell lysis. If cell clumps are present after adding cell lysis solution (occurs when pellet was not well resuspended prior to addition of cell lysis buffer), samples can be incubated for 6–16 h at 37°C. Pre-cooling of precipitation buffer to 4°C can improve precipitation. Incubation at 65°C with hydration solution is critical when pellets are very big.

15. After genomic extraction, prepare the library for next generation sequencing (NGS). The steps below were designed for NGS using the Illumina HiSeq 2500. Primers that amplify the sgRNA/shRNA target region with Illumina adaptor sequences for NGS are listed in the key resources table.

    Note: Other NGS machines can be used, however the library preparation protocol may differ.

    a. PCR1 reaction mix should be prepared as described in the Phusion™ High-Fidelity DNA Polymerase (2 U/μL) manufacturer's protocol https://www.thermofisher.com/order/catalog/product/F530L#/F530L, and the table below. Different barcodes can be used for PCR1 forward primers in order to label different samples. The PCR1 reverse primer sequence varies depending on the library backbone. The amount of PCR1 reactions should be according to the coverage of the screen and assumes that 1 million cells is equivalent to 6 μg of genomic DNA.

    Note: Example of the amount of PCR1 reactions required to maintain a coverage of 1,000× in a screen using a library with a complexity of 2,000 sg/shRNAs: DNA corresponding to 2 million cells should be used for PCR1 reaction (2 million cells correspond to 12 μg of genomic DNA; for PCR1 one reaction should contain 1 μg of DNA template which results in 12 reactions per sample).

PCR1/2 mix for 1 reaction (according to manufacturer's protocol)	
Reagent	Amount
DNA template	1 μg (PCR1) – 2,5 μL (PCR2)
Phusion polymerase 2 units/μL	0.5 μL
dNTPs (10 mM)	1 μL
GC buffer (5×)	10 μL
PCR1 or PCR2 forward primer (Fwd) (100 μM)	0.25 μL
PCR1 or PCR2 reverse primer (Rev) (100 μM)	0.25 μL
DMSO	1.5 μL
ddH$_2$O	X
Total	50 μL

PCR 1/2 cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	16 cycles
Annealing	60°C	20 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

b. After PCR1 is complete, pool all the products from one barcode. Check PCR1 product by running 10 μL on agarose gel. A smeared band should appear around the expected PCR product size (approx. 300 bp).

c. Prepare PCR2 reaction as described in step a. Add 2.5 μL of the pooled PCR1 per PCR2 reaction. Carry out 1 to 2 reactions of PCR2 per barcode. Different barcodes can also be used in PCR2 reverse primer to increase complexity of labeled samples.

Note: Barcodes represent a sequence of six-nucleotide barcodes (e.g. CGTGAT, ACATCG, etc). PCR1 barcode is read as the forward read while PCR2 barcode reads as the illumina indexing read.

d. Pool PCR2 products of one barcode. Check 10 μL of PCR2 by agarose gel electrophoresis. A clear band should appear at the expected PCR product size (approx. 300 bp).

e. Purify PCR2 products using MiniElute PCR Cleanup according to the manufacturer's protocol https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/minelute-pcr-purification-kit/, with one exception - do not add pH indicator to buffer PB, it may affect sequencing. Purify products with the same barcodes separately and elute each in 10 μL molecular grade nuclease-free water.

f. Pool all samples before deep sequencing at the equivalent molarity. Molarity can be checked using the Agilent DNA 7500 Kit in Bioanalyzer 2100. This procedure allows to measure molarity corresponding to PCR products of the right size.

16. Deep sequence with Illumina HiSeq 2500.
17. Analyze screen using MAGeCK (Li et al., 2014).
    a. For hit selection one should filter using the following thresholds:
        i. False discovery rate (FDR $\leq$ 0.1; FDR $\leq$ 0.25).
        ii. Aggregated fold change (median/mean log2Fc $\leq$ −1).

