Published in final edited form as:

Cold Spring Harb Protoc. 2009 March ; 2009(3): pdb.prot5172. doi:10.1101/pdb.prot5172.

Regulating Protein Stability in Mammalian Cells Using Small Molecules

Emily L. Hagan, Laura A. Banaszynski, Ling-chun Chen, Lystranne A. Maynard-Smith, and Thomas J. Wandless¹

Department of Chemical and Systems Biology, Stanford University 318 Campus Drive, Stanford, CA 94305, USA

Introduction

Regulating protein stability using small molecules provides a rapid, reversible and tunable method to study a protein of interest's (POI) role in cells. This protocol explains how to employ an unstable protein domain, named a destabilization domain (DD), fusion to control the biological activity of a POI. In the absence of small molecule ligand, the DD is unstable and directs the fusion protein for degradation. Addition of ligand stabilizes the DD, allowing the fusion protein to accumulate in cells and the POI to exert its biological effect. This ligand is specific for the DD domain and has no detectable off-target effects. By utilizing the specificity of a genetic fusion and the speed of small molecule binding, this technique can provide a better alternative than RNA interference to study a POI's role in cells.

¹Correspondence should be addressed to T.J.W. (wandless@stanford.edu).

Conflicts of Interest: The authors claim no conflicts of interest.

Cautions <!\>:

<!\> Caution Ethanol: Highly flammable. It is harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

<!\> Caution Penicillin/Streptomycin: Both penicillin and streptomycin are highly toxic and may cause cancer. They may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

<!\> Caution Phoenix Cells: Viral supernatants may contain potentially hazardous recombinant virus, depending of the type of insert and packaging cell line. Consult NIH and regional guidelines for proper storage and handling.

<!\> Caution Retroviral vector: Be aware of the BioSafety level (BSL) of your retroviral vector, and the necessary regulations for working at that BSL. Consult NIH and regional guidelines for proper storage and handling.

Recipes <R>:

3T3 media: DMEM, High Glucose 1X (Gibco, 11965)

10% Donor Bovine Serum

2 mM glutamine

100 U/mL penicillin

100 µg/mL streptomycin

PBS (1X): As described in Sambrook, Fritsch, and Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nded.

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

Dissolve in 800 mL ddH₂O. Adjust pH to 7.4 and bring volume to 1 L. Sterilize by autoclaving or filter sterilization.

Phoenix media: DMEM, High Glucose 1X (Gibco, 11965)

10% Fetal Bovine Serum

2 mM glutamine

100 U/mL penicillin

100 µg/mL streptomycin

Related Information

This protocol may be used in conjunction with the CSH protocol “A General Method for Conditional Regulation of Protein Stability in Living Animals.” (Sellmyer, M.A., *et al*).

Materials

Reagents

3T3 media<R>

DMEM, High Glucose 1X (Gibco, 11965)

Donor Bovine Serum (Gibco)

Penicillin/Streptomycin (Gibco)<I>

Ethanol, 200-proof<I>

Lipofectamine2000 (Invitrogen)

NIH3T3 cell line, or other cell line for infection (ATCC)

Optimem (Gibco)

PBS (1X) <R>

Phoenix Ecotropic packaging cell line (Orbigen) <I>

Use the amphotropic packaging line for infection of non-murine cell lines.

Phoenix media<R>

DMEM, High Glucose 1X (Gibco, 11965)

Fetal Bovine Serum (Gibco)

Penicillin/Streptomycin (Gibco) <I>

Polybrene (Sigma-Aldrich), 4 mg/mL in PBS

ProteoTuner system (Clontech)

-or-

Retroviral plasmid, such as pBMN<I>

Plasmids of FKBP-12 mutants

Shield-1 (Clontech, or Cheminpharma), 1 mM stock in 200-proof ethanol

Equipment

Biosafety cabinet, Class II

Incubator (humidified, 37 °C, 5% CO₂)

MiniPrep Plasmid kit (Qiagen)

25 mm, nylon, sterile 0.45 µm syringe filters (FisherBrand)

Tissue culture dishes (Falcon)

Flow cytometer or protein blotting equipment, as necessary to assay POI

Method

Cloning Construct for Use

- 1 Use standard molecular biology techniques to clone a fusion of the destabilization domain and the protein of interest into a retroviral plasmid. F36V should be used as a stable control, and either L106P or E31G R71G K105E as the unstable DD (See Table 1). Appending an epitope tag, such as HA or FLAG, after the protein of interest is often useful, especially when specific antibodies for the protein of interest are not available. In order to later select for cells containing the fusion, it is best to include a marker for infection (drug-resistance or fluorescent protein) behind an internal ribosome entry site (IRES).

