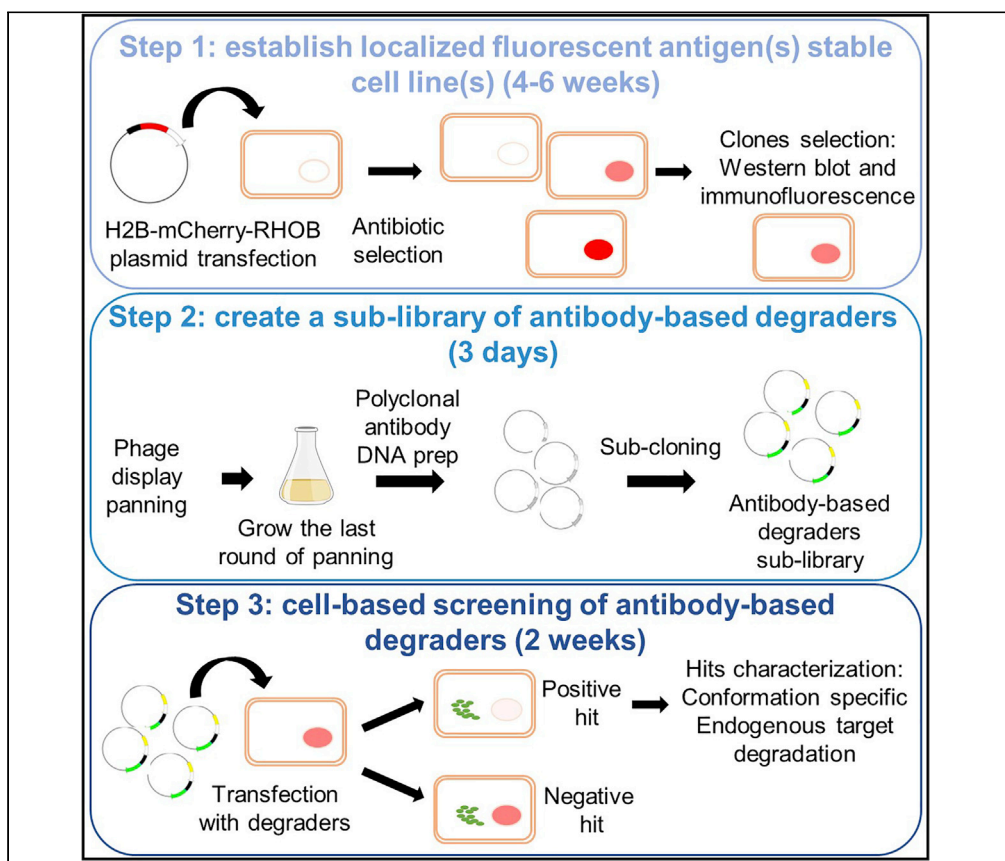


Protocol

Protocol to select conformation-specific intracellular antibodies for targeted protein degradation in an engineered cell line



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Highlights

Antibodies are cloned into a degrader entry vector after a phage display selection

Degraders are screened in a stable antigen-fluorescent protein fusion cell line

This protocol will provide antibody-based degraders to study protein functions

This protocol could be applied to any protein binders and antigens of interest

Here, we provide a protocol for the selection of conformation-specific intracellular antibody degraders using a cell-based screening method. We applied this protocol to select antibody-based degraders targeting the active form of the small GTPase RHOB (i.e., RHOB-GTP) using an engineered H2882 cell line. The protocol can be used to study the function of RHOB active conformation in various cellular settings. This protocol can be broadly applied to select any kind of intracellular antibody degraders, regardless of conformational state.

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Protocol

Protocol to select conformation-specific intracellular antibodies for targeted protein degradation in an engineered cell line

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Summary

Here, we provide a protocol for the selection of conformation-specific intracellular antibody degraders using a cell-based screening method. We applied this protocol to select antibody-based degraders targeting the active form of the small GTPase RHOB (i.e., RHOB-GTP) using an engineered H2882 cell line. The protocol can be used to study the function of RHOB active conformation in various cellular settings. This protocol can be broadly applied to select any kind of intracellular antibody degraders, regardless of conformational state. For complete details on the use and execution of this protocol, please refer to Bery et al. (2019).

Before you begin

This protocol was used in a publication to select intracellular antibodies as macromolecule degraders of the small GTPase RHOB's active conformation to study RHOB-GTP function in cells (Bery et al., 2019a). We set up a cell-based screening that allows the direct monitoring in cells of RHOB-GTP degradation by nanobodies isolated after four rounds of phage display selection. The protocol for selecting conformational RHO antibodies by phage display has been previously described (Chinestra et al., 2012). To quantify the decay reflecting the antigen degradation, clonal stable cell lines with homogenous expression of the antigen or controls of selectivity need to be prepared before starting the screen. In our hands, the establishment of stable cell lines with homogenous expression of the target is mandatory. Notably, we were not successful in a previous attempt to screen the antibody-based degraders by transient transfection, due to high cell to cell heterogeneity in ectopic expression of both the antigen and the degrader. The antigen of interest (here RHOB-GTP) was fused to the histone H2B-mCherry to visualize the degradation by fluorescent microscopy and to localize it to the nucleus where it can accumulate without apparent toxicity. Any antigen of interest can be fused to a fluorescent reporter, such as mCherry, localized or not to a specific sub-cellular compartment (e.g., nucleus).

