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Cell line construction and analysis of agDD-GFP protein aggregates

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We use this protocol and it's working

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Disclaimer

Abstract

Here, we describe protocols for analysis of the inducible protein aggregate agDD-GFP in HeLa-dCas9 and HEK293T cells under a variety of conditions and in various genetic backgrounds. agDD-GFP is stabilized by a small molecule (shield-1, S1) and washout of S1 leads to agDD-GFP aggregation in minutes. By monitoring aggregates after S1 washout by flow cytometry or microscopy, it is possible to observe proteasome-dependent turnover of agDD-GFP proteins. Methods (flow cytometry) and cell lines to observe this turnover are described in this protocol.

Materials

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Chemicals, peptides, and recombinant proteins		
	Puromycin	Sigma-Aldrich	P9620
	G418 (Geneticin)	Invivogen	ant-gn-2
	Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995
	fetal calf serum		
	NLS-Cas9 protein	Berkeley QB3 MacroLab	https://qb3.berkeley.edu/facility/qb3-macrolab/projects/#cas9-nls-purified-protein
	S1	MedChem Express	# HY-112210
	Experimental models: Cell lines		
	HeLa-dCas9 cells	Ref 2	Ref 2
	HEK293T	ATCC	RRID: CVCL_0063
	Recombinant DNA		
	Plasmid expressing agDD-GFP	Ref 1	Addgene #78289
	pCRISPRia-v2	Ref2	Addgene #84832
	pU6-sgRNA EF1a-puro-t2a-mCherry	**unpublished**	**Addgene #217306**
	Oligonucleotide		
	GAPDH: Forward-acgggaagcttgtcatcaat	IDT	qPRC primer

	A	B	C
	GAPDH: Reverse-catcgccccacttgatttt	IDT	qPRC primer
	NRF1: Forward-ggaacagcagtggcaagatctc	IDT	qPRC primer
	NRF1: Reverse-gcaaggctgtagttggtgctca	IDT	qPRC primer
	DDI2: Forward-tgctgaaggaacgcaatccacc	IDT	qPRC primer
	DDI2: Forward-tgctgaaggaacgcaatccacc, Reverse-cagacgaatcctttcttgctctc	IDT	qPRC primer
	UBE3A: Forward-cgaagaatcactgttctctacagc	IDT	qPRC primer
	UBE3A: Reverse-ggattttccatagcgatcatct	IDT	qPRC primer
	UBE3B: Forward-aagctctgcggaactgtcat	IDT	qPRC primer
	UBE3B: Reverse-cggtgttcgctttcagaac	IDT	qPRC primer
	UBE3C: Forward-ctgccaggatgttcagcttc	IDT	qPRC primer
	UBE3C: Reverse-tttcttcctgagtacgatgtaaaaga	IDT	qPRC primer
	PSMD4: Forward-ctggctaaacgcctcaagaagg	IDT	qPRC primer
	PSMD4: Reverse-cactgtcaccagatgagaaccg	IDT	qPRC primer
	PSMB5: Forward-gtgtcccagaagagccaggaat	IDT	qPRC primer
	PSMB5: Reverse-tcttcaccgtctgggaggcaat	IDT	qPRC primer
	UBE3A-GGGCCGCGGCGCAAGACGGG	IDT	Protospacer sequence
	UBE3B-GCCCGGGTCTGGCAGAACTC	IDT	Protospacer sequence

	A	B	C
	UBE3C-GCACAGCTCGGGCCGCTGCA	IDT	Protospacer sequence
	NRF1-GAAGCTCCGGCGCCGAGAGTG	IDT	Protospacer sequence
	DDI2-GACTCACTGAGCGTGTGTGAG	IDT	Protospacer sequence
	Forward gRNA primer sequence for UBE3C knockout: TAATACGACTCACTATAGGAGCTACGAAGACGATGTGG	IDT	Primer for tracr gRNA synthesis
	Reverse gRNA primer sequence for UBE3C knockout: TTCTAGCTCTAAAACCCACATCGTCTTCGTAGCTC	IDT	Primer for tracr gRNA synthesis
	Forward gRNA primer sequence for knockin of RPN13K21R: TAATACGACTCACTATAGGGGCGCCTCCAACAAGTACT	IDT	Primer for tracr gRNA synthesis
	Reverse gRNA primer sequence for knockin of RPN13K21R: TTCTAGCTCTAAAACAGTACTTGTTGGAGGCGCCC	IDT	Primer for tracr gRNA synthesis
	Forward gRNA primer sequence for knockin of RPN13K34R: TAATACGACTCACTATAGGAGTCACGGTGGTCCCCTTC	IDT	Primer for tracr gRNA synthesis
	Reverse gRNA primer sequence for knockin of RPN13K34R: TTCTAGCTCTAAAACGAAGGGGACCACCGTGACTC	IDT	Primer for tracr gRNA synthesis
	ultramer for homology-directed knockin of RPN13K21R;K34R: CTCTTTCCAAGCCTGGTGCCAGGCTCTCGGGGCGCCTCCAACAGGTACTTGGTGGAGTTTCGGGCGGGAAAGATGTCCCTGAGGGGGACCACCGTGACTCCGGATAAG	IDT	gRNA sequence

