Supplementary Materials for

CRL2 Aids Elimination of Truncated Selenoproteins Produced by Failed UGA/Sec Decoding

Hsiu-Chuan Lin, Szu-Chi Ho, Yi-Yun Chen, Kay-Hooi Khoo, Pang-Hung Hsu, Hsueh-Chi S. Yen*

*correspondence to: hyen@imb.sinica.edu.tw

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MATERIALS AND METHODS

Tissue Culture

HEK293T cells were maintained in DMEM with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 6% CO_2 atmosphere otherwise indicated. Selenium is absent from the basal formulation of media, but sufficient quantity of selenium was present in FBS. To adjust selenium availability, HEK293T cells were cultured in FBS-free DMEM containing 10 μg/mL insulin, 5 μg/mL transferrin and sodium selenite (Na_2SeO_3, Sigma-Aldrich, St. Louis, MO, USA) at the indicated concentrations. HeLa, and HepG2 cells were maintained in DMEM with 10% FBS and antibiotics. U2OS, Jurkat, and K562 cells were cultured in McCoy's 5A, RPMI-1640 and IMDM, respectively; all media contained 10% FBS and antibiotics.

To produce lentiviruses, HEK293T cells were transfected with pHAGE, pRev, pTat, pHIV gag/pol and pVSVG using TransIT-293 (Mirus Bio LLC, Madison, WI, USA). To produce retroviruses, HEK293T cells were transfected with pMSCV, pGag/pol and pVSVG. Viruses were harvested 48 hrs after transfection. All tissue culture media and supplements were purchased from Gibco Life Technologies (Carlsbad, CA, USA).

CRL2 is a multi-subunit ubiquitin ligase, and it consists of Cul2, Rbx1, Elongin B/C and an interchangeable BC-box protein. Posttranslational neddylation of Cul2 is required for CRL2 to become active. To block CRL2 function, cells were either treated with 1 μM MLN4924 (Active Biochem, Maplewood, NJ, USA) for 6 hrs, infected with lentiviruses carrying DNCul2 for 38 hrs, or treated with shRNA against Cul2 (shCul2, Santa Cruz Biotechnology, Dallas, TX, USA) for 88 hrs. MLN4924 restricts CRL2 activity by inhibiting Cul2 neddylation. DNCul2 is the N-terminal part of Cul2 that only associates with substrates but not E2 ubiquitin conjugating enzyme. As a result, DNCul2-bound substrates are unable to be ubiquitinated and then degraded. shCul2 inhibits CRL2 by reducing the abundance of Cul2.

Generation of GPS reporter cell lines and GPS assays

To generate GPS reporter constructs, open reading frames from the hORFeome library were cloned into a lentiviral vector carrying the RFP-IRES-GFP GPS cassette using Gateway technology (Life Technologies). To generate GPS reporter cell lines, cells were infected with lentiviruses carrying the GPS reporter constructs. Infection was performed in medium containing 8 μg/mL polybrene (Sigma-Aldrich). To prevent overexpression of GPS reporters from multiple insertion events, the multiplicity of infection (MOI) was controlled to ensure that <10% of cells were RFP-positive (MOI of approximately 0.1). Infected cells were selected by 1 μg/mL puromycin treatment (Clontech Laboratories, Mountain View, CA, USA).

To prepare cells for FACS analysis, cells were trypsinized, resuspended in medium with 10% FBS and analyzed using a BD LSR Fortessa system (BD

Biosciences, San Jose, CA, USA). To enhance the resolution of the GPS results, multiple scaling for the GFP/RFP ratio was recorded. Because the stability varied dramatically among proteins, the plots were scale-adjusted for optimal resolution. The GFP/RFP ratios from separate plots cannot be compared directly. FlowJo (Ashland, OR, USA) was used for FACS data analysis.

Targeting sequence for shRNAs

VHL: sh#1 AGATCTGGAAGACCACCCA

APPBP2: sh#1 GCCTTCAGTTGTGTACTCT; sh#2 GTTATTTAATGATGAGGTA; sh#3 TGATGGATCATGGTGTTAA; sh#4 CAGTTTGATGTTTACTACA; sh#5 GACATCTGGCTTCTTTATA;

KLHDC2: sh#1 CTTGGTGTCTGGGTATATA; sh#2 ACAAGAGTAATCAAGTCAG; sh#3 TGGACTTACTGCATCAGTA; sh#4 AGCCTATAACTACTGGTAA; sh#5 ACACTTACTTCACAGTGTT;

KLHDC3: sh#1 TGGAAAAAGATTGAACCGA; sh#2 GAGATGAATTTGACCTTAT; sh#3 CGTGATTCAGTATAACCTA; sh#4 CCAATGACATTCACAAGCT; sh#5 AGTCTGAAGACTCTGTGCA; sh#6 TGACATTCACAAGCTAGAT; sh#7 TGCTGTATTGTTGGTGACA;

SBP2: sh#1 AGCTCTGTTTAAGAAGAAA; sh#2 ACTCTGTCTCTACCGACATTT; sh#3 TGCTAATGCCGCTACCAATTC;

eEFSec: sh#1 CGGCAAGTTCAAGATCCACAT; sh#2 AAGGTGGTGAAGAAGGTGA;

Cycloheximide-chase, GST pull-down and Western blot

For cycloheximide-chase experiments, cells were treated with 100 μg/mL cycloheximide (Calbiochem, San Diego, CA, USA) and harvested at multiple time points after treatment. Protein levels were then measured by Western blotting. Protein samples for Western blotting were prepared by lysing cultured cells in SDS sample buffer followed by sonication. ImageJ (National Institute of Health, Bethesda, MD, USA) was used for blot quantification.

The GST pull-down assays were performed on HA-tagged single copy 293T stable cell lines generated in a similar manner to that of GPS reporter cells. Cells were transfected with GST-tagged constructs for 42 hrs following 6 hrs of treatment with 10 μM proteasome inhibitor MG132 (Calbiochem) to block protein degradation. Cells were lysed by RIPA buffer (150 mM NaCl, 1.0% IGEPAL®CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with protease and phosphatase inhibitors (Roche, Basel, CH), mixed with binding buffer (1% CA630 in 1xPBS) and then incubated with Glutathione Sepharose 4B (GE Healthcare Life Science, Little Chalfont, UK) for 16 hrs at 4°C. The beads were washed 3 times with binding buffer and boiled in SDS sample buffer for Western blot analysis. Antibodies to the following epitopes and proteins were purchased from the

indicated vendors: HA (16B12, Covance, Princeton, NY, USA), GFP (JL-8, Clontech), GAPDH (GTX110118, GeneTex, Irvine CA, USA), Tubulin (Ab-2, Thermo Fisher Scientific, Waltham, MA, USA), GST (27457701, GE Healthcare), SBP2 (12798-1-AP, Proteintech, Chicago, IL, USA) and eEFSec (sc-79717, Santa Cruz Biotechnology).