Various negative control cell lines were also produced to enable the selection of conformation-specific RHOB-GTP degraders. We used a constitutively active mutant of RHOB (RHOB^{Q63L}) to mimic RHOB-GTP conformation and an inactive mutant (RHOB^{T19N}) to mimic RHOB-GDP conformation. This protocol has a broad applicability as it can be applied to select any kind of intracellular antibody degraders (conformation-specific or not). In fact, we previously implemented this protocol as proof-of-concept to isolate (non-conformational) anti-GFP antibody-based degraders already identified as



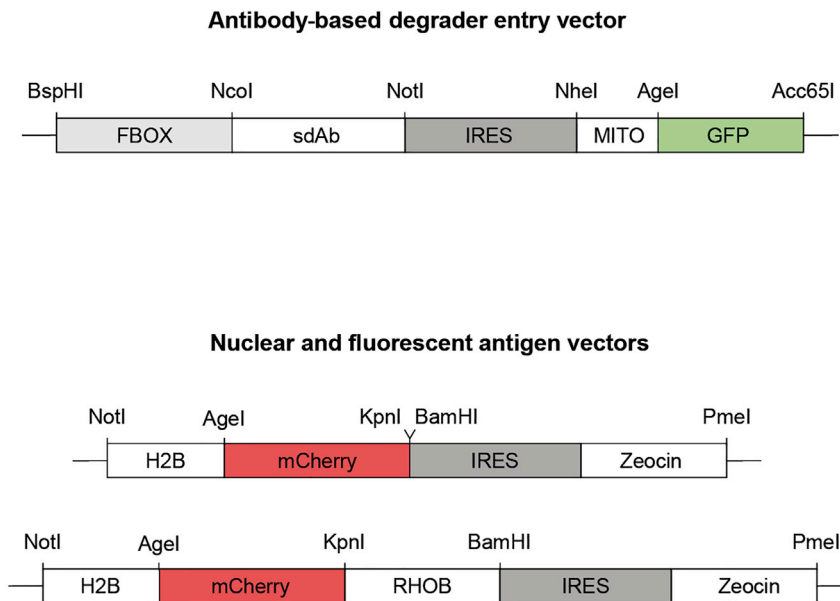


Figure 1. Schematic diagrams of the vectors used in this protocol

(Top) FBOX-sdAb-IRES-MITO-GFP is the antibody-based degrader entry vector (top vector). NcoI/NotI are the restriction sites used to insert any antibody of interest in fusion with the FBOX domain. GFP could be replaced by any other fluorescent protein of interest by using AgeI/Acc65I restriction sites. sdAb: single domain antibody. (Bottom) H2B-mCherry-IRES-Zeocin is the antigen entry vector to express the antigen (here RHOB) in fusion to the histone H2B and the red fluorescent protein mCherry (middle and bottom vectors). Any antigen of interest can be inserted in this vector using KpnI/BamHI restriction sites. The different restriction sites used to construct these vectors are shown on the schematic diagrams.

functional intracellular antibodies (i.e., that bind to GFP fusion in cells). We checked their ability to degrade H2B-GFP antigen in HeLa S3 H2B-GFP stable cell line (Moutel et al., 2016). Furthermore, this protocol is not limited to the antibody format and it could be applied to protein binders with a different scaffold such as DARPin (Binz et al., 2003, Bery et al., 2019b), monobodies (Koide et al., 1998, Spencer-Smith et al., 2017) or affimers (Tiede et al., 2017).

Experimental considerations

In this protocol, two entry vectors are used and were described elsewhere (Bery et al., 2019a). A schematic diagram of the antibody-based degrader entry vector is shown in Figure 1 (top panel). This construct is a fusion between the SlmbFBOX domain from *Drosophila* and an antibody of choice (or any other kind of protein binders). IRES-MITO-GFP is used as fluorescent reporter to visualize transfected cells. MITO is a mitochondrial targeting sequence (MTS) from subunit VIII of human cytochrome c oxidase fused to a GFP (Green Fluorescent Protein).

The other vector employed in this protocol is the nuclear red fluorescent antigen entry vector (RHOB is the antigen of interest here). The red fluorescent protein mCherry is in fusion with histone H2B and therefore linked to the chromatin (nuclear signal). Schematic diagrams of the different vectors are shown Figure 1 (bottom panel). Any antigen of interest can be inserted between KpnI and BamHI restriction sites to generate a nuclear and red fluorescent-bound antigen. Stable clones can be selected in eucaryote cells via the Zeocin resistance marker.