	A	B	C
	CGGAAAGGGCTGGTGTAC ATTCAGCAGACGGAC		
	UBE3C MiSeq Forward- TAATACGACTCACTATAGG AGCTACGAAGACGATGTGG	IDT	MiSeq
	UBE3C MiSeq Reverse- TTCTAGCTCTAAAACCCAC ATCGTCTTCGTAGCTC	IDT	MiSeq
	RPN13 MiSeq Forward- ACACTCTTTCCCTACACGA CGCTCTTCCGATCTCTTTC AGGATGACGACCTCAG	IDT	MiSeq
	RPN13 MiSeq Reverse- GTGACTGGAGTTCAGACGT GTGCTCTTCCGATCTTGAA TGTACACCAGCCCTTTC	IDT	MiSeq

Cell line maintenance

1 Maintain HeLa-dCas9 and HEK293T cells in complete DMEM (Dulbecco' Modifies Eagles Medium (DMEM) with 10% fetal bovine serum and optional 1% penicillin-streptomycin).

2 agDD-GFP (Addgene Plasmid #78289) is integrated into HeLa-dcas9 and HEK293T cells using the piggy-bac transposase, grown for 10 days, and then sorted for GFP positive cells. *After integration maintain cells in complete DMEM that contains shield-1 ligand at a final concentration of 1 µm.*

Expression of CRISPRi targeting vectors in HeLa-dCas9 cells

3 Prepare vectors for expression of gRNAs. Single sgRNA expression vectors were individually cloned by annealing complementary synthetic oligonucleotide pairs (Integrated DNA Technologies) for each sgRNA with flanking BstXI and BlpI restriction sites and ligating the resulting double-stranded segment into either BstXI/BlpI-digested pCRISPRi-v2 (Addgene #84832) [marked with a puromycin resistance cassette and BFP as described (2)] or BstXI/BlpI-digested pU6-sgRNA EF1a-puro-t2a-mCherry Addgene #217306) (marked with a puromycin resistance cassette and RFP). *Oligonucleotide sequences are provided in the materials table.*

4 The resulting sgRNA expression vectors are individually packaged into lentivirus.

5 These sgRNA vectors were transduced into HeLa^{dCas9} +agDD-GFP cells at MOI < 1 (20 – 40% infected cells).

6 Select cells with puromycin (1 microgram/ml) for 48 hours, then allow additional 48 hours of recovery.

7 Allow 5-8 days for knockdown [measured by qPCR] before assaying phenotypes.

Gene editing of UBE3C knock-out and RPN13 knock-in in HEK293T cells

8 Forward and reverse oligonucleotides that contain CRISPR target sequences for UBE3C or RPN13 were ordered from IDT, which were used to generate gRNA by Precision gRNA Synthesis kit (Thermo Fischer, A29377). Briefly, the gene-specific primers were coupled with Phusion DNA polymerase, Tracr Fragment and T7 primer mix to generate the full-length gRNA DNA template, followed by *in vitro* transcription and gRNA purification.

Gene-specific primers for UBE3C and RPN13 are as follows:
Forward gRNA primer sequence for UBE3C knockout:
TAATACGACTCACTATAGGAGCTACGAAGACGATGTGG
Reverse gRNA primer sequence for UBE3C knockout:
TTCTAGCTCTAAAACCCACATCGTCTTCGTAGCTC

Forward gRNA primer sequence for knockin of RPN13K21R:
TAATACGACTCACTATAGGGGCGCCTCCAACAAGTACT
Reverse gRNA primer sequence for knockin of RPN13K21R:
TTCTAGCTCTAAAACAGTACTTGTTGGAGGCGCCC

Forward gRNA primer sequence for knockin of RPN13K34R:
TAATACGACTCACTATAGGAGTCACGGTGGTCCCCTTC
Reverse gRNA primer sequence for knockin of RPN13K34R:
TTCTAGCTCTAAAACGAAGGGGACCACCGTGACTC

9 Electroporation was performed using the Neon Electroporation system (Invitrogen, MPK5000) and the Neon Transfection Kit (Invitrogen, MPK1096B). 1.2 micrograms of gRNA and 6 micrograms of Cas9 protein were mixed in 10ul buffer R and were incubated for 10 minutes to allow the RNP complex to form. Cells were detached and resuspended in buffer R (a component of the Neon transfection kit) at a concentration of 2×10^5 cells per 5ul. 10ul of cell suspension was mixed with RNP complex, which is enough for 2 transfections. To make RPN knock-in cells, 78 micromol of the ultramer was added right before adding cells to the mixture

Ultramer for homology-directed knockin of RPN13 K21R and K34R:
CTCTTTCCAAGCCTGGTGCCAGGCTCTCGGGGCGCCTCCAACAGGTACTTGGTG
GAGTTTCGGGCGGGAAAGATGTCCCTGAGGGGGACCACCGTGACTCCGGATAAGCGGAAA
GGGCTGGTGTACATTCAGCAGACGGAC

To create the K21R:K34R double knock-in cells, sequential targeting first with the K34R guide and then with the K21R guide was performed.