Targeted mass spectrometry for the analysis of truncated form of selenoproteins

Sample Preparation

HEK293T cell pellets were lysed in ice-cold 25 mM ammonium bicarbonate buffer containing 6M urea and protease inhibitor cocktail. After removal of cell debris by centrifugation at 16,100*g* for 10 min, protein sample was reduced and alkylated by 5 mM DTE and 25 mM IAM, respectively, at 37°C for one hour, and then centrifuged through *Amicon™ Ultra-0.5 Centrifugal Filters* (50 kDa cut-off filter for SELK, 30kDa cut-off filter for SEPHS2) to reduce complexity. The collected filtrates were digested with trypsin (for SELK) and Arg-C (for SEPHS2) at 37°C overnight, then desalted using *C18* Zip-tips prior to LC-MS/MS analysis.

Mass Spectrometry and Data Analyses

For SELK target peptide, LC-SRM-MS analyses were performed on a Thermo Scientific LTQ-Orbitrap Elite mass spectrometer equipped with a Waters NanoAcquity LC system. For SEPHS2 target peptide, LC-PRM-MS analyses were operated on a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer equipped with a Thermo Scientific UltiMate 3000 RSLCnano system. SRM specific information including the monitored precursor-to-fragment ion transitions were determined from LC-MS/MS analysis of standard peptide and HEK 293T cell lysates containing overexpressed truncated form of SELK. Isolation width was kept at 2 Th, acquired with narrow product ion scan ranges and normalized collision energy at 30%. XICs for each transition were manually generated by Qual Browser using a ±13 ppm mass tolerance and 11-point Gaussian smoothing. In PRM experiments, a full mass spectrum at 120K resolution (AGC target 5×10^4, 100 ms maximum injection time, *m/z* 10-1500) was followed by one PRM scan at 15000 resolution (AGC target 1×10^4, 250 ms maximum injection time). Isolation width was set at 0.7 *m/z* and normalized collision energy at 30%.