Determine the concentration of antibiotic for the stable cell line establishment (antibiotic kill curve)

⌚ Timing: 2 weeks

1. Plate 40,000 H2882 cells per well of a 12-well plate with RPMI medium supplemented with 10% FBS.
2. The next day, change the medium with 1 mL of medium containing the following concentrations of Zeocin ($\mu\text{g mL}^{-1}$): 0; 50; 100; 200; 300; 400; 500; 600; 700; 800; 900; 1,000.
3. Change the medium every 3 to 4 days with fresh antibiotic.
4. After 10 to 14 days of antibiotic selection, choose the concentration of antibiotic which is twice the one that kills all the cells (e.g., at $50 \mu\text{g mL}^{-1}$ of Zeocin all H2882 cells were dead after 14 days, therefore we choose $100 \mu\text{g mL}^{-1}$ as concentration of Zeocin for the selection of H2882 stable cell lines).

Alternatives: Any antibiotic working in eucaryote cells can be used here depending on the backbone of the vector (e.g., Neomycin/G418, blasticidin, hygromycin, puromycin, ...).

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-RHOB	Santa Cruz Biotechnology	Cat#sc-180; RRID: AB_2179110
Rabbit polyclonal anti-mCherry	Biovision	Cat#5993-100, RRID: AB_1975001
Mouse monoclonal anti-RHOB	Santa Cruz Biotechnology	Cat#sc-8048, RRID: AB_628219
Goat anti-mouse Alexa Fluor 488	Thermo Fisher	Cat# A-11001 RRID: AB_2534069
Mouse monoclonal anti- α -tubulin	Sigma-Aldrich	Cat#T9026; RRID: AB_477593
Bacterial and virus strains		
TG1	Euromedex	Cat#LU-60502-1
DH5 α	New England Biolabs	Cat#C29871
Chemicals, peptides, and recombinant proteins		
Zeocin	Invitrogen	Cat#R25001
Mowiol 4-88	Sigma	Cat#81381-50G
Paraformaldehyde, 16% solution	MP Biomedicals	Cat#02199983-CF
Critical commercial assays		
JetPrime transfection reagent	Polyplus Transfection	Cat#114-15
NucleoSpin Plasmid (Miniprep Kit)	Machery Nagel	Cat#740588.250
NucleoBond Xtra Midi Plus (Midiprep Machery Nagel Kit)	Machery Nagel	Cat#740412.50
Experimental models: cell lines		
Human: H2882	(Sato et al., 2007)	RRID: CVCL_5158
Recombinant DNA		
p-H2B-mCherry-RHOB ^{Q63L} Δ CAAX-IRES-Zeo	(Bery et al., 2019a)	N/A
p-H2B-mCherry-RHOB ^{T19N} Δ CAAX-IRES-Zeo	(Bery et al., 2019a)	N/A
p-H2B-mCherry-IRES-Zeo	(Bery et al., 2019a)	N/A
p-FBOX-sdAb-6xHis-myc-IRES-MITO-GFP	(Bery et al., 2019a)	N/A
Software and algorithms		
NIS Elements v3	Nikon	https://www.nikon.com/products/microscope-solutions/support/download/software/imgsfw/nis-br_v3221432.htm

Materials and equipment

Cell culture

Here we specify the reagent used for the cell culture of H2882 cells (wild-type and stable clones):

Note: Cell culture is performed in a sterile tissue culture hood under aseptic conditions and cells are maintained in a humidified 37°C incubator with 5% CO₂.

H2882 Cell Medium: RPMI supplemented with 10% FBS with or without 100 µg mL⁻¹ Zeocin.3.7%

Paraformaldehyde (PFA) solution

Reagent (stock concentration)	Final concentration	Amount
Paraformaldehyde (16%)	3.7%	5.55 mL
PBS (1×)	n/a	18.45 mL
Total	n/a	24 mL

PFA is stored at 22°C for 1 week.

△ **CRITICAL:** PFA is a carcinogen, mutagen and toxic reagent and should be handled under a chemical fume hood and waste should be discard in a specific bin.

Mowiol preparation

Reagent (stock concentration)	Final concentration	Amount
Glycerol (100%)	25%	6 g
Mowiol (powder)	100 g L ⁻¹	2.4 g
ddH ₂ O	n/a	6 mL
Tris-HCl pH 8.5 (0.2 M)	0.1 M	12 mL
Total	n/a	24 mL

Add 6 mL of milli-Q water to the glycerol and Mowiol powder and leave 2 h at 22°C.

Add 12 mL of 0.2 M Tris-HCl pH 8.5.

Incubate 3 h in a water bath at 60°C, mixing regularly.

Centrifugation of the solution to remove any non-solubilized element (5 min at 5,000 × g).

Make aliquots in 1.5 mL Eppendorf tubes.

Store at -20°C for several months.

Equipment for the screen

MoFlo Astrios (Flow cytometer, post-screen analysis of the antibody-based degraders)

Alternatives: Any flow cytometer that has a green and a red laser can be used for the post-screen analysis.

