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Knockoff: Druggable Cleavage of Membrane Proteins

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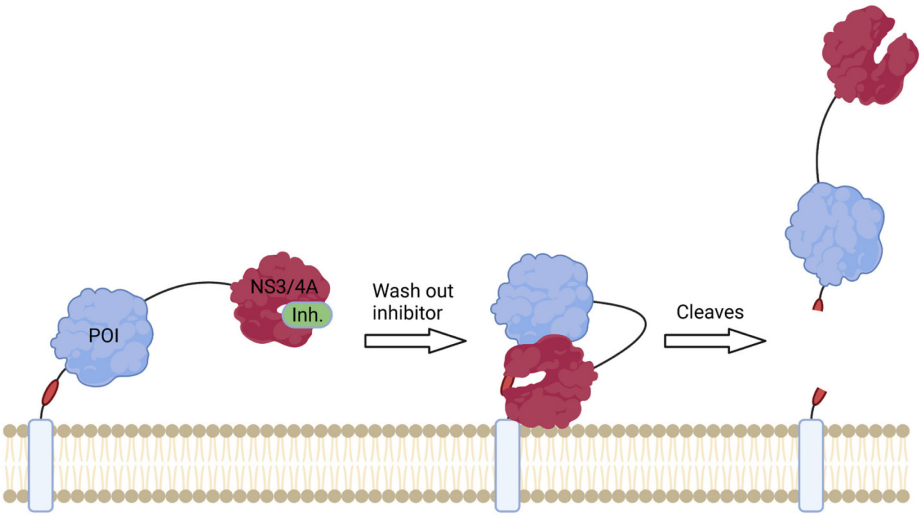
[Abstract] Comparative cell biology relies on methods that disrupt protein function. Traditional approaches target the gene that encodes the protein of interest via conventional knockout (KO) methodology, conditional Cre-lox system, or recently, flexible protocols based on CRISPR/Cas9. However, these technologies lack precise temporal control (hours), whereby the slow half-lives of proteins may confound measurements, possibly resulting in misleading phenotypes. Targeting the protein itself bypasses issues pertaining to protein half-life, resulting in more acute disruption. An ideal system would enable controllable protein disruption, dependent on the presence or absence of a small molecule, with high temporal control achieved through washout/addition of the small molecule. Here, we outline the use of knockoff, a general method to disrupt membrane proteins based on the NS3/4A protease of the hepatitis C virus. This technique has been used in post-mitotic cells to study the function of long-lived integral membrane proteins and is suitable for the study of other membrane-bound proteins.

Graphic abstract:



Removal of the protease inhibitor induces cleavage from the membrane.
General model of knockoff method. Inh, Inhibitor; POI, Protein of Interest; NS3/4A, Hepatitis C viral protease.

Keywords: Knockoff, Degron, Protease, Protein degradation, Membrane proteins

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[Background] Removing a protein from a biological system is a common approach in the study of its function. Traditionally, this removal has been achieved through deletion of the gene encoding the protein of interest (POI), which prevents new copies of the protein from being made. However, drawbacks to this technique limit its usefulness. Constitutive genetic knockouts necessitate the development of an organism without the POI and may promote compensatory mechanisms, thus confounding any observed phenotype. Some control can be achieved through application of a Cre-lox system. This system uses a sequence-specific recombinase (Cre) to allow for a time-controllable genetic knockout (Sauer, 1987). Yet, this system still functions on the genetic level, which leaves the researcher at the mercy of the natural half-life of their POI. Indeed, half-lives of many presynaptic proteins range from many days to over a week (Dörrbaum *et al.*, 2018).

These drawbacks to genetic interruption have necessitated the generation of new technologies to acutely and directly disrupt protein function. There are several strategies for protein degradation, including heterobifunctional chemical degraders (Toure and Crews, 2016) and antibodies (Clift *et al.*, 2017) that act by binding the target protein and recruiting endogenous degradation machinery, resulting in protein ubiquitination and elimination. Unfortunately, these techniques require substantial engineering for each different target molecule to ensure binding and specificity. Other adaptor-small molecule-based methods, like the auxin inducible degron (AID) (Natsume *et al.*, 2016), have proven useful in some settings but have not seen widespread application in post-mitotic cells. Indeed, our group has observed substantial leak of the AID system in neurons (Vevea and Chapman, 2020). Most importantly, membrane proteins are inherently difficult to disrupt because heavily ubiquitinated proteins must first be sorted away from their 'home' membrane for elimination. A method for membrane protein control that is applicable to many different targets would be useful for scientists studying protein function, particularly for hard to target membrane proteins in post-mitotic cells, such as neurons, which have slower rates of protein turnover.