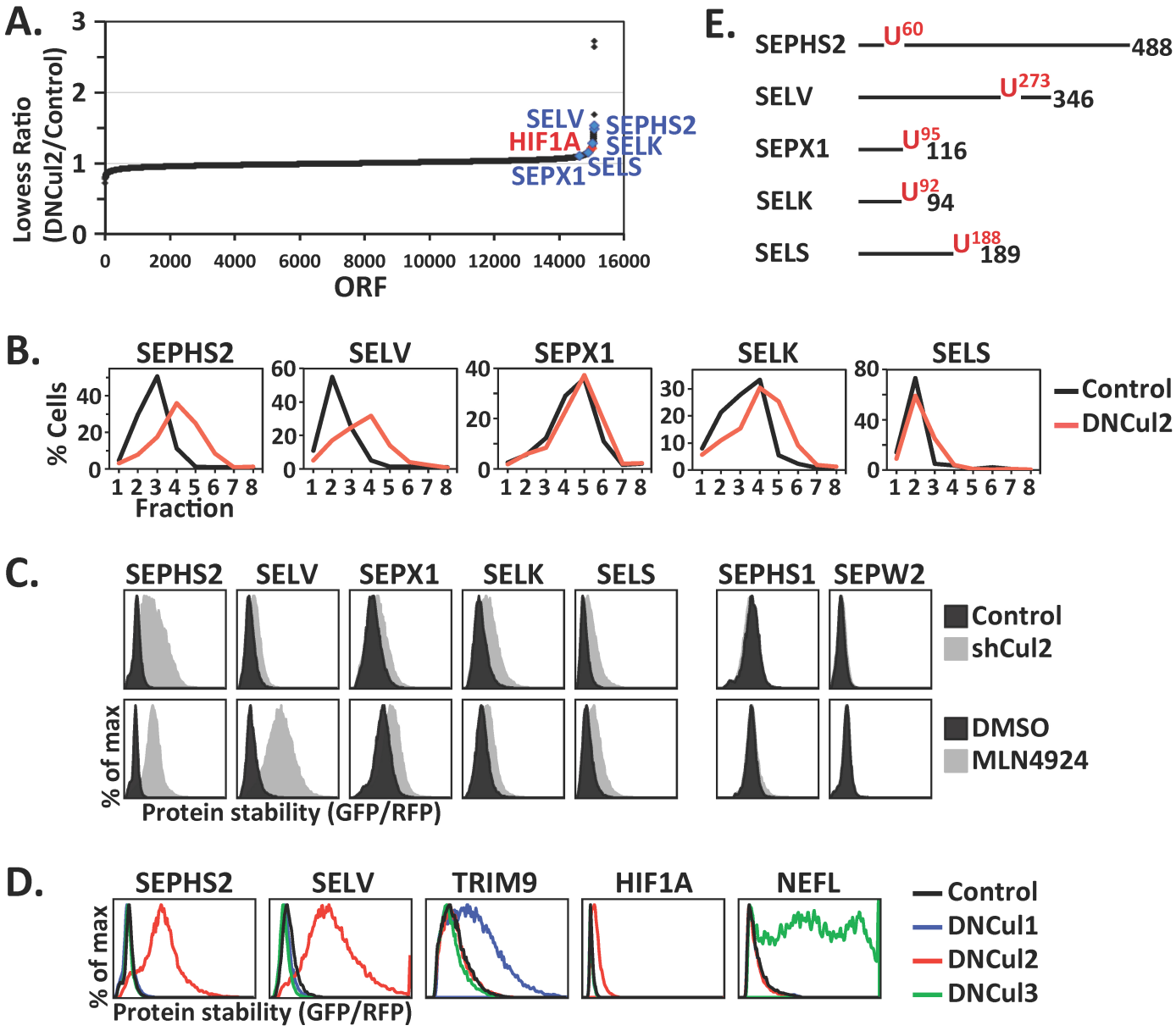

Fig. S1. Identification of CRL2 substrates by a GPS screen. (A) The HEK293T GPS cell library was treated or not treated with DNCul2 and then compared by GPS profiling. Lowess ratio (DNCul2/control) = 1 means the stability of examined protein does not change after blocking CRL2 function. The Lowess ratio (DNCul2/control) for CRL2 substrates should be greater than 1. The Lowess ratios for HIF1A, SEPHS2, SELV, SEPX1, SELK and SELS were 1.21, 1.49, 1.53, 1.10, 1.29 and 1.15, respectively. (B) The result of CRL2 GPS screen. There were a total of 16 selenoproteins in the GPS library, and we identified five of them. The z-score was 14.77 for the proportion test, which means that selenoproteins were significantly enriched. (C) HEK293T GPS reporter cells expressing selenoproteins from the UTR construct were treated or not treated with shRNA against Cul2 (shCul2) or MLN4924 and then analyzed by GPS. (D) GPS reporter cells were treated with dominant-negative (DN) Cul1, Cul2 or Cul3, and then analyzed. TRIM9, HIF1A and NEFL are known substrates of CRL1, CRL2 and CRL3 ubiquitin ligases, respectively. (E) A schematic representation of the selenoproteins. The positions of the Sec/U residues and lengths of the full-length proteins (number of amino acids) are as indicated.

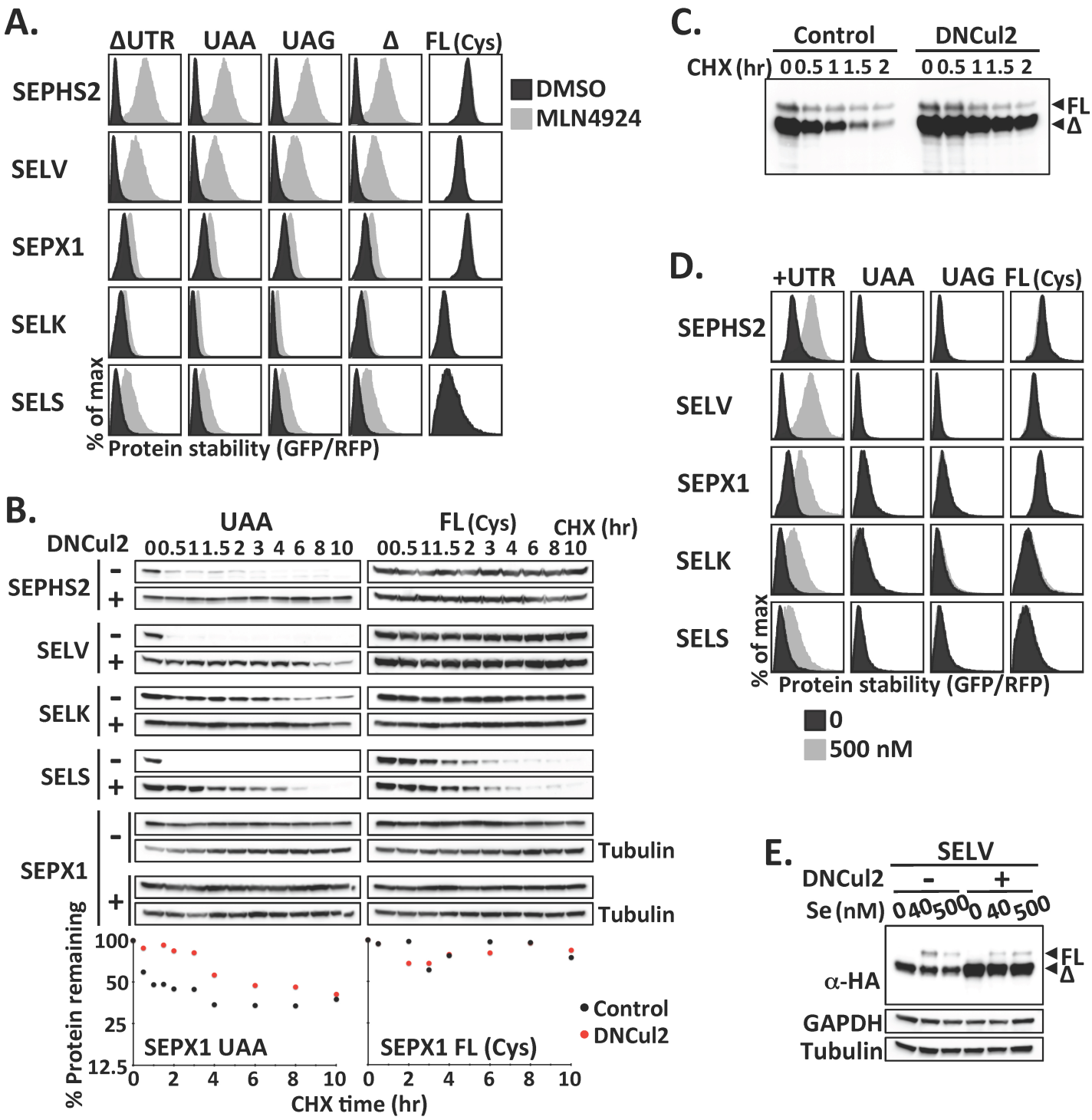

Fig. S2. Characterization of selenoprotein degradation. (A) GPS reporter cells expressing selenoprotein mutants were treated with DMSO or MLN4924 and then analyzed. (B) The stability of selenoproteins expressed from the UAA and UGU constructs was subject to cycloheximide (CHX)-chase analysis. (C) The stability of HA-tagged SELV proteins expressed from the UTR construct was analyzed by CHX-chase analysis. (D) Cells were cultured in serum-free medium with or without sodium selenite supplement, and then analyzed. (E) Cells carrying a single copy of HA-tagged SELV expressed from the UTR construct were cultured in medium with various concentrations of sodium selenite, treated or not treated with DNCul2, and analyzed by Western blotting.