Nikon Eclipse 90i: used for the immunofluorescence of the stable cell lines.

Inverted Nikon Eclipse Ti: used for the cell-based screen.

Alternatives: Any epifluorescence microscope device can be used for the immunofluorescence analysis.

Step-by-step method details

Transfection of H2882 cell lines

⌚ Timing: 4 days

Transfecting H2882 cells that are *RHOB*^{-/-} cells with H2B-mCherry-RHOB^{Q63L}ΔCAAX plasmid will allow the selection of clones stably expressing this fluorescent localized antigen (see [Before you begin](#) section and [Figure 1](#), bottom panel). This will make the screen easier and more reliable than transient transfection of the antigen. If the degrader needs to be specific of a conformation or an isoform, then several stable cell lines can be done. Before starting the antibiotic selection of the cell line, an antibiotic kill curve needs to be performed to determine the concentration of antibiotic to use that kill all the cells (see [Before you begin](#) section).

1. Plate 40,000 cells per well of a 24-well plate (in 0.5 mL of RPMI supplemented with 10% FBS) to get the cells at 80% confluence the next day.
2. 24 h later, transfect the cells with the JetPRIME reagent and the plasmid(s) of your choice (i.e., nuclear red fluorescent antigen entry vector). Add 0.75 μg of DNA to 50 μL of JetPRIME buffer, vortex 10 s and centrifuge 3 s at 2,000 × g. Add 1.5 μL of JetPRIME reagent, vortex 10 s and centrifuge 3 s at 2,000 × g. Incubate at 22°C for 10 min and then add the mix drop by drop on the cells.
3. 4 h later, change the medium with 0.5 mL of fresh medium.

Note: Any cell lines of interest that can be easily transfected (for the cell-based screen) could be used to make stable clones such as HEK293T or HeLa cells. Any suitable transfection reagent can also be used.

Selection of H2882 stable clones

⌚ Timing: 3–4 weeks

Once the cells are transfected, the antibiotic selection process can start. Generally, after 3 to 4 weeks of antibiotic selection (depending of the antibiotic used), clones should appear and be picked.

4. 72 h after the transfection, detached the cells.
 - a. Aspirate the medium, wash with 0.5 mL of PBS and add 0.3 mL of trypsin.
 - b. Leave the cells at 37°C for 3–5 min, until they are detached. Add 0.7 mL of complete medium and resuspend the cells by pipetting up and down carefully (1 mL of resuspended cells).
5. Plate 1/50 (i.e., 20 μL of the 1 mL of resuspended cells), 1/150 (6.7 μL of the 1 mL) and the rest of the cells (973.3 μL) in 10 mL of medium containing your antibiotic at the concentration determined with the killing curve in 100 mm plates.
6. Every three / four days, change the medium of the plates with fresh antibiotic.
7. After 3 to 4 weeks (at least with Zeocin), clones (i.e., colonies of cells) should appear on the plate. When clones are 3–6 mm large, use sterile 8 mm cloning cylinders to pick the colonies.
 - a. Aspirate the medium, wash the 100 mm plate with 5 mL of warm PBS.
 - b. Put the cloning cylinder around the colony of cells and press gently on the top of the cylinder to create a seal between the cylinder and the plate.
 - c. Add 50 μL of trypsin and wait under the tissue culture hood for 3 to 5 min, until the cells detached. You can pipet carefully up and down to force the cells to detach.
 - d. Prepare a 96-well plate with 200 μL of complete medium with antibiotic.
 - e. Once detached, pipet the 50 μL of trypsinized cells and add it into one well of the 96-well plate. One clone per well.
8. Amplify the clones by increasing the surface of culture every time they are confluent: from 96-well to 48-well to 12-well, until a dozens of aliquots (2 × 10⁶ cells per vial) can be frozen as stock and stored in liquid nitrogen.