In vivo validation of target

⏱ Timing: 4 days

This step follows target modification as defined in transduction and selection of mouse mammary tumoroids (graphical abstract step 1B) and describes preparation of tumoroid samples for orthotopic transplantation and *in vivo* treatment. Note, all animal experiments need to be performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

18. After target modification and validation, dissociate tumoroids into single cells as described in preparation steps 10 and 11 and count the cells. Troubleshooting 4.
19. Pellet the required cell number by centrifuging at 1,000 × *g* for 5 min, discard supernatant and resuspend in 1:1 BME/ADDF6+ (40–50 μL per mammary gland/sample). Per gland, the number of tumoroid cells can vary between 20,000 and 500,000 depending on the tumoroid line (e.g.,
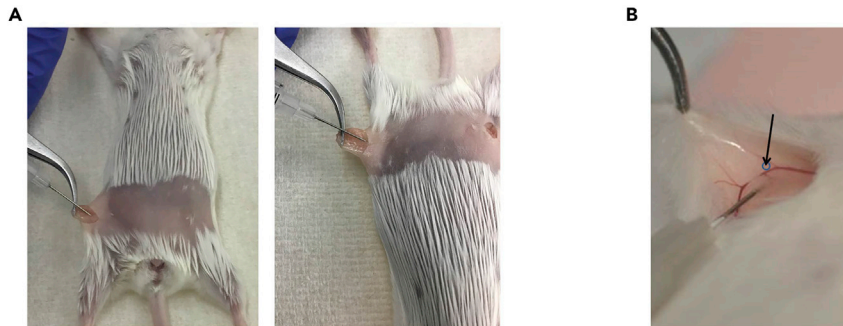
Figure 2. Orthotopic transplantation of tumoroids
(A) Injection of tumoroid suspension into the mouse mammary fat pad.
(B) Mouse mammary fat pad. Arrow indicates the site of the lymph node to avoid.

for tumor latencies of 2–3 weeks: ORG-KB1P4N.1, 40,000 cells; ORG-KB1P4R.1, 10,000 cells; ORG-KPM1, 500,000 cells).

20. Proceed to orthotopic transplantations (Figure 2).
    a. Make a small incision (approx. 0.5 cm) just below the nipple using iris scissors.
    b. Inject cells into the mammary gland fat pad. Avoid the lymph node while injecting.
    c. Suture the skin, place the mouse back in its cage, and monitor recovery until it is completely awake.

Optional: Different protocols for intramammary injections can be followed. With our protocol, we use isoflurane (3% (start) → 2%) as anesthesia and rimadyl (0.26 mL in 200 mL of drinking water) for analgesia.

⚠ CRITICAL: Tumoroid lines derived from KB1P(M) and KP(M) mice can be transplanted into isogenic FVB mice, while tumoroids derived from KB2P mice should be transplanted into (FVB/N X 129/Ola)F1 mice (Duarte et al., 2018). All tumoroids lines can be transplanted into immunodeficient mice. Transplantation of tumoroids modified with Cas9 should be carried out in immunodeficient or Cas9-tolerant mice to avoid immunogenic rejection of the graft.

21. Initiate treatment upon tumor outgrowth (75 mm^3). Troubleshooting 5.
    a. Mammary tumor size can be determined by caliper measurements, and tumor volume can be calculated by the following formula: $0.5 \times length \times width^2$.
    b. Vehicle (20% (2-Hydroxypropyl)-β-cyclodextrin) and olaparib (100 mg/kg) treatment is carried out for 28 consecutive days. This is done via intraperitoneal injection (5 mg/mL stock solution → 20 μL/g injected; max. 1 mL/mouse).
       i. Pre-heat the treatment substances to body temperature before injecting.
       ii. Inject gradually on the left side of the median, towards the groin area and between the fourth and third nipple.
       iii. Insert the needle maximally 0.5 cm, with a 45° angle and the bevel of the needle facing up.
       iv. Remove the needle gradually and put the mouse back in the cage.

Note: The site of injection can be alternated to avoid irritation from consecutive injections in the same site. Macroscopically, we do not observe irritation or swelling, although microscopic examination occasionally reveals skin irritation.

