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A General Method for Conditional Regulation of Protein Stability in Living Animals

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INTRODUCTION

The ability to rapidly and reversibly perturb protein levels in living animals is a powerful tool for researchers to determine protein function in complex systems. We recently designed a small protein domain, based on the 12-kDa FKBP protein, that can be fused at either the N- or C-terminus of a protein of interest. This destabilizing domain (DD) confers instability to fusion protein partners, allowing targeted degradation of the protein of interest. A small molecule called Shield-1 binds to the DD and protects the fusion protein from degradation. Small-molecule mediated post-translational regulation of protein stability affords this system rapid, reversible and tunable control of protein levels and function in a variety of model

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CONFLICTS OF INTEREST

The authors claim no conflicts of interest.

<!-- Caution

Anesthesia; Isoflurane

Chemicals are harmful if inhaled, ingested, or skin absorbed. Wear gloves and appropriate personal protective equipment.

<!-- Caution

Animal Treatment

All animals must be treated in accordance with your Institutional Guidelines for Animal Care and Use.

<!-- Caution

N,N Dimethylacetamide (DMA)

Intense or continued but not chronic exposure could cause temporary incapacitation or possible residual injury.

<!-- Caution

Trypsin with .05% EDTA

Chemicals are harmful if inhaled, ingested, or skin absorbed. Wear gloves and appropriate personal protective equipment.

<!-- Caution

Tween 80 (Polysorbate 80)

Exposure may cause irritation with only minor residual injury.

<!-- Caution

Streptomycin

Streptomycin is a suspected carcinogen and mutagen. It is also known for ototoxicity.

Streptomycin is harmful if inhaled, ingested, or skin absorbed. Wear gloves and appropriate personal protective equipment.

<R> Recipe

Cell culture media for HCT116 cells

DMEM with 2 mM L-glutamine (Gibco)

10% FBS (Gibco)

<!-- 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco)

systems. In this protocol we present methods and techniques for delivering Shield-1 to regulate destabilized proteins in mice.

RELATED INFORMATION

This protocol was adapted from Banaszynski and Sellmyer *et al.* Chemical control of protein stability and function in living mice. *Nat Med* (2008) vol. 14 (10) pp. 1123-7.

This protocol should be used in conjunction with the CSH protocol for using destabilizing domains in cultured cells (Hagan, E.L., *et al.*).

MATERIALS

Reagents

<!--Anesthesia; isoflurane (Webster Veterinary)
 <R><!--Cell culture media (DMEM with L-glutamine, Invitrogen)
 Cells (HCT116, ATCC)
 D-luciferin (30 mg/mL in PBS; Biosynth)
 Fetal Bovine Serum (Gibco)
 Lipofectamine 2000 (Invitrogen)
 <!--Mouse Strain (*nu-/nu-*, should be age, breed, and sex matched)
 <!--N,N-dimethylacetamide (DMA) (Sigma)
 PBS pH 7.2 (Invitrogen)
 <!--PEG400 (Sigma)
 <!--Penicillin/Streptomycin (Gibco)
 Protein detection reagents (ELISA kits, immuno-histochemistry reagents, immunoblotting reagents)
 ProteoTuner System (Clontech)
 Shield-1 (Clontech or Cheminpharma)
 <!--Trypsin with .05% EDTA (Gibco)
 <!--Tween 80 (Sigma)

Equipment

Cooled CCD camera (e.g. IVIS, Caliper)
 ELISA kit (BD Biosciences)
 Incubator, preset to 37 °C and 5% CO₂.
 Living Image Software (Caliper)
 Tissue culture dishes (Fisher)

METHOD

A variety of transgene delivery methods (e.g., viral, liposomal, or stem cell delivery) can be imagined for the analysis of a DD fusion protein in an animal model. In this protocol, we

discuss using tumor xenografts in mice as a mechanism for delivery of our destabilized protein.