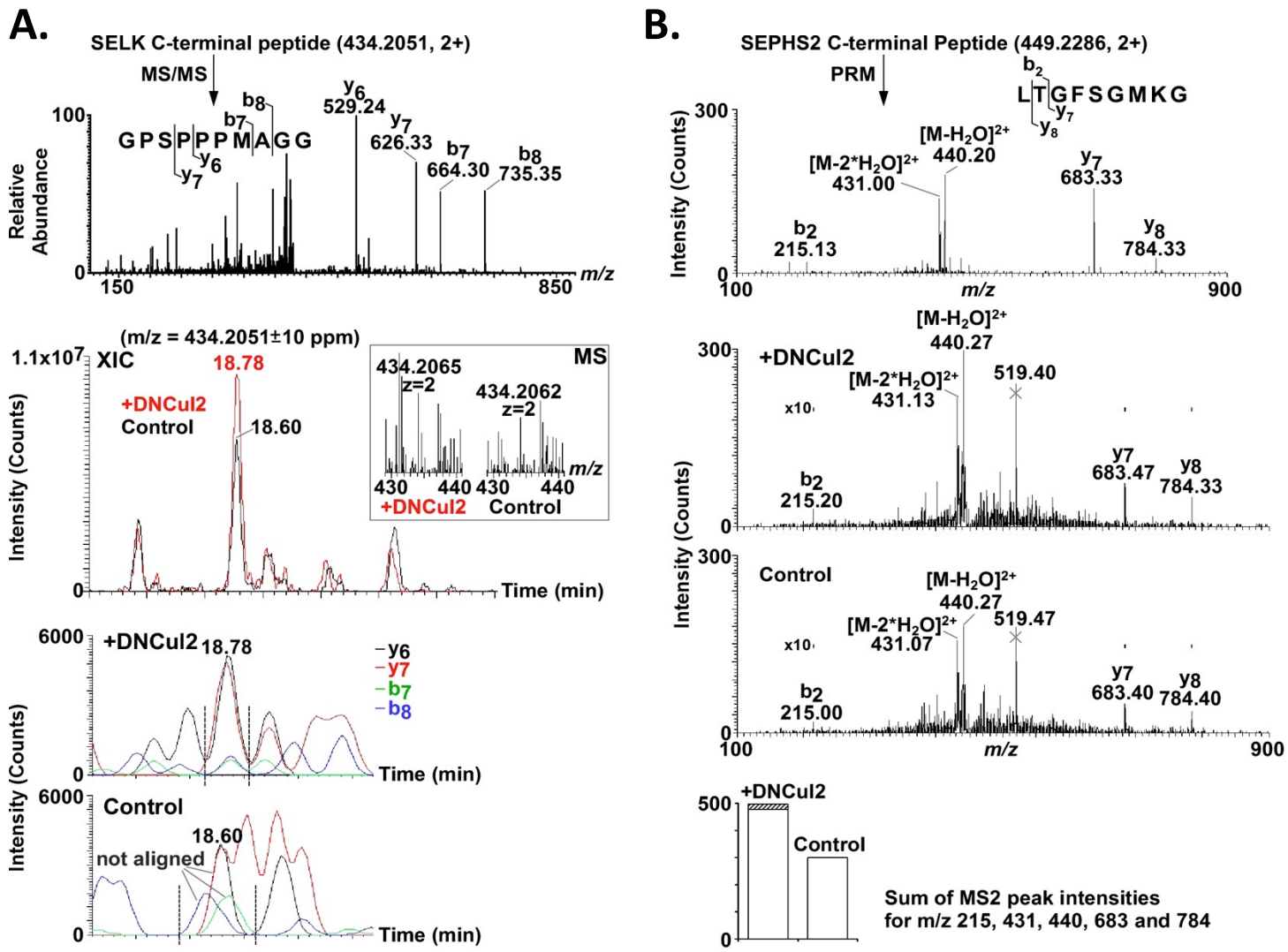

Fig. S3. Identification of endogenous C-terminal peptides from UGA-terminated SELK and SEPHS2 by targeted LC-MS/MS analyses. (A) The target SELK peptide from DNCul2 treated HEK293T cells could be detected at MS level (middle panel) and confirmed by selected reaction monitoring (lower panel, upper trace) of 4 MS^2 fragment ions at the expected ratio with reference to that afforded by synthetic standard (upper panel). A lower intensity peak of similar *m/z* was also detected for the untreated control sample (middle panel) but the monitored MS^2 transitions did not align correctly (lower panel, lower trace) to confirm its identity, thus indicating a lower amount or absence of this target C-terminal peptide in sample not treated by DNCul2. (B) The target SEPHS2 peptide from HEK293T cells was not detectable at MS level but parallel reaction monitoring of the expected MS^2 ions as compared to the standard (upper panel) allowed its positive identification in both DNCul2 treated and control samples (middle panel). The sum of peak intensity for the 5 assigned MS^2 ions, with and without normalization to an internal reference peak derived from GAPDH, indicates a significantly lower amount of the target SEPHS2 C-terminal peptide in the DNCul2 untreated control (lower panel).