Here, we use the viral NS3/4A protease for druggable cleavage (separation) of a protein from its resident membrane, resulting in protein degradation through a newly exposed degron. The NS3/4A protease has several potent inhibitors that are nontoxic to neurons, which can be used to inhibit protease activity, thus preserving protein function until a specified time. Upon washout of the inhibitor, the protease cleaves a small (10 amino acid) inserted substrate site. The resultant cleavage generates a new amino terminus (*i.e.*, degron), which will lead to rapid destruction through endogenous protein degradation pathways. The procedure for the application of knockoff detailed here includes possible caveats and important notes, and compares the apparent dissociation rate of two nontoxic protease inhibitors. The inhibitor Paritaprevir (PRV) dissociates rapidly, while the inhibitor Glecaprevir (GCV) dissociates slowly, thus yielding different rates of protein disruption. We find this technique works in HEK293T cells and primary neurons with no signs of toxicity when using PRV, GCV, or another inhibitor, Danoprevir (DNV). Importantly, the inhibitors seem to be stable for weeks at 37°C in both culture conditions. Broadly, this technique requires some structural information or assumptions, so is not a 'plug-and-play' technique akin to fluorescent protein tagging. The use of this technique also relies on a constitutive or conditional knockout background, as the endogenous protein is not targeted, only the

engineered one. However, the ability to target long-lived membrane proteins in post-mitotic cells for acute disruption is an unmet need and is therefore valuable.

Materials and Reagents

1. Tissue Culture (TC)-treated culture dishes 150 mm × 25 mm (Thermo Fisher Scientific, catalog number: 08-772-24)
2. Polypropylene Konical tube, 25 × 89 mm (Beckman Coulter, catalog number: C14291)
3. 24-well cell culture plates (Genesee Scientific, catalog number: 25-107)
4. Steriflip sterile disposable vacuum filter units, 0.22 µm (Thermo Fisher Scientific, catalog number: SCGP00525)
5. Steriflip sterile disposable vacuum filter units, 0.45 µm (Thermo Fisher Scientific, catalog number: SE1M003M00)
6. HEK-293T cells (ATCC, catalog number: CRL-3216)
7. pFUGW was a gift from David Baltimore (Addgene #14883; Lois *et al.*, 2002) (storage at 4°C)
8. pCD/NL was a gift from Jakob Reiser (Addgene #17531; Zhang *et al.*, 2004) (storage at 4°C)
9. pLTR-G was a gift from Jakob Reiser (Addgene #17532; Reiser *et al.*, 1996) (storage at 4°C)
10. pF(UG) hSyn SYT1-A'Q-4xGS-NS34a-FLAG (codopt) (SYT1-SELF: Addgene # 158771; Vevea and Chapman, 2020) (storage at 4°C)
11. DMEM, high glucose (Thermo Fisher Scientific, catalog number: 11-965-118) (store at 4°C)
12. Fetal bovine serum (Atlanta Biologicals, catalog number: S11550H) (store at -20°C)
13. Penicillin-streptomycin (Fisher Scientific, catalog number: SV30010) (store at -20°C)
14. NaCl (Thermo Fisher Scientific, catalog number: S271-10) (store at room temperature)
15. KCl (Sigma, catalog number: 746436-12KG) (store at room temperature)
16. Na_2HPO_4 (Sigma, catalog number: S3264-500G) (store at room temperature)
17. D-(+)-Glucose (Fisher, catalog number: 50-712-745) (store at room temperature)
18. HEPES (7.4) (Fisher Scientific, catalog number: H75030-5000.0) (store at room temperature)
19. 37% HCl for adjusting pH (Sigma-Aldrich, catalog number: 320331-500ML) (store at room temperature)
20. $CaCl_2$ (Sigma-Aldrich, catalog number: 223506-500G) (store at room temperature)
21. Filter sterilized, double-distilled, and autoclaved water
22. Sucrose (Sigma-Aldrich, catalog number: 84097-1KG) (store at room temperature)
23. EDTA (Sigma, catalog number: E5134-500G) (store at room temperature)
24. DPBS, no calcium, no magnesium (Thermo Fisher Scientific, catalog number: 14190136) (store at 4°C)
25. Poly-D-Lysine hydrobromide (Millipore Sigma, catalog number: P0899-500MG) (store at -20°C at 0.5 mg/ml, use at 0.05 mg/ml)
26. Hibernate A medium (BrainBits, catalog number: HA) (store at 4°C)
27. Trypsin EDTA 1× (Corning, catalog number: 25-053-CI) (store at 4°C)