Note: While a cycle of 28 days is standard for olaparib treatment, 56 days can also be used in order to observe a better window between the different lines. For hypersensitization

experiments (increase response in already responsive lines/combination experiments), 50 mg/kg olaparib may be ideal (Paes Dias et al., 2021b).

22. Sacrifice animals with CO_2 when the tumor volume reaches 1500 mm^3.

Optional: Sterile collection and cryopreservation of multiple tumor pieces can be carried out for future engrafting experiments. Fresh tumor samples can be preserved by snap-freezing them in tubes on dry ice (possibly mixed with methanol) for DNA, RNA or protein extraction, by freezing them in OCT for the generation of cryosections, or by fixing them for 24 h in 4% formaldehyde solution in PBS (wt/vol) for the generation of formalin-fixed paraffin-embedded biobanking and (immuno-)histochemical stainings.

EXPECTED OUTCOMES

This protocol details the steps for carrying out functional genetic dropout screens using mouse mammary tumoroids. While this protocol can be applied to other research aims, we focused on the use of PARPi-resistant tumoroids for the identification of genes whose loss reverts resistance to PARPi, as in Paes Dias et al. (2021b).

During the routine culture of tumoroids, the size of each tumoroid within the same well tends to be uniform. However, throughout the screen, the size of tumoroids within the same well will differ more than the parental lines, as different tumoroids will express different sgRNA/shRNAs targeting different genes (Figure 3A). This is similar to the size variation that is also seen in 2D culture screens. Additionally, at the end of the screen, it is expected that drug-treated tumoroids will look less confluent and display increased cell death compared to untreated controls (Figure 3A).

There are multiple ways to present screen analysis data. Dispersion of sgRNA/shRNAs as plotted in Figure 3B helps to determine how each sgRNA/shRNA scores. sgRNA/shRNAs plotted on the left extreme of the plot might be underrepresented (too few counts), whereas sgRNA/shRNAs on the bottom region of the cloud represent putative hits that are depleted in the treated versus untreated condition. Thus, candidate targets should ideally have corresponding sgRNA/shRNAs that fall within the bottom right area of the scatter plot (Figure 3B). If only a small portion of sgRNA/shRNAs are observed in the bottom right of the cloud, this indicates that few or no dependencies are present or that the screen set up was not optimal. At the gene level, one can plot all genes based on their p value as in Figure 3C. While this is helpful to rank all genes and evaluate significance, this plot does not depict the strength of the depletion, which is better plotted in Figure 3D.

Following target identification, we describe the steps for using tumoroids for the validation of candidates *in vivo*. Olaparib-treated mice bearing ORG-KB1P4N.1 tumors should respond to treatment, with a survival of 40–60 days. In contrast, olaparib-treated mice bearing ORG-KB1P4R.1 tumors do not respond to therapy and have a similar survival as vehicle-treated mice. Olaparib-treated mice bearing ORG-KB1P4R.1 with target modification are expected to show similar survival curves as mice bearing ORG-KB1P4N.1 tumors. If mice bearing target-modified ORG-KB1P4R.1 tumors already display increased survival in vehicle-treated conditions, this can indicate that target modification affects tumor progression independently of drug treatment.