10 After 48 hours of cell recovery, cells were single cell sorted into 96-well plates by FACS to isolate single
clones. After 1-2 weeks, single cells that grew to colonies were split into two sets: one for Illumina MiSeq
sequencing analysis, the other for western blotting.

11 Clones are identified as knockouts by Illumina MiSeq sequencing and further confirmed by western blotting.

agDD-GFP analysis by flow cytometry

12 Cells were maintained in S1 to maintain agDD-GFP in a soluble folded form (1 µm S1).

13 To initiate aggregation, S1-containing mediate was aspirated from the cells, and conditioned media containing 5 µm $FKBP12^{F36V}$ (5) was added to cells.

14 After aggregation timecourse, cells were treated with trypsin and quenched with Phenol red free-DMEM. Cells were filtered through a cell strainer cap tube and analyzed on an Attune NxT (Thermo Fisher). At least 10,000 single, healthy cells were analyzed for each condition. Median fold changes of GFP were calculated and plotted using FlowJo Software.

Immunoblotting of NRF1

15 Cells were cultured in the presence of the corresponding stress to 60-80% confluency in 6-well plates, 10 cm or 15 cm dishes. After removing the media, the cells were washed with DPBS three times. Cells were resuspended in lysis buffer (50 mM TRIS 7.5, 150 mM NaCl, 0.5% NP-40, containing mammalian protease inhibitor cocktail (Sigma), Phos-STOP, and 20 unit/ml Benzonase (Millipore)). Cell lysates were cleared by centrifugation (15000 rpm, 10 min at 4 oC).

16 The concentration of the supernatant was measured by BCA assay. 20ug of cell lysate [treated with endoH or untreated] was denatured by the addition of LDS sample buffer supplemented with 100 mM DTT, followed by boiling at 95oC for 5 minutes and loaded onto the 8% or 4-12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific), followed by SDS-PAGE with MOPS SDS running buffer (Thermo Fisher Scientific).

17 The proteins were electro-transferred to nitrocellulose membranes and then the total protein was stained using Ponceau (Thermo Fisher Scientific). The membrane was then blocked with LI-COR blocking buffer at room temperature for 1h. Then membranes were incubated with the indicated primary antibodies (4oC, overnight), washed three times with TBST (total 30 min), and further incubated either with fluorescent IRDye 680RD Goat anti-Mouse IgG H+L, or IRDye 800CW Goat anti-Rabbit IgG H+L secondary antibody at (1:10,000) at room temperature for 1h.

17.1 After thorough wash with TBST for 30 min, near infrared signal was detected using OdysseyCLx imager and quantified using ImageStudioLite (LI-COR).

Over expression of NRF1

18 NRF1 (Addgene #181917) and NRF1 18ND (Addgene #181918) (10) overexpression plasmids from were packaged into lentivirus followed by transduction of HeLa^{dCas9} agDD-GFP^{HIGH} cells.

19 48 hours after infection, cells were selected with puromycin (1 microgram/ml) for 48 hours, recovered for an additional 48 hours.

20 Aggregation is started for various lengths of time and measured by flow cytometry in control and over expression cell lines. Ectopic expression of NRF1 or the 18ND mutant is verified by immunoblotting of cell extracts.

Analysis of gene knockdown efficiency and NRF1 pathway transcription by qPCR

21 Harvest cells that have been selected with puromycin and recovered [5-8 days post infection] for knockdown analysis. For NRF1 pathways transcription experiments, plate selected cells and perform aggregation timecourse. *qPCR primers for GAPDH, UBE3A, UBE3B, UBE3C, NRF1, DDI2, PSMD4, and PSMB5 are provided in the materials table.*

22 Total RNA was isolated from ~1 million frozen cell samples using Direct-zol RNA MiniPrep kit as described by the manufacturer.

23 Reverse-transcription was carried using SSIII Reverse-transcriptase (Thermo Fisher Scientific) with oligodT primer (Thermo Fisher Scientific, SO124) in the presence of RNaseIN Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific).

24 Quantitative PCR (qPCR) (in technical triplicates) was performed with Kappa Sybr Fast qPCR 2x Mix with low ROX (Roche), according to the manufacturer's instructions on Thermo pro quant 7.

Protocol references

1. Y. Miyazaki *et al.*, A method to rapidly create protein aggregates in living cells. *Nat Commun* **7**, 11689 (2016).
2. L. A. Gilbert *et al.*, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442-451 (2013).