28. Neurobasal-A Medium (Thermo Fisher Scientific, catalog number: 10888022) (store at 4°C)
29. GlutaMax Supplement (Fisher Scientific, catalog number: 35-050-061) (store at 4°C)
30. B27 Supplement (50×) (Thermo Fisher Scientific, catalog number: 17504044) (store at -20°C)
31. Conditioned neuronal media (see Note 2)
32. Paritaprevir (PRV) (MedChemExpress, catalog number: HY-12594) (store at -20°C)
33. Glycaprevir (GCV) (MedChemExpress, catalog number: HY-17634) (store at -20°C)
34. DMEM Media (DMEM+2) (see Recipes)
35. HBS (see Recipes)
36. Sucrose Buffer (see Recipes)
37. Neuronal Culture Medium (NBMA+3) (see Recipes)

Equipment

1. Incubator at 37°C and 5% CO_2
2. Centrifuge (Eppendorf Centrifuge 5430)
3. Ultra-Centrifuge (Beckman Coulter Optima L90K Ultracentrifuge) and Rotor (SW28)

Software

1. ImageJ (Fiji) for Western Blot Analysis
2. Prism Software (GraphPad 9.0.0) for Western Blot Analysis

Procedure

Design of Knockoff Constructs:

The knockoff construct for a given protein must include the NS3/4A protease, a 10 amino acid substrate site (amino acids ADVVCC | QMSY, where | marks cleavage), and the functional POI. In this design process, a published structure for the POI may assist in the decision of where to place the substrate site. Above all, the POI must be amenable to addition of the protease tag and insertion of the substrate site. This can be checked by making these modifications (protease tag and substrate site insertion) separately and comparing the modified construct to the conventional KO phenotype.

Flexible linkers between the POI and the protease help preserve the function of the POI once tagged and allow the protease freedom to reach the substrate site. The length of the linker should be approximated based on the distance between the protease and the substrate site using published structure information. We have had success using flexible linkers of varying lengths, composed of Gly-Ser-Ser-Ala repeats. We estimate the length of the linker using the approximate length of a peptide bond, which is 0.36 nm (Ainavarapu *et al.*, 2007).

The protease should also be on the same side of the membrane as the substrate site to allow access. Regarding the position of the 10-residue substrate cleavage site, it is helpful if this site is in a flexible,

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accessible region of the protein, again to allow the protease and substrate site to come together. For example, in the synaptotagmin 1 knockoff construct (SYT1-SELF), the substrate site is in the juxtamembrane region between the transmembrane domain and the Ca^{2+}-binding domains (Vevea and Chapman, 2020). If needed, small flexible linkers could be included on either side of the inserted substrate site to ensure that the cleavage site is accessible by the protease.

We found that the best way to express these constructs is in a lentiviral vector. An example lentiviral vector expressing SYT1-SELF is shown in Figure 1. Lentiviruses can easily be made 'in-house,' store well in -80°C freezers, have high transduction rates, and allow a broad range of titratable protein expression with minimal toxicity.