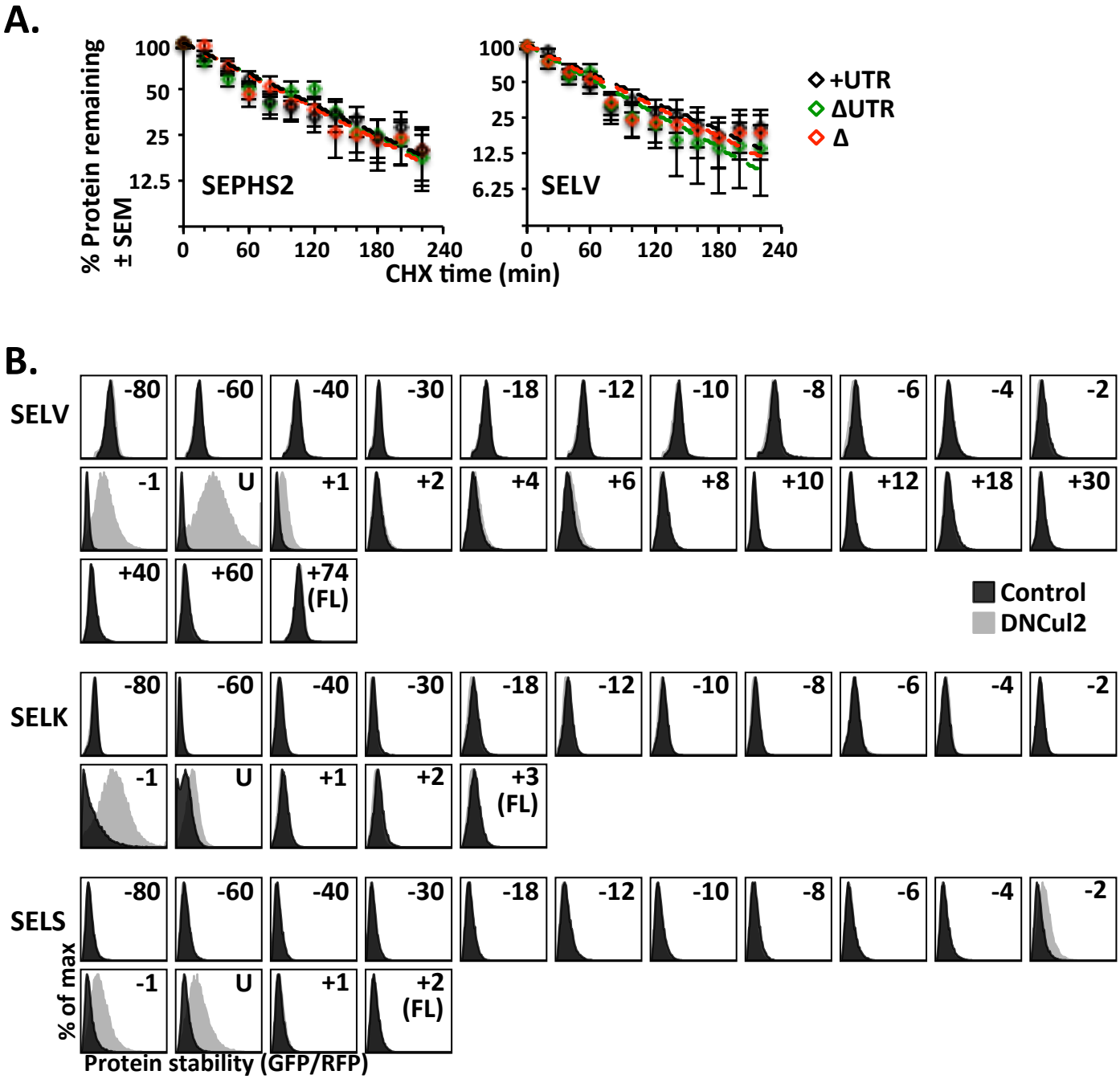

Fig. S4. Characterization of CRL2-dependent selenoprotein degradation. (A) CHX-chase analysis of truncated SEPHS2 or SELV from various transcript constructs. The experiments were performed in triplicate. (B) GPS reporter cells carrying various lengths of selenoproteins were treated or not treated with DNCul2 and then analyzed. Truncations longer than the UGA-terminated proteins carried the UGA to UGU mutation to force translational read-through. The truncation sites relative to Sec are as indicated.

