Supplementary materials

Endoplasmic reticulum-associated degradation is required for systemic water homeostasis

Guojun Shi[1,8], Diane Somlo[2,6,8], Geun Hyang Kim[1,8], Cristina Prescianotto-Baschong[3], Shengyi Sun[2,7], Nicole Beuret[3], Jonas Rutishauser[3], Qiaoming Long[4], Peter Arvan[5], Martin Spiess[3], Ling Qi[1,5]*

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Supplementary Table 1. Analysis of WT and *Sel1L*^{IKO} urine at day 21 post-tamoxifen.

Urinalysis Parameter	WT	IKO	
pH	8.75 ± 0.50	8.25 ± 0.87	(p = 0.36)
Protein (mg/dL)	75 ± 50	40 ± 42	(p = 0.33)
Glucose (mg/dL)	0 ± 0	0 ± 0	
Ketones (mg/dL)	0 ± 0	0 ± 0	
Bilirubin (mg/dL)	0.25 ± 0.50	0 ± 0	(p = 0.36)
Heme	none	none	
WBC	none	none	
RBC	none	none	
Epithelial Cells	none	none	
Fat Drops	none	none	
Casts	none	none	
Crystals	Struvite	Struvite	

WT, n = 4. IKO, n = 4. Females, 5 months. Data are mean ± SD.

Supplementary Figures and Figure Legends

Figure S1. AQP-2 translocation in response to dDAVP and induced Sel1L-deficient mice lack histologically evident kidney pathology.
(A) Higher resolution reveals intercalated cells as well as principal cells in kidney collecting duct histology. Confocal images of AQP-2 (red) in kidney from *Sel1L*$^{f/f}$ mice either injected with dDAVP or control with nuclei stained by DAPI (blue). 1 and 2 show high magnification from insets. Structures traced in inset; Whole tubules outlined in white. Yellow lines trace principal cells (AQP2-positive). Cyan lines trace intercalated cells (AQP2-negative). (B) H&E staining of kidneys of *Sel1L*$^{f/f}$ and *Sel1L*^{ERCre} mice 2 weeks after tamoxifen treatment. Regions of cortex, medulla and collecting ducts shown, with noted absence of medullary collecting duct dilation seen in mouse models of nephrogenic diabetes insipidus (1). n = 4 mice each group.