LIMITATIONS

Retaining crucial features of the original tumor, displaying small size combined with 3D growth, and easy genetic modification make GEMM-derived mammary tumoroids especially suitable for genetic (and pharmacologic) screens. High complexity screens can require high numbers of tumoroids in order to keep the ideal screen coverage. However, after multiple passages, proliferation of tumoroids can plateau and original tumor characteristics can be lost. Hence, the library size might be limited by how many tumoroids need to be propagated. Although less straightforward, one could overcome
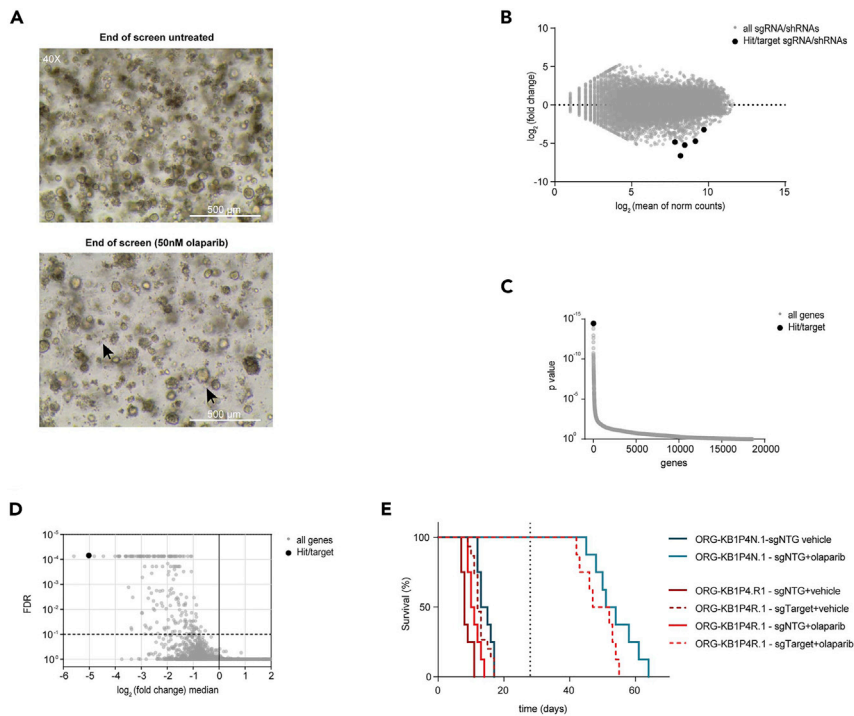
Figure 3. Expected outcome of tumoroid-based dropout screen and *in vivo* validation

(A) Example of ORG-KB1P4R.1 tumoroids at the end of the dropout screen. Left arrow points at dead cells, right arrow points at large tumoroid. Images taken with 40× magnification, scale bar: 500 μm.

(B) Example of plot of $\log_2$ ratio (fold change (treated versus untreated)) versus abundance (mean of normalized (norm) counts) of sgRNA/shRNAs.

(C) Example of plot of distribution of the one-sided p value (gene dropout) for all genes targeted by the shRNA/sgRNA library.

(D) Example of plot of distribution $\log_2$ ratio (fold change (treated versus untreated)) median for all genes versus false discovery rate (FDR).

(E) Example of expected Kaplan–Meier survival curves of mice transplanted with ORG-KB1P4S.1 and ORG-KB1P4R.1 tumoroids lines, and treated with 100 mg/kg olaparib. End of treatment (28 days) is indicated by a dotted line.

this problem via orthotopic transplantations of the original tumor pieces back in mice and subsequently derive 3D cultures from these tumors right before library transduction. This results in higher amounts of cells without extensive passaging of tumoroid lines.

Another potential disadvantage are the elevated costs of the components required to culture tumoroids (e.g., BME, B27). Additionally, we observed that the variability between BME batches can affect experimental outcomes. We recommend testing BME between new batches by measuring proliferation/survival of the same chosen lines cultured with old vs. new batches.

Screens in 3D tumoroids have the advantage of using cells that closely resemble *in vivo* characteristics. A drawback of all *in vitro* culture systems, including 3D tumoroid lines, is the lack of stroma. This prevents screening for factors involved in tumor microenvironment interactions, which has been found to influence treatment response in breast cancer (reviewed in Mcmillin et al., 2013). One could overcome this by using co-culture systems with stromal elements such as fibroblasts and/or immune cells.

Finally, in this protocol we describe the use of mouse tumoroid lines that resemble triple-negative breast cancer. However, we have not been able to successfully establish long-term mouse tumoroids from GEMMs of invasive lobular carcinoma (Boelens et al., 2016; Derksen et al., 2006). Hence, the approach described in this protocol might not be feasible for every breast cancer subtype.

