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Supplemental Information

Cul5-type Ubiquitin Ligase KLHDC1 Contributes to the Elimination of Truncated SELENOS Produced by Failed UGA/Sec Decoding

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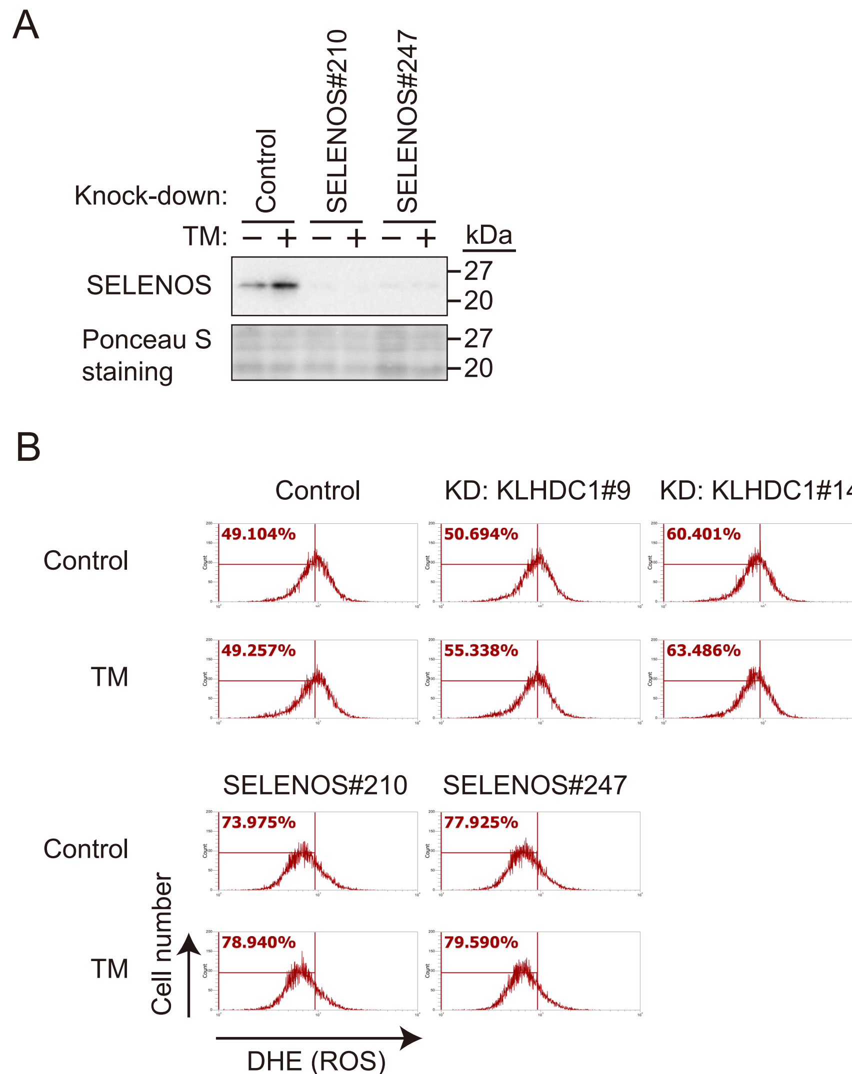

Figure S1. Reactive oxygen species (ROS) production is decreased by the downregulation of SELENOS in U2OS cells. Related to Figure 6. (A) Establishment of SELENOS-knockdown U2OS cells. Control or SELENOS-knockdown cells (#210 or #247) were treated with tunicamycin (TM; 1 μg/mL) for 24 h, and cell lysates were subjected to immunoblotting (IB) with an anti-SELENOS antibody. Ponceau S staining was used as a loading control. (B) SELENOS but not KLHDC1 is involved in ROS production. Control or KLHDC1-knockdown (#9 or #14) U2OS cells, as well as SELENOS-knockdown cells (#210 or #247), were treated with TM (1 μg/mL) for 24 h, followed by incubation with dihydroethidium (DHE; 2 μM) for 30 min. The cells were washed and subjected to FACS analysis. Thresholds and percentages are shown for comparisons of the samples. DHE staining intensity was quantified to measure the ROS levels in the cells. Representative data from three independent experiments are shown.