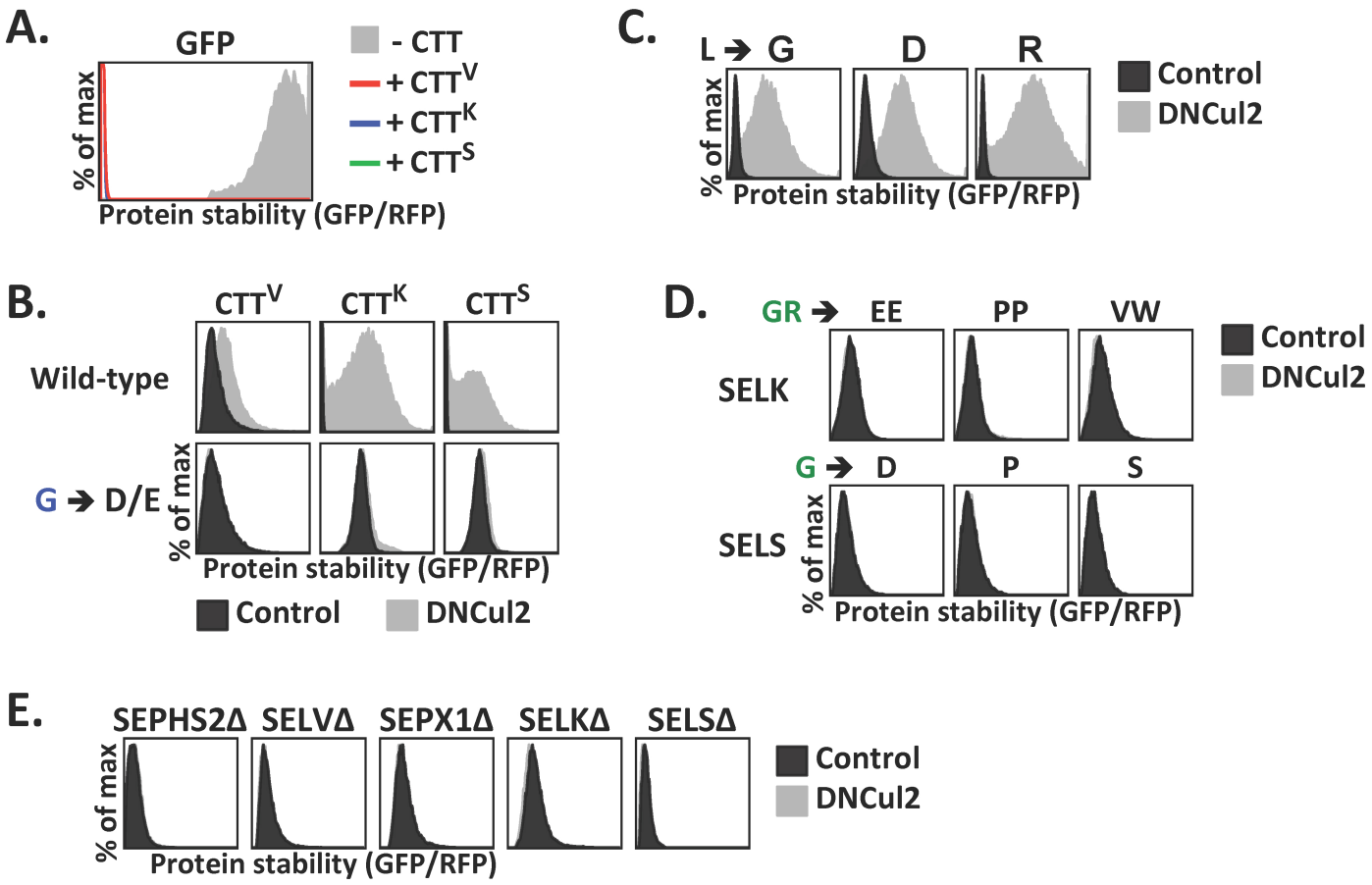

Fig. S5. Identification of the determinants for CRL2-mediated selenoprotein degradation. (A) The stability of GFP without or with the CTT tag was analyzed. CTT^V, CTT^K, CTT^S represent the CTT of SELVΔ, SELKΔ and SELSΔ, respectively. (B) The stability of GFP tagged with wild-type or mutant CTTs. The glycine at the -1 position of CTT^K or CTT^S was mutated to glutamic acid, while that at the -2 position of CTT^V was mutated to aspartic acid. (C) GPS analysis of UGA-terminated SELV with mutations in the leucine immediately N-terminal to Sec. (D) GPS analysis of full-length SELK or SELS with mutations C-terminal to the Sec residue. (E) GPS analysis of UGA-terminated selenoproteins fused with GFP at the C-terminus.

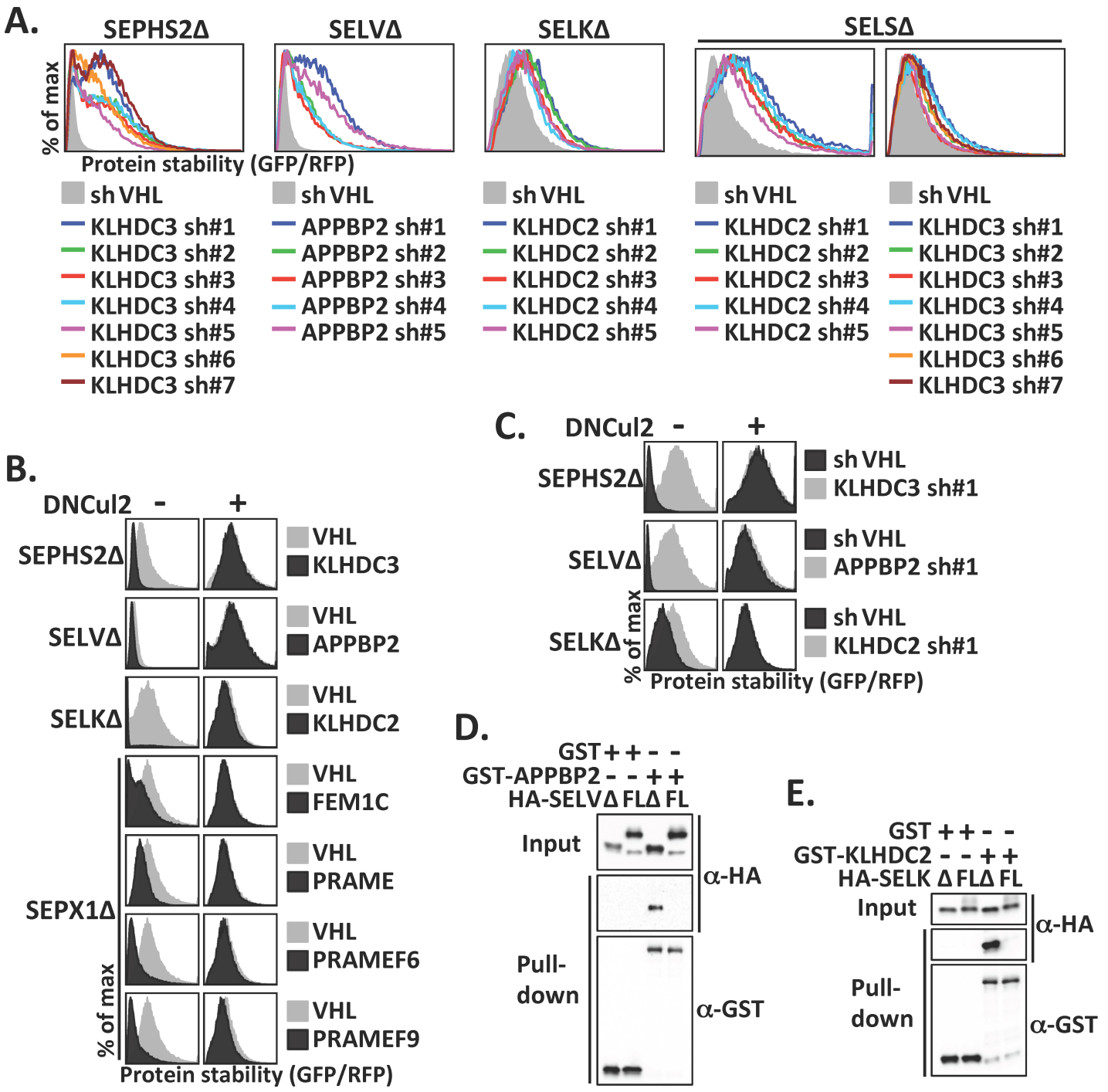

Figure S6. Identification of BC-box proteins involved in recognizing various selenoproteins. (A) GPS reporter cells expressing UGA-terminated selenoproteins were treated with shRNAs against APPBP2, KLHDC2 or KLHDC3 and then analyzed. (B) GPS assay of UGA-terminated selenoproteins in cells treated with viruses expressing DNCul2 and various BC-box proteins. (C) The stability of UGA-terminated selenoproteins in cells treated with DNCul2 and shRNAs against various BC-box proteins as indicated. (D) GST pull-down using lysates expressing HA-tagged SELV and GST or GST-tagged APPBP2. (E) GST pull-down using lysates expressing HA-tagged SELK and GST or GST-tagged KLHDC2.