Characterization of H2882 stable clones

⌚ Timing: 1 week

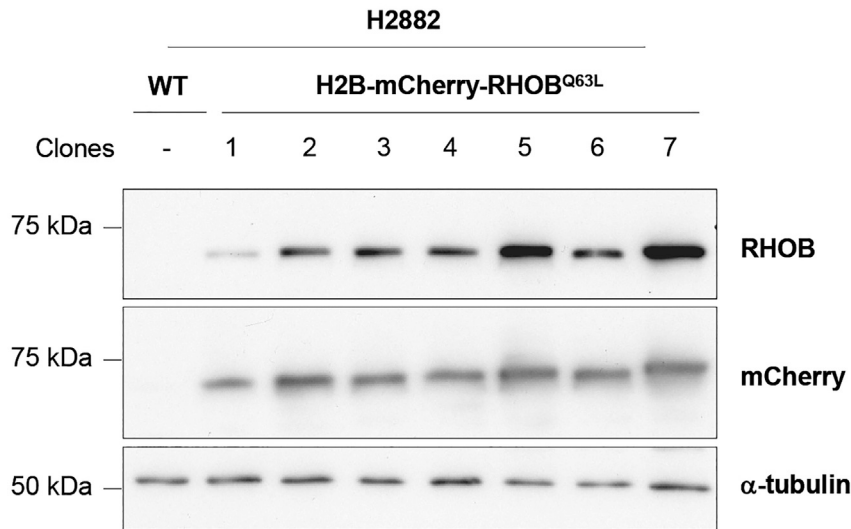


Figure 2. Analysis of the expression of the antigen (RHOB) by western blot

Wild-type (WT) and stable H2882 clones transfected with the H2B-mCherry-RHOB^{Q63L} expression vector and selected with antibiotics were lysed and analyzed by western blot to check the expression of the antigen (RHOB) and the fluorescent reporter (mCherry) using anti-RHOB and anti-mCherry antibodies. α -Tubulin is the loading control (incubated 1 h at 1/10,000 dilution).

Clonal stable cell lines have to be checked for the proper expression and localization of the antigen in cells by immunofluorescence and western blot.

9. Check the expression of the antigen by western blot (using either anti-RHOB, 1/500, and/or anti-mCherry, 1/500, antibodies incubated 18 h) (see [Figure 2](#)).
10. Check the correct localization of the fluorescent antigen (here nuclear mCherry-RHOB) by immunofluorescence (see [Figure 3](#)).
 - a. Plate cells on a coverslip, the day after, fix the cells with 3.7% PFA, permeabilize the cells, block them and incubate the appropriate primary (mouse anti-RHOB: 1/50, 3 h incubation at 22°C) and secondary antibodies (goat anti-mouse Alexa Fluor 488, 1/400, 1 h incubation at 22°C) and mount the coverslip on a slide with mounting medium containing DAPI (Mowiol, see [Materials and equipment](#) section).

△ CRITICAL: The clone used for the cell-based screen should ideally express a medium level of fluorescent antigen: enough to detect it easily by microscopy but not too high to be still able to observe a degradation by microscopy (here H2B-mCherry-RHOB, see clones 2–4 and 6 on [Figure 1](#) as good examples).

Creation of a sub-library of intracellular antibody-based degraders

⌚ Timing: 3 days

The phage display technology selects antibodies specific for an antigen. The protocol for selection and enrichment of conformational antibodies targeting GTP-bound RHO by phage display has been previously described elsewhere ([Chinestra et al., 2012](#)). Usually three to four rounds of panning are sufficient to get specific antibodies according to the stringency of the competitive or washing steps ([Haque and Tonks, 2012](#)). Then, antibody fragments are usually tested first as recombinant antibody fragments by various *in vitro* methods such as ELISA, western blot, or immunofluorescence. Then, some hits are further sub-cloned into a mammalian expression vector to be expressed as an

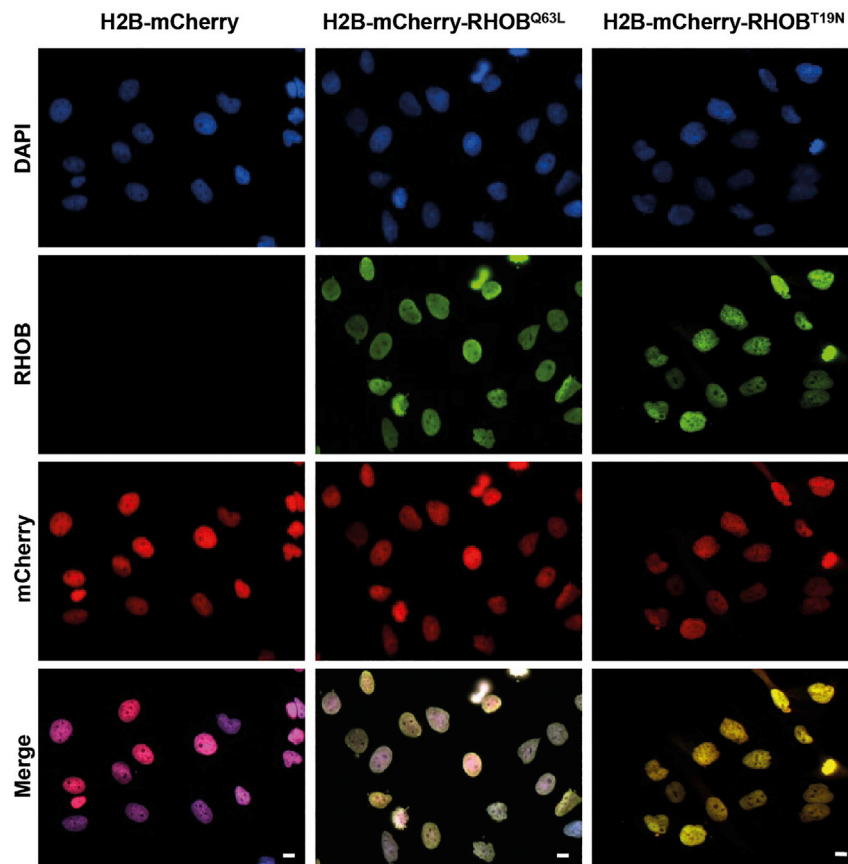


Figure 3. Localization of the antigen in cells by immunofluorescence

H2882 cells stably expressing the histone H2B-mCherry, H2B-mCherry-RHOB^{Q63L}, or H2B-mCherry-RHOB^{T19N} were stained with an anti-RHOB primary antibody and an Alexa-488 secondary antibody (green channel). DAPI is used to stain the DNA (blue channel) and mCherry is directly visualized on the red channel. Scale bars, 10 μ m.