This technology will work with transient transfections of viral and non-viral plasmids, but it requires careful titration of DNA into cells to achieve the proper tunability. Therefore, we recommend stable integration of the construct for cleaner expression of fusion and maintenance of construct. Order of fusion (N-/C-terminal DD) and presence/absence of a linker will be dictated by folding constraints of the protein of interest.

- 2 Mini Prep enough bacteria to get at least 10 µg DNA of the sequence-verified plasmid.

Packaging Into Viral Particles (4 days; Day 1–4)

- 3 In the evening of Day 1, plate 2×10^6 Phoenix cells into a 10 cm plate per construct. Culture cells in Phoenix media at 37 °C.
This amount should provide around 80% confluency at time of transfection.
- 4 The morning of Day 2, transfect 10 µg DNA with 25 µL Lipofectamine2000 in 3 mL Optimem into Phoenix cells. Prepare the DNA:lipid complexes as described in Lipofectamine2000 product literature.
- 5 After four hours of transfection, replace Optimem media with 10 mL Phoenix media.
- 6 In the evening of Day 3, plate 1×10^6 NIH3T3 cells per 10 cm plate to be infected.

For other cell lines, plate at a cell density to be approximately 30–40% confluent at time of infection.

- 7 48 hours after transfection, harvest the viral particles by filtering the media through a 0.45 µm syringe filter. Add 1000× polybrene in PBS to a final concentration of 4 µg/mL. Proceed immediately to creation of cell line (recommended), or freeze viral supernatant at –80 °C.

Freezing viral supernatants creates a loss of approximately 50% viral titre.

Creation of Cell Line (variable 3–10 days; Day 4–12)

- 8 Aspirate the media off NIH3T3 cells and add 3 mL filtered viral supernatant.
Extra supernatant can be frozen at –80 °C for later use.
- 9 After four hours of infection, replace the viral media with 10 mL 3T3 media.
- 10 48 hours after infection, select cells with stable integration of the DD -POI construct using a flow cytometry sorter and a fluorescent marker or drug selection.

If using drug selection, grow cells under selection for at least seven days. If using flow cytometry, can use sorted cells as soon as enough numbers for downstream experiments are achieved.

We typically conduct experiments with heterogeneous sorted populations. If clonal populations are desired, screen several clones to account for variation in transgene expression. Comparing clones can be especially useful for POIs with very low functional thresholds, such as transcription factors.

Regulating Protein Stability in Mammalian Cells (2 days for validation, then ongoing)

- 11** To test for ligand-dependent control of protein stability, plate equal numbers of DD-POI cell lines in two plates. Add Shield-1 to a final concentration of 1 μ M in one plate, and an equal volume of ethanol to the other plate.

- 12** 24 hours later, assay the protein stability by method of choice (flow cytometry or protein blotting).

In our system, 24 hours and 1 μ M Shield-1 provide steady-state levels of protein stability. These numbers may need to be adjusted depending on the cell type and POI.

- 13** To more-fully characterize the DD-POI fusion, perform a dose-response curve with Shield-1 between 1 nM and 3 μ M to measure the EC₅₀ and maximum stabilization. Assay stabilization kinetics with growth and decay time courses after addition and removal of drug, respectively.

Dose cells with Shield-1 simply by adding drug to the media. Remove Shield-1 by washing the cells three times with media.

Reference Banaszynski et al. for more information on kinetics experiments.