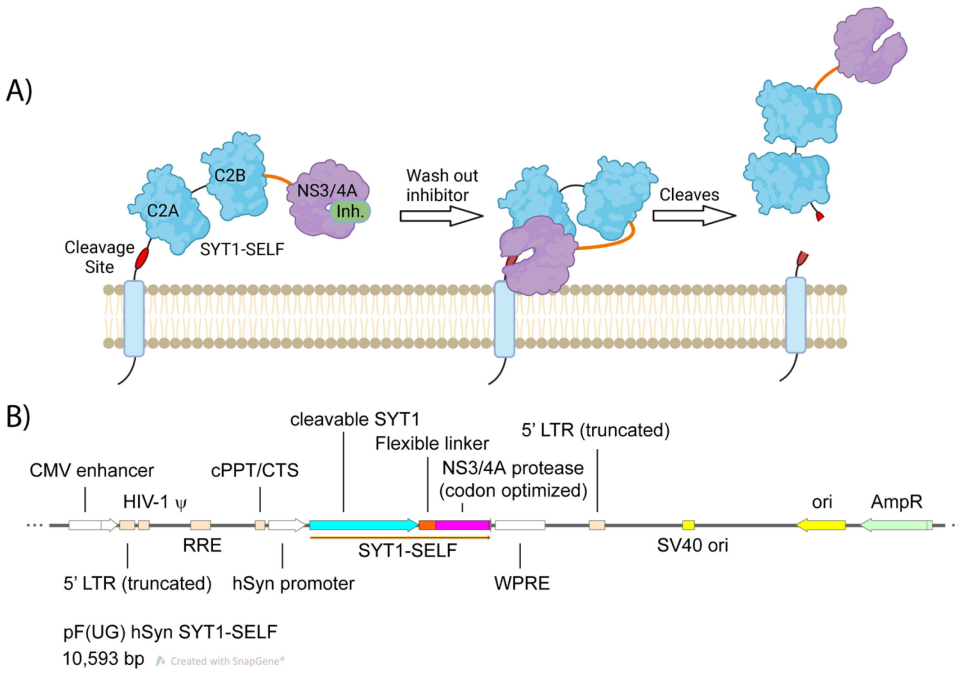

Figure 1. Design of SYT1-SELF Knockoff Construct.

A. Diagram depicting the SYT1-SELF knockoff construct. Made with Biorender. B. Vector map of the plasmid used for lentiviral transduction of the SYT1-SELF construct. This is the third generation lentiviral vector pFUGW, modified to use a human Synapsin promoter to ensure expression only in neurons. Virus titer should be adjusted to match expression to endogenous protein levels.

A. Lentivirus Production (adapted from Kutner *et al.*, 2009)

1. Seed 15 cm dishes with HEK-293T cells and use at 30-40% confluence at transfection.
2. Use $CaPO_4$ transfection to transfect the HEK-293T cells with 27 µg of pFUGW based vector, 20 µg of pCD/NL, and 6 µg of pLTR-G per 15 cm dish, as described previously (Kutner *et al.*, 2009) (see Recipe 3 for 2× HBS).
3. After 16 h, replace the DNA-containing media with 22 ml of fresh DMEM+2 (see Recipe 1).
4. Forty-eight hours later, collect the supernatant media from the tissue culture dishes.

5. Centrifuge the media at 500 × *g* for 10 min at room temperature. Filter this supernatant through a 0.45 µm PES (low-protein binding) bottletop filter.
6. Move all virus-containing media to the ultra-centrifuge tubes, then carefully add 4 ml of sucrose buffer (see Recipe 2) to the bottom of the ultra-centrifuge tube so that the dense sucrose buffer can filter the virus out of the media during ultracentrifugation.
7. Centrifuge the media and sucrose buffer at 82,250 × *g* for 2 h at 4°C.
8. Resuspend the viral pellets in 100 µl of PBS without Mg^{2+}/Ca^{2+}. The virus can be stored at -80°C until use.

B. Preparation of Neuronal Cultures
1. Add Poly-D-Lysine to 12-well culture dishes for 1 h at room temperature. Wash the wells twice with sterile water.
2. Sacrifice a pregnant rat at day 18 of gestation with asphyxiant gas (CO_2).
3. In a laminar flow hood, remove the embryos, and extract the hippocampi in Hibernate A medium.
4. Incubate hippocampi in trypsin/EDTA for 30 min at 37°C, inverting every 10 minutes.
5. Wash the hippocampi twice with 1 ml of DMEM+2 media, and then triturate the cells no more than ten times to dissociate the hippocampi.
6. Plate the neurons at 1.25 × 10^5 cells per ml per well in a 12-well plate, or half that per well in a 24-well plate.
7. One hour after plating, remove the DMEM+2 media and replace with 1 ml per well of NBMA+3 (see Recipe 4).
8. Maintain the neurons by adding 300 µl of fresh NBMA+3 (for a 24-well plate) twice per week after the first week.

C. Transduction and Inhibitors
1. When the neuronal cultures reach 2-6 days in vitro (DIV), add the appropriate amount of lentivirus to each well. The appropriate amount will depend on the normal amount of POI expression; be careful not to overexpress the modified protein.
   Note: Viral titer will need to be adjusted for each batch of virus that is created. Titrate to endogenous expression levels and be cognizant of protein over-expression.
2. On this same day, add the NS3/4A protease inhibitor to the cells so that its final concentration is 0.5 µM.
3. When adding media to maintain the neurons, add media with inhibitor to keep the concentration consistent. We have not observed any loss of PRV over the course of two weeks in culture.

D. Removal of Inhibitors and assessment of protein degradation
1. Remove all the media from the well, and briefly wash with conditioned NBMA+3 twice, then once for 5-10 min at 37°C. The post-wash incubation should also be with conditioned NBMA+3 media.