TROUBLESHOOTING

Problem 1

Some tumoroid lines tend to sink to the bottom of the matrix droplet/well and start adhering to the plate as 2D lines (Figure 4). We have observed this mainly in tumoroid lines derived from epithelial-to-mesenchymal transition (EMT)-like tumors (also known as claudin-low), such as ORG-KB2P, ORG-KP(M) and ORG-WB1P-Myc lines. This may also happen when tumoroids are very big.

Potential solution

Keeping organoids at an ideal size is important to keep the 3D culture going. Flipping the plate upside down during the 30 min incubation at 37°C while BME solidifies helps tumoroids to stay at the top of the matrix droplet and avoids 2D expansion. 2D cells can also be a result of residual stromal cells. This can happen in freshly-derived tumoroids. In this case we advise a short treatment with 5 μM Nutlin-3a (5–7 days). Nutlin-3a kills P53-proficient cells and, as the lines we describe in this paper are P53-deficient, treatment with Nutlin-3a will make sure to eliminate non-tumor cells.

Problem 2

When tumoroids are larger, it can be difficult to dissociate them into single cells.

Potential solution

To help dissociate bigger tumoroids, one can use a fire-polished glass pipette during incubation with TrypLE. By pipetting up and down with the glass pipette, tumoroids are subjected to mechanical dissociation. The use of a fire-polished glass pipette can also be helpful in case a tumoroid line does not respond well to the frequent use of TrypLE. We have not observed increased cell death as a result of this procedure. Note that it is important to coat the glass pipette with medium or BSA as described in preparation step 5. Coating of the glass pipette is important as tumoroids tend to stick to the pipette surface.

Problem 3

Difficult transduction of tumoroids can happen. Bigger constructs tend to be more difficult to transduce, especially in tumoroids. This can be observed when no or few cells survive antibiotic selection. To confirm the problem is not low viral titer, transduction of the same virus should be tested in 2D cells.

Potential solution

For difficult constructs, we found the use of a high virus titer to be crucial. For this, good viral production efficacy as well as viral concentration are important.

Problem 4

Engrafting of some tumoroid lines can be less efficient. This can result in a longer time before tumor outgrowth and/or in reduced penetrance.

Potential solution

In this protocol we describe the dissociation of tumoroids before transplantation. Transplantation of tumoroids that are not single cells but comprising 150–200 cells each, as previously reported in
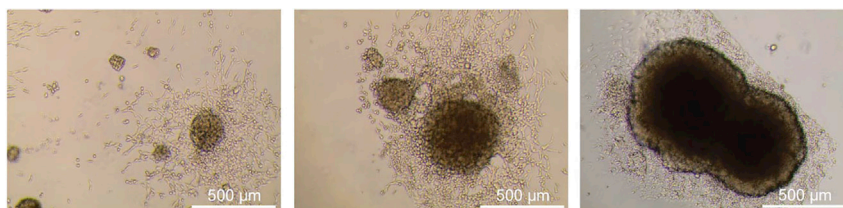

Figure 4. Problem 1 – Tumoroids spreading as 2D cells in the bottom of the plate
Images taken with 40× magnification, scale bar: 500 μm.

Duarte et al., can improve the engrafting of tumoroids. In this case, cell counting is carried out by dissociating and counting a representative well and by extrapolating cell number in the identical non-dissociated wells. This method could result in less consistent cell counting and consequently in slightly higher variability in tumor growth speed between different animals.

Problem 5

For some tumoroid lines tumor outgrowth is highly accelerated and can rapidly achieve the humane endpoint tumor size (1500 mm^3) before the treatment has an effect.

Potential solution

For these lines (e.g., ORG-KB1P4R.1) we suggest starting treatment at a smaller tumor size (e.g., 40–50 mm^3). The amount of tumoroid cells transplanted can also be adjusted to optimize the growth curve.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jos Jonkers (j.jonkers@nki.nl).

Materials availability

Materials associated with this study are available upon request from the lead contact.

Data and code availability

This paper does not report datasets or original code.

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AUTHOR CONTRIBUTIONS

M.P.D. wrote the manuscript and prepared figures. S.R. and J.J. supervised the project and reviewed/edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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