Troubleshooting

(step 10) Problem: Low infection observed when creating cell lines. Solutions:

- a.** Verify that polybrene was added to the viral supernatant before infection. Polybrene is a cationic lipid that helps diminish the electrostatic repulsion of the viral particle with the cell surface, thus aiding binding.
- b.** Try concentrating the viral supernatant with a 100,000 MWCO Amicon spin concentrator before addition of polybrene. Concentrating from 10 mL to 3 mL will increase the viral titre.
- c.** Cleaning up and concentrating the vector DNA, with a Midi- or Maxi-prep, can increase the efficiency of the lipofection in step 4. We strive for DNA concentrations of at least 800 ng/ μ L.

(step 12) Problem: No signal for drug-dependent stabilization of the DD-POI fusion by protein blotting.

Solution: The POI may not tolerate a fusion on that terminus of the protein. Try changing the fusion protein to place the DD at the other terminus of the POI. A linker is not usually necessary, but may help the POI properly fold. Using the F36V domain as a stable control can help identify if the DD-POI fusion is properly expressed.

(step 12) Problem: Fusion protein is not destabilized.

Solution: Again, moving the DD to the other terminus of the POI can change fusion protein stability. However, this technique has not been validated for every single protein, so it is

possible some proteins are not amenable to the DD system. E31G R71G K105E is a more effective destabilizer than L106P at the C -terminus of proteins.

Discussion

Modulating a protein of interest's stability with a destabilization domain provides the user with unparalleled control of protein function. This technique is faster than RNA interference (RNAi), reversible and tunable unlike the Cre/lox system, and more specific than most chemical perturbants (Banaszynski *et al.*; Maynard-Smith *et al.*). Notably, addition of 1 μ M Shield-1 has no detectable off-target effects as judged by microarray (Maynard-Smith *et al.*). To date, this system has successfully regulated examples of intracellular, nuclear, and secreted proteins (Banaszynski and Sellmyer *et al.*). However, it is unlikely that this technology will work for every protein in a cell. The POI may lose its functionality when in the fusion context, especially if the active state is a higher-order multimer. The DD placement, however, is very flexible. Destabilization domains have been shown to work at both termini as well as in an internal loop of the POI (Chu *et al.*). Lastly, a large intracellular pool of unmodified POI may swamp out the DD-POI effect and obscure a phenotype for ligand-dependent control of protein stability. For this reason, we recommend targeting for regulation monomeric proteins with low endogenous levels or with a dominant phenotype.

References

1. Chu BW, Banaszynski LA, Chen LC, Wandless TJ. Recent Progress with FKBP-derived Destabilizing Domains. *Bioorg Med Chem Lett*. 2008; 18:5941–5944. [PubMed: 18815033]
2. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AGL, Wandless TJ. A Rapid, Reversible, and Tunable Method to Regulate Protein Function in Living Cells Using Synthetic Small Molecules. *Cell*. 2006; 126:995–1004. [PubMed: 16959577]
3. Banaszynski LA, Sellmyer MA, Contag CH, Wandless TJ, Thorne SH. Chemical Control of Protein Stability and Function in Living Animals. *Nat Med*. 2008; 14:1123–1127. [PubMed: 18836461]
4. Maynard-Smith LA, Chen LC, Banaszynski LA, Ooi AGL, Wandless TJ. A Directed Approach for Engineering Conditional Protein Stability Using Biologically Silent Small Molecules. *J Biol Chem*. 2007; 282:24866–24872. [PubMed: 17603093]
5. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY, USA: 1989.
6. Sellmyer MA, Thorne SH, Banaszynski LA, Contag CH, Wandless TJ. A General Method for Conditional Regulation of Protein Stability in Living Animals. *CSH Protocols*. 2008 submitted.

Figure 1. Destabilization Domain Scheme

Genetic fusion of the destabilization domain (DD) to a protein of interest (POI) confers instability to the entire fusion product, resulting in protein degradation. Addition of a small molecule ligand, Shield-1, rescues the fusion and allows the POI to exert its normal biological effect.

Table 1

Destabilization Domains

FKBP-12 Mutation (s)	Stability	Use at
F36V	Stable (Control)	N- and C-termini of POI
F36V L106P	Unstable	N- and C-termini of POI
F36V E31G R71G K105E	Unstable	C-terminus of POI