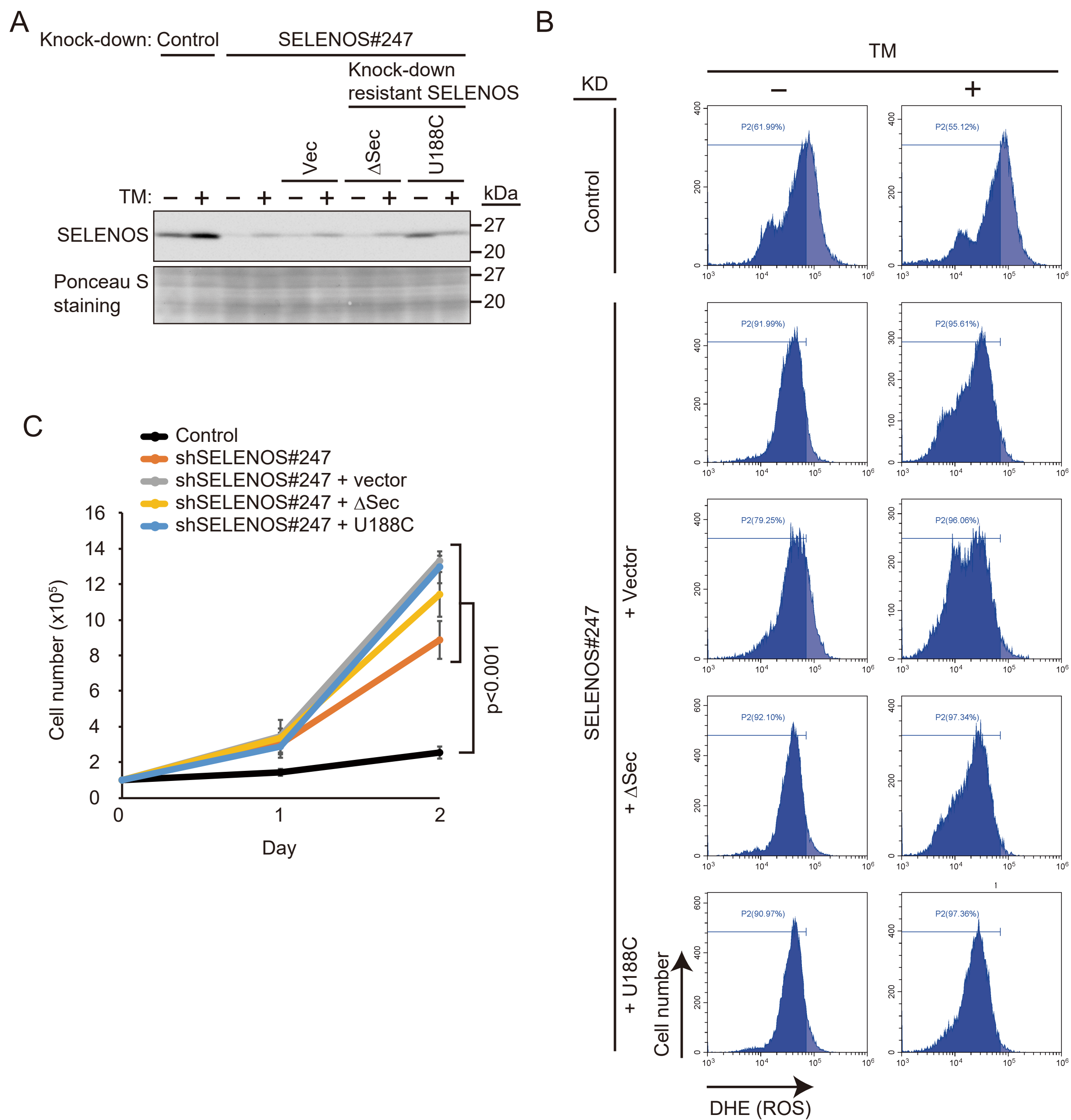

Figure S2. SELENOS(U188C) does not rescue SELENOS knockdown. Related to Figure 6. (A) SELENOS-knockdown U2OS cells were rescued with SELENOS(ΔSec) or SELENOS(U188C). Control, SELENOS-knockdown cells (#247), and non-tagged SELENOS(ΔSec)- and SELENOS(U188C)-stably expressing cells were treated with tunicamycin (TM; 1 μg/mL) for 24 h, and cell lysates were subjected to immunoblotting (IB) with an anti-SELENOS antibody. Ponceau S staining was used as a loading control. Forced expression of SELENOS(ΔSec) was weak and at the level of that in control cells. SELENOS(U188C) expression was stronger than that of SELENOS(ΔSec) but decreased upon TM treatment. (B) SELENOS(U188C) does not rescue SELENOS knockdown. Control, SELENOS-knockdown cells (#247), and non-tagged SELENOS(ΔSec)- and SELENOS(U188C)-stably expressing cell lines were treated with TM (1 μg/mL) for 24 h, followed by incubation with dihydroethidium (DHE; 2 μM) for 30 min. The cells were washed and subjected to FACS analysis. The thresholds and percentages are shown for comparisons of the samples. The DHE staining intensity indicates the reactive oxygen species (ROS) level in the cells. Representative data from three independent experiments are shown. (C) Cell growth is accelerated upon SELENOS knockdown. Control or SELENOS-knockdown cells (#247) and non-tagged SELENOS(ΔSec)- and SELENOS(U188C)-stably expressing cells were seeded in a 6-well culture plate (1×10^5/well) and cultured for 2 days. Cell numbers on day 0, 1, and 2 are shown. Data represent the mean ± SD of three independent experiments. Statistical significance of differences was not considered, as described in the methods section.