intracellular antibody but only few antibodies performed best both in *in vitro* assays and as intrabodies. To directly identify the best intracellular antibody-based degraders, namely that express well in the reducing intracellular environment and remain functional (i.e., that deplete specifically their target antigen), we set up a cell-based screen after the last round of phage display by sub-cloning the library of antibodies obtained after this last round of phage display panning in an antibody-based degrader entry vector (described in [Before you begin](#) section and in [Bery et al., 2019a](#)). Importantly, this protocol is not limited to phage display and could be applied to other similar panning enrichment methods involving large libraries of protein binders (e.g., single domain antibodies, DARPins, monobodies, ...).

- From the last round of phage display, directly grow TG1 *E. coli* containing the modified pHEN2 vector from NaLi-H1 library ([Moutel et al., 2016](#)) in 100 mL of LB with ampicillin and 0.5% glucose for 5 h at 37°C (until Optical Density (OD) \approx 0.1–0.2).

△ CRITICAL: It is important to not grow 16–18 h, and be careful that OD not exceed 0.2 to avoid overgrowth of some antibody clones and loss of diversity.

- Make a high purity medium scale preparation of plasmid DNA from the culture using any mid-prep kit (i.e., Machery Nagel or Qiagen).

13. Digest 2–3 μg of DNA by NcoI and NotI enzymes 2 h at 37°C. This will digest the coding sequence of the antibodies from the modified pHEN2 vector.
 - a. Run a 1% agarose gel to separate the digested DNA fragments
 - b. Purify the NcoI/NotI digested DNA (band around 400 bp) with a gel extract DNA purification kit (i.e., Machery Nagel or Qiagen).
14. Digest the entry vector pFBOX-X-IRES-MITO-GFP by NcoI and NotI (see [Figure 1](#)).
15. Ligate the polyclonal pool of DNA fragments coding for the antibodies selected after the last round of phage display into the pFBOX-X-IRES-MITO-GFP vector using the T4 DNA ligase.
16. Transform competent DH5 α bacteria or any cloning strain with the ligation mix and plate on several agar plates with 100 $\mu\text{g mL}^{-1}$ ampicillin.

▮▮ Pause point: A 50% glycerol stock of each bacterial colony can be done by growing for 18 h the cells in a 96-well plate (one colony per well of a deep-well 96-well plate) containing 500 μL of LB complemented with 100 $\mu\text{g mL}^{-1}$ of ampicillin. The next day, add 500 μL of 100% glycerol in each well, mix each well to homogenize and store the plate at -80°C . Several plates can be done and be used as starting point for the miniprep step described in the next step.

Cell-based screening of intracellular antibody-based degraders

⌚ Timing: 2 weeks

The polyclonal pool of single domain antibodies (sdAb) selected after the last round of phage display is now in fusion with the FBOX domain along with a fluorescent reporter MITO-GFP. This reporter will allow the monitoring of transfected cells (useful for flow cytometry or microscopy analyses for instance). Each clone will be transfected for 48 h in the stable cell line expressing the fluorescent antigen of choice (here H2882 cells expressing H2B-mCherry-RHOB^{Q63L} mutant). Each well will be observed using an inverted epifluorescence microscope. The cells expressing a FBOX-sdAb fusion will have green mitochondria, and in these cells, the red fluorescent nuclear signal will be examined.

17. The next day, start as many minipreps as needed for the screen by inoculating a colony into 3 mL of LB medium with 100 $\mu\text{g mL}^{-1}$ ampicillin. A total of 300 minipreps was performed in ([Bery et al., 2019a](#)). This can be scaled up or down (see [Limitations](#) section).
 - a. Miniprep are done using a miniprep kit (Machery Nagel or Qiagen).
 - b. To check the diversity of the sub-library, 50 clones have been sent for sequencing.

▮▮ Pause point: Minipreps can be stored at -20°C and the screen can be performed over weeks if necessary.

18. In each well of 24-well plates, seed 30,000 H2882 cells stably expressing the target antigen H2B-mCherry-RHOB^{Q63L}.
19. The next day, transfect 0.75 μg of plasmid DNA of interest (coding for FBOX-sdAb-IRES-MITO-GFP, see [Figure 1](#), top panel) with 1.5 μL of JetPRIME reagent. One plasmid per well, as many wells as needed for the screen (i.e., 300 clones will require 300 wells).