Note: Conditioned media is neuronal culture media that has been used to culture healthy cortical neurons and therefore already contains growth factors that are secreted by the neurons. This media can be generated from wells that are not used on the same plate or on other neurons that are grown at the same time. To store conditioned neuronal culture media, remove it from the cells at 14-21 DIV, filter sterilize, and store at 4°C for use within weeks or freeze (from -20°C to -80°C) for later use.

2. Efficiency of cleavage can be assessed by western blot, as shown in Figure 2. This should be used to determine if the cleavage is occurring within the construct and confirm sensitivity to inhibitors.

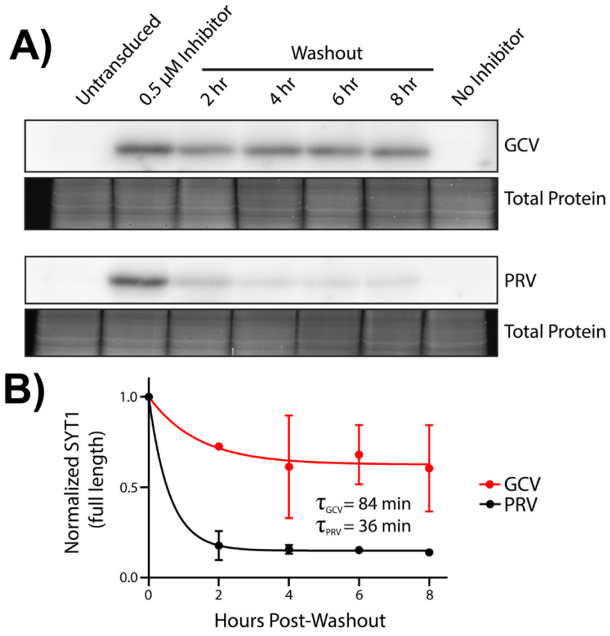

Figure 2. Observed cleavage rates of SYT1-SELF knockoff expressing neurons treated with Paritaprevir or Glecaprevir.

A. Representative immunoblots using an anti-FLAG antibody showing cleavage of SYT1-SELF in rat cortical neurons. The total protein is revealed by trichloroethanol (TCE) in-gel staining. GCV-treated neurons have a slower SYT1-SELF rate of cleavage relative to PRV-treated neurons, likely owing to a slower dissociation rate of GCV. B. Quantification of blot in (A), mean ± SEM, N=2. PRV appears to dissociate from the protease quickly while GCV does not, effectively preventing cleavage for hours. $Plateau_{PRV}$ = 0.15; $Plateau_{GCV}$ = 0.63.

E. Downstream applications

Once the inhibitor is removed, the neurons (or other cell type) are ready for interrogation with the desired method. Note the cleavage rate calculated from Procedure D to guide downstream applications.

Data analysis

1. Protein levels were quantified from immunoblots using ImageJ.
2. Values for tau and plateaus were calculated with a nonlinear regression from GraphPad Prism.

Notes

1. Here, we observe a significantly shorter τ than what we observed previously for this construct (Vevea and Chapman, 2020). One possible reason is the use of rat cortical wild-type neurons here and mouse hippocampal SYT1KO neurons previously.
2. For full immunoblot procedure, see Vevea and Chapman (2020). Many antibodies can be used to verify knockoff. An antibody specific to the researcher's POI is most useful, but the anti-NS3 antibody 1B6 or the anti-FLAG we use here are also very useful.

Recipes

1. DMEM Media (DMEM+2)
   440 ml DMEM
   10 ml (1×) Penicillin Streptomycin
   50 ml Fetal Bovine Serum
2. Sucrose Buffer
   In filter sterilized, double-distilled, and autoclaved water.
   0.2 g/ml Sucrose
   100 mM NaCl
   20 mM HEPES
   1 mM EDTA
3. HBS
   In 500 ml filter sterilized, double-distilled, and autoclaved water.
   8.0 g NaCl
   0.37 g KCl
   0095 g Na_2HPO_4
   1.35 g Glucose
   5.0 g HEPES
   Add HCl until pH is 7.0-7.05
4. Neuronal Culture Medium (NBMA+3)
   480 ml Neurobasal A Medium
   10 ml (1×) Penicillin Streptomycin
   5 ml (1×) GlutaMax Supplement
   5 ml (1×) B-27 Supplement

Acknowledgments

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Competing interests

The authors declare no competing financial interests.

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