Transparent Methods

Reagents

Protein A Sepharose was purchased from GE Healthcare Bio-Sciences. MG132 was purchased from Calbiochem. Tunicamycin was purchased from Santa Cruz Biotechnology. Cycloheximide was purchased from Nacalai Tesque. Ethylenediaminetetraacetic acid (EDTA), cOmplete EDTA-free protease inhibitor cocktail, 4′,6-diamidino-2-phenylindole (DAPI), polybrene, Ponceau S, triton X-100, and tween 20 were purchased from Merck. Can Get Signal was purchased from Toyobo. Ni-NTA agarose was purchased from Fujifilm Wako Pure Chemical Corporation. Sodium selenite was purchased from Fujifilm Wako pure chemical corporation.

Plasmid construction

Human *KLHDC1* (GenBank/EBI accession number: NM_172193), *KLHDC2* (NM_014315), *KLHDC3* (NM_057161), and *SELENOS* (NM_018445) cDNA was PCR-amplified from a HEK293T cell cDNA library and subcloned into pcDNA3-puro containing a 3×FLAG sequence or pCI-neo containing a 3×HA sequence. 3×FLAG-KLHDC1(ΔCul5 box) was constructed by deleting the BC and Cul5 boxes, comprising 339 amino acids. PCR was used to construct 3×HA-SELENOS(U188C) with primers encoding the U188C mutation, which was subcloned into pCI-neo. pMT107 expresses N-terminal hexa-histidine (His_6)-tagged-ubiquitin (Okumura et al., 2004).

Gene knockdown with short hairpin RNA (shRNA)

The pMX-puro II vector was constructed by deletion of the U3 portion of the 3′ long terminal repeat of pMX-puro. The mouse U6 gene promoter followed by DNA corresponding to an shRNA sequence was subcloned into the *Not* I and *Xho* I sites of pMX-puro II, yielding pMX-puro II-U6/siRNA. The DNA for the shRNA encoded a 19-21 nucleotides hairpin sequence specific to the mRNA target, with a loop sequence (-TTCAAGAGA-) separating the two complementary domains, and contained a tract of five T nucleotides to terminate transcription (Kamura et al., 2004a). Double-stranded oligo DNA for the shRNA was ligated into the pMX-puro II vector. Human KLHDC1#9 and #14 target sequences were 5′-GAAATTACTCTCAAGAACTTC-3′ and 5′-GACACAAAGACACAGACTTGG-3′, respectively, and those for human SELENOS#210 and #247 were 5′-GGAACCTGATGTTGTTGTTAA-3′ and 5′-GCAGCTGCTCGACTGAAAAT-3′, respectively (Noda et al., 2014). The non-specific target sequence was 5′-CAGTCGCGTTTGCGACTGG-3′ (Ryther et al., 2004). Retroviruses encoding the shRNAs were

produced by transfecting pMX-puro II and pCL-10A1 into HEK293T cells. Two days after transfection, the culture medium containing recombinant retroviruses was collected (Okumura et al., 2010). U2OS cells were cultured in retrovirus-containing culture medium in the presence of polybrene (10 μg/mL) for 2 days and then selected with puromycin (3 μg/mL) for 1 week. Knockdown-resistant SELENOS contained silent mutations in the region targeted by shRNA#247 (GCAGCTGCaaGgCTGAAAAT, lower-case letters represent silent mutations). Knockdown-resistant SELENOS cDNA was ligated into pMSCV-hygro (Takara Bio Inc.) and was utilized for retrovirus production using the retrovirus packaging vector pCL-10A1.

Cell culture and transduction

HEK293T and U2OS cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (043-30085, Fujifilm Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL). HEK293T and U2OS cells were transfected with expression plasmids using polyethyleneimine (MW-25K; Polysciences Inc., Warrington, PA) at a mass ratio of 1:3. For SELENOS knockdown, U2OS cells were infected with retroviruses and selected with hygromycin (400 μg/mL) for 1 week. MG132 (2 μM) was added to the culture medium for the indicated period. U2OS cells were seeded in a 6-well culture plate (1×10^5/well) and the cells were counted daily for 2 days.