⚠ CRITICAL: It is important to include a negative control (antibody not binding the antigen of interest) in the screen to assess the effect of an antibody non-degrading the antigen (as shown in [Figure 4](#)).

20. 48 h later, the cells are fixed 15 min with 3.7% of PFA, rinse three times with PBS and observed under an inverted microscope. See [Figure 4A](#) left-hand side panel for representative images expected from such a screen.

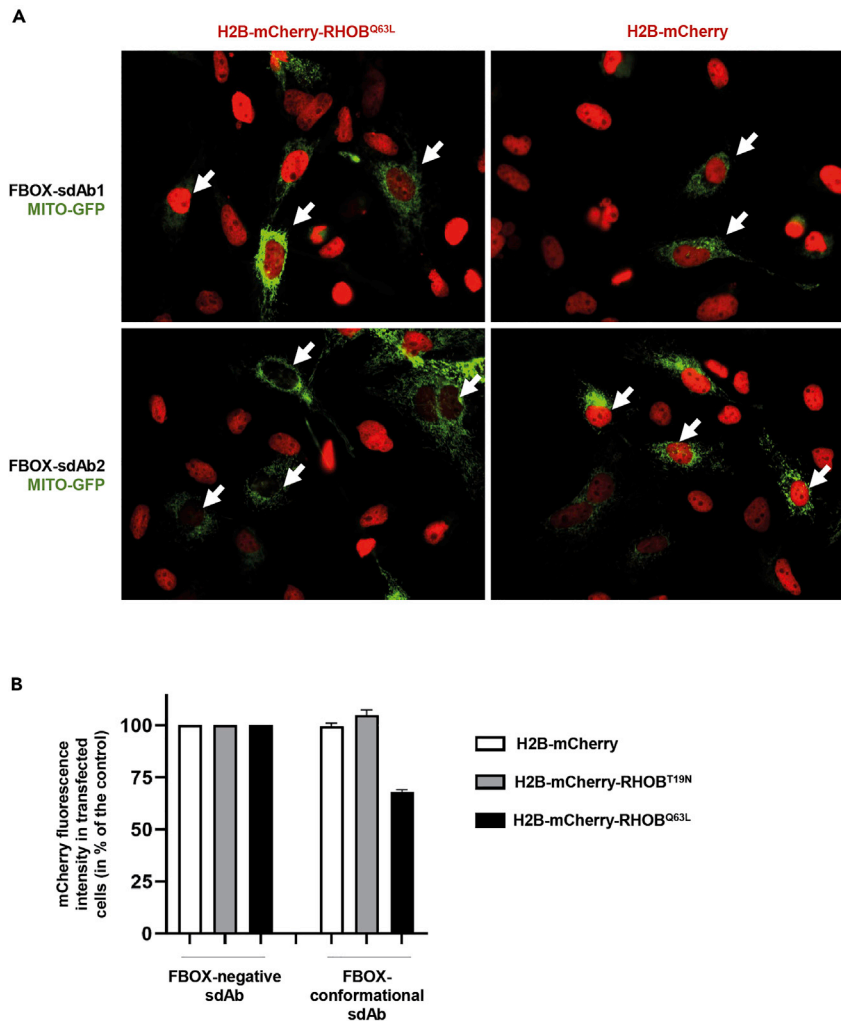


Figure 4. Expected results from the cell-based screening of conformational antibody-based degraders

(A) Representative images obtained from the screen after transfection of the single domain antibody (sdAb)-based degrader plasmid (FBOX-sdAb-IRES-MITO-GFP) into the stable cell lines H2B-mCherry-RHOB^{Q63L} and the negative stable cell line H2B-mCherry. White arrows indicate transfected cells (green mitochondria) where a decrease of the mCherry fluorescence is only observed with the FBOX-sdAb2 in the H2B-mCherry-RHOB^{Q63L} stable cell line (FBOX-sdAb1 is a negative control).

(B) Analysis of the conformation of the positive hits by flow cytometry. H2882 stables clones: H2B-mCherry (white bars), H2B-mCherry-RHOB^{T19N} (gray bars), and H2B-mCherry-RHOB^{Q63L} (black bars) are transfected with a negative FBOX-sdAb (not degrading) or a positive FBOX-sdAb (degrading). The intensity of mCherry fluorescence in the green transfected cells is analyzed by flow cytometry and compared to the normalized negative control. Data represented in (B) are means \pm standard deviation (SD) of three independent experiments.

21. All the positive hits are subsequently screened against the negative stable cell lines expressing H2B-mCherry to confirm the specificity of the antibody-based degraders. See [Figure 4A](#) right-hand side panel.
22. To check the conformational specificity of the hits, a counter-screen on a negative cell line (here the H2B-mCherry-RHOB^{T19N} cell line) is performed by flow cytometry. See [Figure 4B](#).
23. Finally, positive hits which efficiently degrade the target but not the controls, are selected for further characterization on their capacity to efficiently degrade the endogenous antigen in more physiological cellular models.