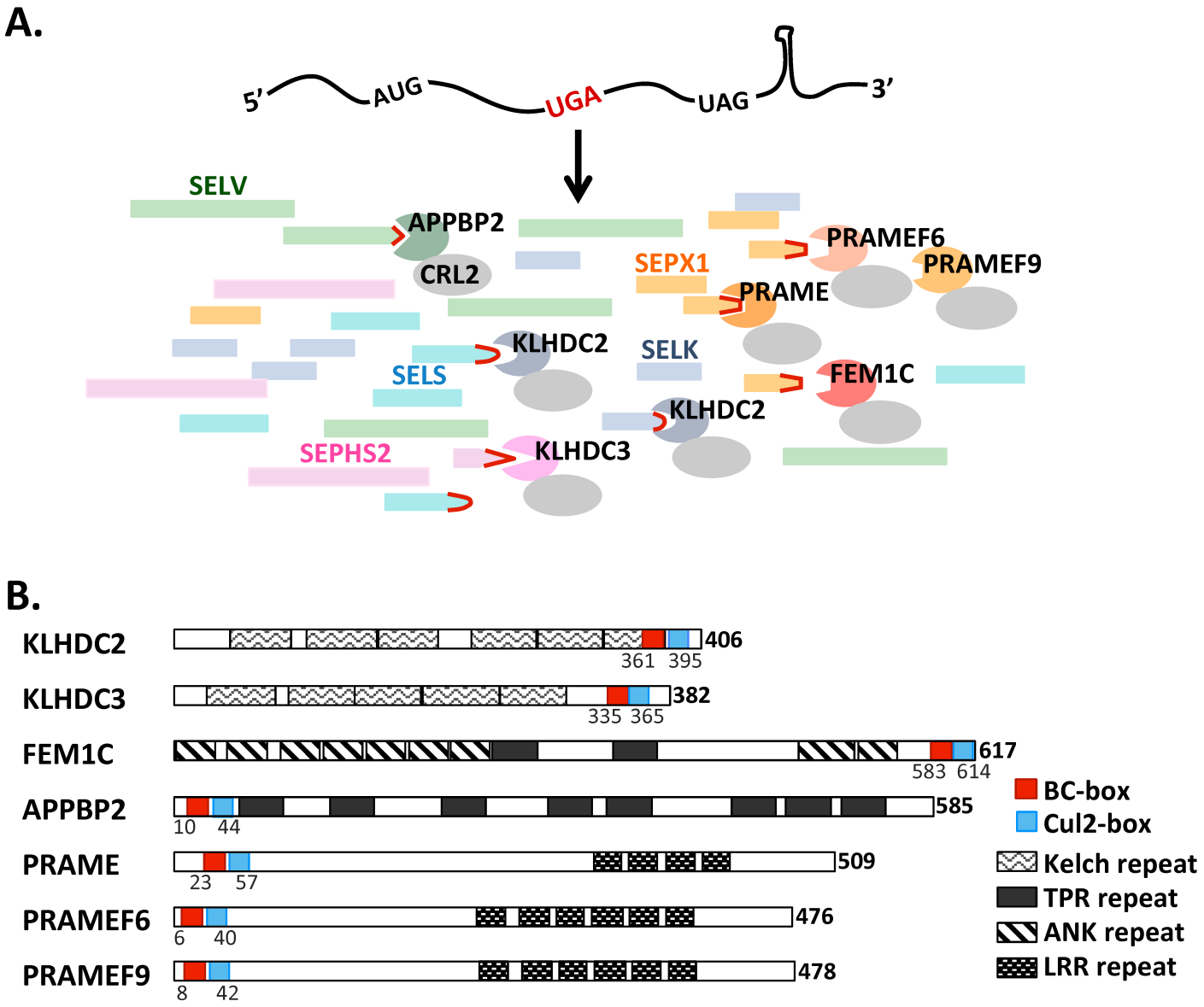

Fig. S7. (A) Model of CRL2-mediated selenoprotein quality control. (B) The domain organization of BC-box proteins.