*Before using an animal model system, ligand-dependent stability of the fusion protein of interest should be validated first in heterogeneous or clonal cell populations in tissue culture (see the related CSH protocol – Hagan, E. L., et al). Always confirm that the protein of interest can retain its function either an N- or C-terminal fusion. Briefly, create a stable cell line carrying the DD fused to a protein of interest (POI). This cell line should be tested for Shield-1 dependent protein levels by performing a dose-response experiment using varying concentrations of Shield-1 (from 3 μ M to 1 nM) and a time course assay. Typical *in vitro* assays for protein levels such as immunoblotting or ELISA and a functional assessment of the DD fusion protein should be used. Maximum stabilization typically has been observed using 1 μ M Shield-1, with maximum protein levels achieved after anywhere from 4 to 24 hours, depending on the protein of interest. Upon removal of Shield-1, protein is degraded to background levels within 2–4 hours. Shield-1 stabilization results in over a 50-fold increase in mean fluorescence intensity of yellow fluorescent protein (Banaszynski *et al.*) and a 6-fold increase in luminescence of a thermo-stable luciferase (tsLuc, Tisi *et al.*) *in vitro*.*

Shield-1 stabilization displays similar dynamic ranges in living mice, however it is reasonable to expect that a large increase in dosage is necessary to achieve sufficient levels of Shield-1 at the xenograft site. For animal experiments, a range of Shield-1 dosages (1–10 mg kg⁻¹) should be tested for optimal stabilization of destabilized transgenes. Maximum stabilization of DD-tsLuc is typically seen 8–24 hours after Shield-1 injection. Destabilized proteins return to basal levels by 36–48 hours after injection. *It is advisable to run a pilot experiment with fewer groups and low numbers (2–4) of animals per group to determine a suitable Shield-1 dosage regimen and to optimize transgene detection assays.*

Day 1: Plate Cells

(1) Plate cells containing a stably integrated destabilized transgene in a culture flask (175 mm³). Grow the cells to 80% confluence and count number of cells. Adjust the number of cells to implant per animal depending on the growth of the cell line in animals. Typically 100,000–5 million cells per animal should be sufficient for detection of the DD-POI after a few days. Normal experimental group sizes (n = 6–8 mice) should allow for statistically significant comparisons.

Day 2: Transplant Cells

(2) Trypsinize, quench with complete media, and spin cells (5 min, 500 g, swing-bucket rotor). Wash cells three times with PBS, spinning as above. Resuspend cells in 100 μ L (10,000 cells per μ L) of DMEM (no FBS) per animal.

(3) Xenograft cells subcutaneously (or at desired location) into mice anesthetized with isoflurane (2%).

Day 5: Shield-1 Treatment

(4) Wait several days to allow cells to form stable grafts and begin experimental protocol. Reconstitute Shield-1 in DMA at various concentrations up to 10 mg mL⁻¹. This stock solution may be kept for several months at –20 °C. Make up a fresh solution of 9:1 PEG400:Tween 80 before each injection. Inject Shield-1 i.p. at a concentration of 10 mg kg⁻¹ or 3 mg kg⁻¹ using a 1:1 mixture of 10 mg mL⁻¹ or 3 mg mL⁻¹ Shield-1 in DMA stock solution with the fresh 9:1 PEG: Tween mix. Inject control mice with DMA/PEG/Tween vehicle alone. *Shield-1 may be injected intravenously, however intraperitoneal injections often produce more reliable results.*

Day 6 and later: Experimental Analysis

- (5) Assay for experimental DD-POI stabilization. For direct protein measurement, remove tumors, standardize tumor tissue amount (typically 1 g per mouse), homogenize tissue and assay for protein levels *via* ELISA or immunoblotting.
- (6) Continue dosing with Shield-1. To maintain high levels of DD-POI, dose every 48 hours.
- (7) Periodically assay directly for DD-POI stabilization and for the phenotypic or functional effects of protein stabilization. For instance, we assayed for tumor xenograft regression based on Shield-1 stabilization of the secreted IL-2 protein. We monitored subcutaneous tumor size via caliper measurements.