Expected outcomes

This protocol will allow the isolation of antibody-based degraders that works in the intracellular environment. Hence, it will be possible to use the antibody without the E3 ligase as tracking or inhibitory tools rather than only being a degrader. In addition, the antibody-based degraders can be expressed in cell lines of interest by lentivectors or other virus-based vectors, with inducible promoter if required, to study the function of the specific conformation of the target.

Quantification and statistical analysis

Images from the cell-based screen described in this protocol (see [Figure 4A](#)) could be quantified by using software such as ImageJ or Photoshop. By determining the fluorescence intensity in the nucleus of transfected cells versus cells transfected with a negative control, a percentage of degradation could be obtained.

We rather quantified the degradation percentage using flow cytometry after the cell-based screen, which allows the quantification of a large number of cells (more than 10, 000) in a very fast fashion. We normalized the intensity of the mCherry fluorescence from GFP positive cells (i.e., green mitochondria) transfected with an antibody-based degrader to the intensity of the mCherry fluorescence from the GFP positive cells transfected with a non-degrader negative control antibody (see [Figure 4B](#) as example).

Limitations

A limitation of this protocol would be the level of the screen's throughput described here. It is a relatively low throughput screen and could be turned into a medium throughput screen using automation. Minipreps can be performed in 96 wells, followed by 96 well reverse transfection, and screening could be performed with a high content imager, such as Operetta or Arrayscan, or a flow cytometer, both in 96-well plate format. In these cases, quantification should be performed using the device dedicated software. Such screening methods would further provide a direct quantification of the degradation for each clone tested.

The number of clones to screen to get a degrader depends on various parameters such as the amino acid sequence diversity of the clones obtained after the phage display (i.e., number of clones with a different amino acid sequence) but also on the antibody library used for the phage display (e.g., favorable or not for the selection of intracellular antibodies). Of note, the clones' diversity will also be influenced by the phage display panning conditions, the nature of the antigen used, ... We screened 300 clones for 30 positive clones but only 8 hits had a different amino acid sequence which corresponds to a $\approx 2.5\%$ hit rate. Therefore, using a higher throughput technique will increase the number of clones screened and potentially the number of positive hits.

Importantly, while the antibody-based degraders isolated with this screen degrade their target in an engineered stable cell line, further depletion of their endogenous untagged target in a model cell line or organism could give rise to several issues: (i) the cell line of interest should be easy to transfect or transduce, (ii) the cell line should express an antigen at a level easily revealed with a quantitative assay, (iii) the cell line should express the E3 ligase complex that associate with the Fbox domain for efficient degradation. Accordingly, we and others have shown that the type of E3 ligase used for antibody-based degraders is important ([Bery et al., 2020](#), [Shin et al., 2015](#), [Lim et al., 2020](#)), therefore, it might be possible to improve the degradation efficiency by changing the FBOX with another E3 ligase such as the VHL ([Fulcher et al., 2016](#)) or SPOP ([Shin et al., 2015](#)).

Troubleshooting

Problem 1

Too many clones in the establishment of stable cell lines (related to Selection of H2882 stable clones, steps 4 and 5).

Potential solution

Make more dilutions of the cells (1/200, 1/500, ...).

Problem 2

No clones appear in the establishment of stable cell lines after 3 to 4 weeks of antibiotic selection (related to Selection of H2882 stable clones, steps 4 and 5).

Potential solution

Check the transfection efficiency by microscopy (fluorescent reporter) before adding the antibiotic. Use an easy to transfect cell line such as HEK293T or HeLa.

If it does not work, lentivectors could be used to efficiently and stably express an antigen of interest.

Problem 3

No detectable MITO-GFP transfection reporter for some clones in the cell-based screen.

Potential solution

Increase the concentration of the plasmid miniprep by doubling the bacterial culture volume, and reducing the miniprep elution volume to the minimum according to manufacturer recommendation. Choose a more efficient transfection reagent.

Problem 4

No positive degraders selected (related to Cell-based screening of intracellular antibody-based degraders, steps 19 and 20).

Potential solution

Increase the number of antibody-based degraders screened and check the diversity of the antibodies selected after the phage display by sequencing. If the diversity is too low (over representation of few clones), then use the antibodies from the previous round of phage display after checking the diversity of that round by sequencing.

Problem 5

The antibody-based degraders work in the engineered stable cell line but not on the endogenous target in a cell line of interest (related to Cell-based screening of intracellular antibody-based degraders, step 23).

Potential solution

Switching FBOX domain to the one of a more efficient E3 ligase in the cell line used (e.g., VHL or SPOP) could resolve this issue.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Aurélien Olichon (aurelien.olicchon@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No data or code were generated in this study.

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Author contributions

Conceptualization: N.B., G.F., and A.O.; Methodology: N.B., G.F., and A.O.; Writing: N.B., G.F., and A.O.; Supervision and Funding Acquisition: A.O. and G.F.

Declaration of interests

N.B., G.F., and A.O. are co-inventors on the patent PTC/EP2016/052136, concerning the discovery of RHO-GTP single domain antibodies and their applications.

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