Antibodies

A rabbit polyclonal antibody to recombinant KLHDC1 was generated in our laboratory by immunizing rabbits with His_6-tagged recombinant human KLHDC1 and was purified by affinity chromatography. The working concentration was 1 μg/mL in Can Get Signal. A rabbit polyclonal antibody to HA was generated in our laboratory and used for IB and immunohistochemistry (1 μg/mL in PBS). Antibodies against the following proteins were also used: FLAG (1 μg/mL in PBS; M2; Sigma-Aldrich), HA (1 μg/mL in PBS; 12CA5; Sigma-Aldrich, used for IP), Cul5 (1 μg/mL in Can Get Signal; sc-373822; Santa Cruz Biotechnology), Cul2 (1 μg/mL in PBS; sc-166506; Santa Cruz Biotechnology), SELENOS (1 μg/mL in PBS; V6639; Merck), poly-ubiquitin (1 μg/mL in Can Get Signal; FK2; Cosmo Bio), His_6 (1 μg/mL in PBS; MAB050; R&D systems), VCP (1:2000 dilution in PBS; MA3-004; Thermo Fisher Scientific).

IP and IB analyses

Cells were lysed in lysis buffer containing 50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1% triton

X-100, 0.4 mM Na_3VO_4, 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, and cOmplete EDTA-free protease inhibitor cocktail (according to manufacturer's protocol). The lysates were incubated with 0.5 μg of antibody overnight and then with 20 μL of Protein A Sepharose beads at 4 °C for 1 h. The beads were washed three times with lysis buffer. Immunoprecipitated proteins were eluted with SDS-PAGE sample buffer with boiling, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated in 3% skim milk in PBS at room temperature for 15 min and then with primary antibodies at 4 °C overnight. The membrane was washed three times with tris-buffered saline with tween-20 (TBST) containing 50 mM tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% tween 20 at room temperature for 15 min, followed by incubation with horseradish peroxidase-conjugated anti-goat IgG (1:4000 dilution in 3% skim milk in PBS; sc-2020; Santa Cruz Biotechnology), anti-mouse IgG (1:8000 dilution in 3% skim milk in PBS; A4416; Merck), or anti-rabbit IgG (1:8000 dilution in 3% skim milk in PBS; A6154; Merck) at room temperature for 1 h. The membrane was washed three times with TBST for 15 min. Protein levels were analyzed using enhanced chemiluminescence reagent (Luminata Forte, Merck or Chemi-Lumi One L, Nacalai Tesque), and an ImageQuant LAS 400 Mini (GE Healthcare) or a SOLO.7S.EDGE (Vilber, France) system. Images were processed using Photoshop.

Ni-agarose pull-down

HEK293T cells cultured with or without MG132 (2 μM for 15 h) in 6-cm dishes and were sonicated (UD-211; Tomys Seiko) in PBS containing 8 M urea and 10 mM imidazole. After centrifugation, the supernatant was incubated with 10 μL of Ni-NTA agarose beads at 4 °C for 1 h. The beads were washed three times with the aforementioned buffer. His_6-ubiquitinated proteins were eluted with SDS-PAGE sample buffer containing 300 mM imidazole with boiling.

Immunofluorescence staining

U2OS cells were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min and washed three times with PBS. The cells were incubated with antibodies (1 μg/mL) in PBS containing 0.1% triton X-100 and 0.1% bovine serum albumin at 4 °C overnight and then washed three times with PBS. The cells were incubated with Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse antibodies (both at a 1:2000 dilution; Invitrogen) in PBS containing 0.1% triton X-100 and 0.1% bovine serum albumin in the dark at room temperature for 1 h. Then, the cells were incubated with 0.1 μg/mL DAPI in PBS for 1 min, washed extensively with PBS, and photographed using a charge-coupled device camera (Axio Observer Z1; Zeiss).

FACS

Flow cytometry was conducted with an Attune NxT (Thermo Fisher Scientific) or CytoFLEX (Beckman Coulter) flow cytometer. Briefly, cells treated or not with TM (1 μg/mL for 24 h) were harvested by trypsinization and washed with culture medium and PBS once. The cells were suspended in 85 μL of Annexin V binding buffer (10 mM HEPES-NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), and 10 μL of Annexin V-FITC (4700-100; Medical & Biological Laboratories) and 5 μL of PI (100 μg/mL) were added. The cells were incubated in the dark at room temperature for 15 min, and 400 μL of Annexin V binding buffer was added. The cells were subjected to flow cytometry according to the manufacturer's instructions. For DHE staining, cells were incubated in culture medium containing DHE (2 μM) for 30 min, harvested by trypsinization, washed once with culture medium and three times with PBS, and subjected to flow cytometry.

Statistical analysis

Data are reported as the mean ± SD of three independent experiments. Means of different groups were compared by one-way ANOVA. We did not consider statistical significance even if the p value < 0.05 because"statistical significance" has today become meaningless (Amrhein et al., 2019; Andrade, 2019; Wasserstein et al., 2019).

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