Interpretation of Results

(8) A negative control group receiving a transplant of xenografted cells that do not contain DD-POI but are dosed with Shield-1 will help attribute observed results to stabilization of the transgene, and not any nonspecific effects of the ligand, vehicle, or xenograft procedure. A negative control group of mice receiving xenografted cells containing DD-POI that are given vehicle alone will show the background level of destabilized protein activity and provides a comparison for groups in which the DD-POI is stabilized by Shield-1. A positive control group in which mice receive cells containing unregulated POI will allow observation of transgene effects without temporal and tunable ligand control. Different doses of Shield-1 (10 mg kg⁻¹ and 3 mg kg⁻¹) can be used to determine any concentration-dependent activities of the protein of interest. Also, different dosages can affect the systemic diffusion of a secreted transgene. For example, 10 mg kg⁻¹ Shield-1 can stabilize secreted IL-2 such that it can be detected systemically, while at a dose of 5 mg kg⁻¹, IL-2 is only locally detectable at the xenograft site.

DISCUSSION

We have presented steps to control protein stability in mice. Modifications to this protocol should be used for other model systems (*e.g.* rat, zebrafish) or organisms (Herm-Götz *et al.*; Armstrong *et al.*). The advantages of the destabilizing domain technology are the speed of protein destabilization after drug removal, the ability to tunably regulate protein levels with Shield-1, and the extremely low basal protein levels in the absence of Shield-1. We have observed that the DD-POI dynamic range of stabilization for IL-2 and tsLuc in mice are similar to *in vitro* studies. We have determined the kinetics of stabilization and destabilization for only DD-tsLuc and it may be necessary to test this for other fusion proteins especially if a tight temporal window of stabilization is desired. If stabilization of a protein over an extended time period is desired, we have dosed mice with Shield-1 every 48 hours and seen maintenance of DD-POI levels. Researchers should consider the financial costs of long-term use of Shield-1 in animals and be aware that other FKBP ligands are capable DD stabilization (Banaszynski *et al.*). Additionally prolonged treatment with Shield-1 appears to be innocuous in cells (Maynard-Smith *et al.*) and whole animals. We have treated nude mice with Shield-1 for 2 months every 48 hours and have not observed gross signs of toxicity (*e.g.* changes in feeding behavior, grooming, or activity levels).

TROUBLESHOOTING

Problem

Transgene protein levels are not detectable after Shield-1 administration. [Step 5]

Solution

Depending on the location of cell transplant, different levels of Shield-1 may be necessary to reach the target tissue. Increasing the dose of Shield-1 (up to 10 mg kg⁻¹) may increase the stabilization of the transgene to locally and even systemically detectable levels.

Additionally, try repeated injections of Shield-1. It is possible that Shield-1 is injected into the bowel of the animal and may not reach significant concentration in the bloodstream.

A good test for whether Shield-1 is reaching the targeted tissue is to express or co-express DD-tsLuc in grafted cells. This provides an optical reporter for Shield-1 stabilization at the target tissue. Briefly, 8–24 hours after Shield-1 administration, inject 3 mg of D-luciferin (100 µL of a 30 mg mL⁻¹ stock) i.p. and wait 5 minutes before imaging anesthetized mice (isoflurane 2%) with a cooled CCD camera (IVIS, Caliper). Compare the luciferase output of Shield-1 injected mice to control mice. Quantitate the signal by selecting the xenografted area as the region of interest (R.O.I) and calculate luminescence in photons/sec/cm²/sr using image analysis software (Living Image, Caliper). If Shield-1 is reaching the tissue, luciferase signal should be approximately six fold above background.

Finally, check the maintenance of the destabilized transgene after xenograft. Harvest or biopsy implanted cells and stroma that have been passaged through the animals and plate in cell culture media. Cells that have maintained the destabilized transgene will be responsive to Shield-1 treatment.

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Table 1

Experimental Animal Groups

Group	Cells implanted	Shield-1 treatment
Negative Control	Cells no DD-POI	10 mg kg ⁻¹
Negative Control	DD-POI	Vehicle
Experimental	DD-POI	10 mg kg ⁻¹
Experimental	DD-POI	3 mg kg ⁻¹
Positive Control	POI	Vehicle
Positive Control	DD-tsLuc	10 mg kg ⁻¹
Positive Control	DD-tsLuc	Vehicle