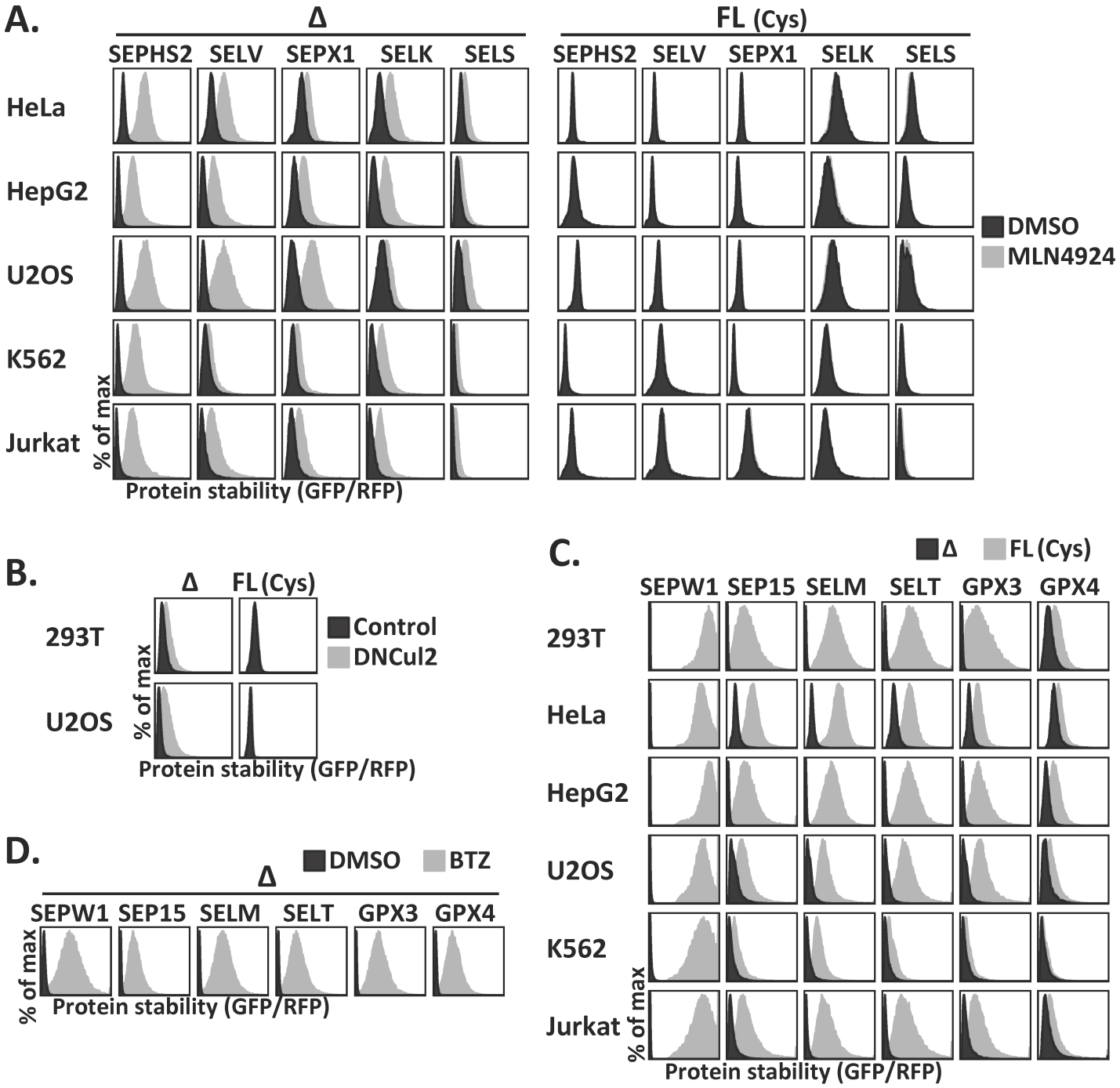

Fig. S8. Selenoprotein degradation in various cell lines. (A) Various cell lines expressing truncated or full-length versions of selenoproteins from the GPS cassette were treated with DMSO or MLN4924 and analyzed by GPS. (B) GPS assay for truncated and full-length SEPW1. Full-length and truncated SEPW1 are presented using different x-axis scales. (C) Various cell lines expressing the truncated or full-length versions of selenoproteins were analyzed by GPS. (D) GPS assay for UGA-terminated selenoproteins from cells treated with DMSO or bortezomib (BTZ).

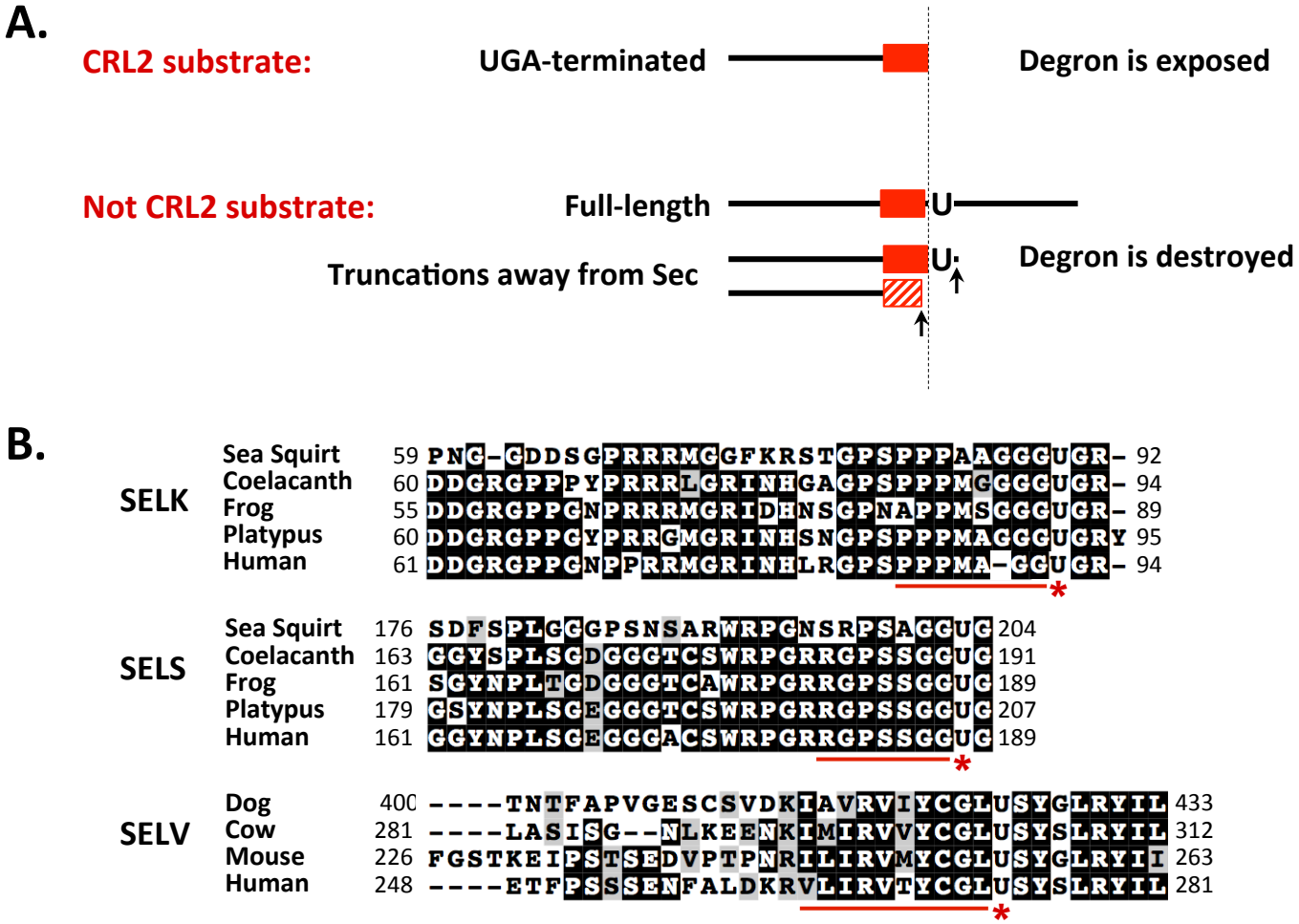

Fig. S9. (A) The "end degron" model to explain the accuracy of CRL2-mediated selenoprotein surveillance. (B) Clustal multiple sequence alignments between *Homo sapiens* (Human), *Mus musculus* (mouse), *Bos taurus* (cow), *Canis familiaris* (dog), *Ornithorhynchus anatinus* (platypus), *Xenopus tropicalis* (frog), *Latimeria chalumnae* (coelacanth) and *Ciona intestinalis* (sea squirt). SELV only exists in mammals.

AUTHOR CONTRIBUTIONS

H.C.S Yen designed and performed experiments, analyzed data, supervised the project and wrote the paper; H.C. Lin performed experiments and analyzed data; S.C. Ho performed initial BC-box screens; Y.Y. Chen and P.H. Hsu performed mass spectrometry experiments; K.H. Khoo supervised mass spectrometry experiments.