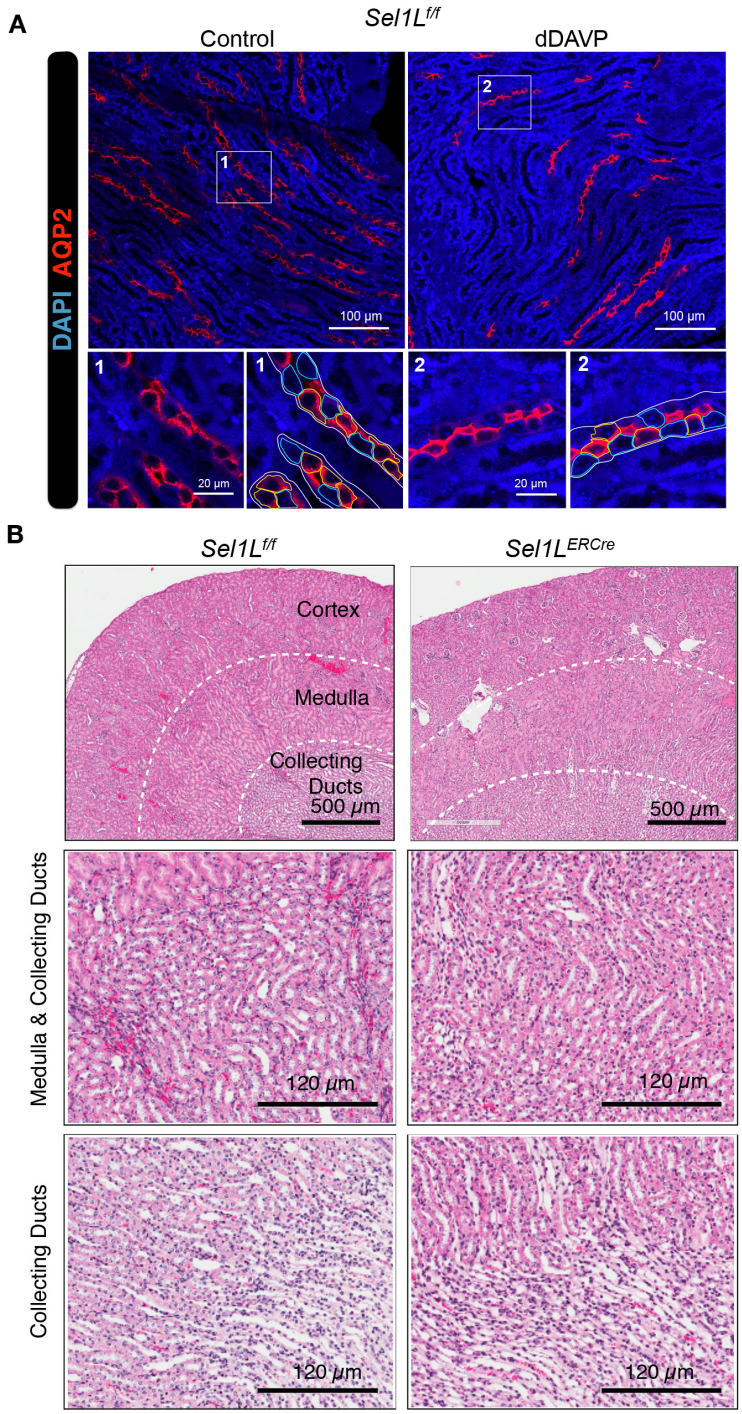


Figure S2. Expression of Sel1L and Hrd1 in vivo and in certain regions of the brain.
(A) Gene expression of *Sel1L* and *Hrd1* in various mouse tissues. Data is based on cDNA profiling data from BioGPS online database [lookup: Sel1L, Organism: *Mus musculus,* Probeset 1436774_at; Hrd1 (Syvn1), Organism: *Mus musculus,* Probeset 1443609_s_at]. Colored bars, tissues directly involved in systemic water balance. **(B)** Representative fluorescent staining of Hrd1 in the adult mouse brain (the same as the one shown in Fig. 2A) with insets highlighting cortex (areas 1,3) and hippocampus (area 2). Of note, Sel1L antibody does not work for immunostaining.

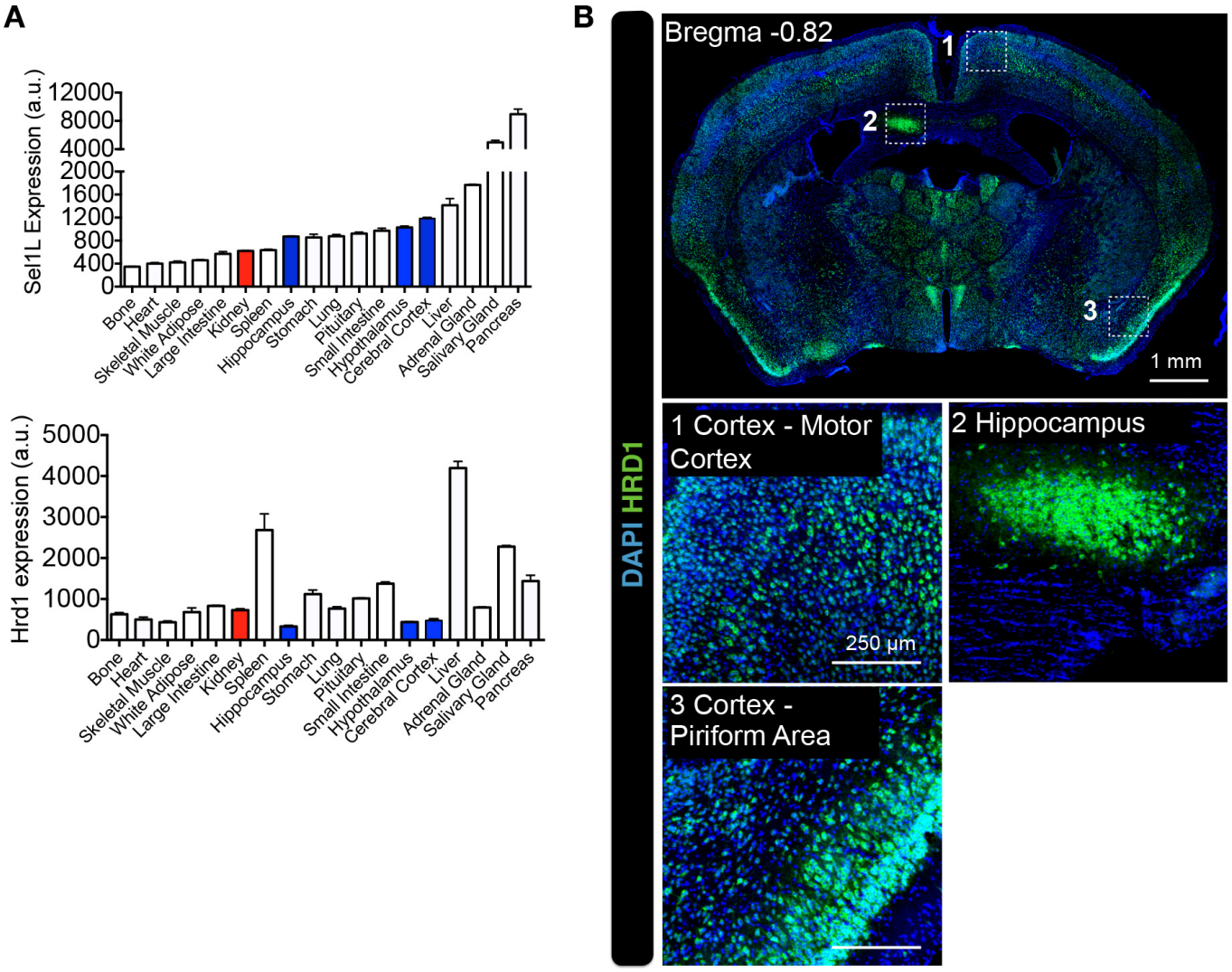


Figure S3. Sel1L deficiency in AVP neurons leads to diabetes insipidus but not neuronal cell death. **(A)** Quantitation of fluorescent staining of Hrd1 in the PVN of *Sel1L*$^{f/f}$ and *Sel1L*^{ERCre} mice 6 days after tamoxifen injections as shown in Figure 4A. (n = 3 mice each genotype). **(B-C)** In-situ hybridization detection of AVP mRNA in PVN of *Sel1L*$^{f/f}$ and *Sel1L*^{ERCre} mice 1 week after tamoxifen injection. Dashed line outlines the PVN. Quantification of AVP mRNA intensity per cell is shown in (c). **(D)** TUNEL staining of brain sections including PVN from *Sel1L*$^{f/f}$ and *Sel1L*^{AVP} mice at the age of 6 weeks (n=2 each). DNase I-treated arcuate nucleus (ARC) section was used as a positive control.

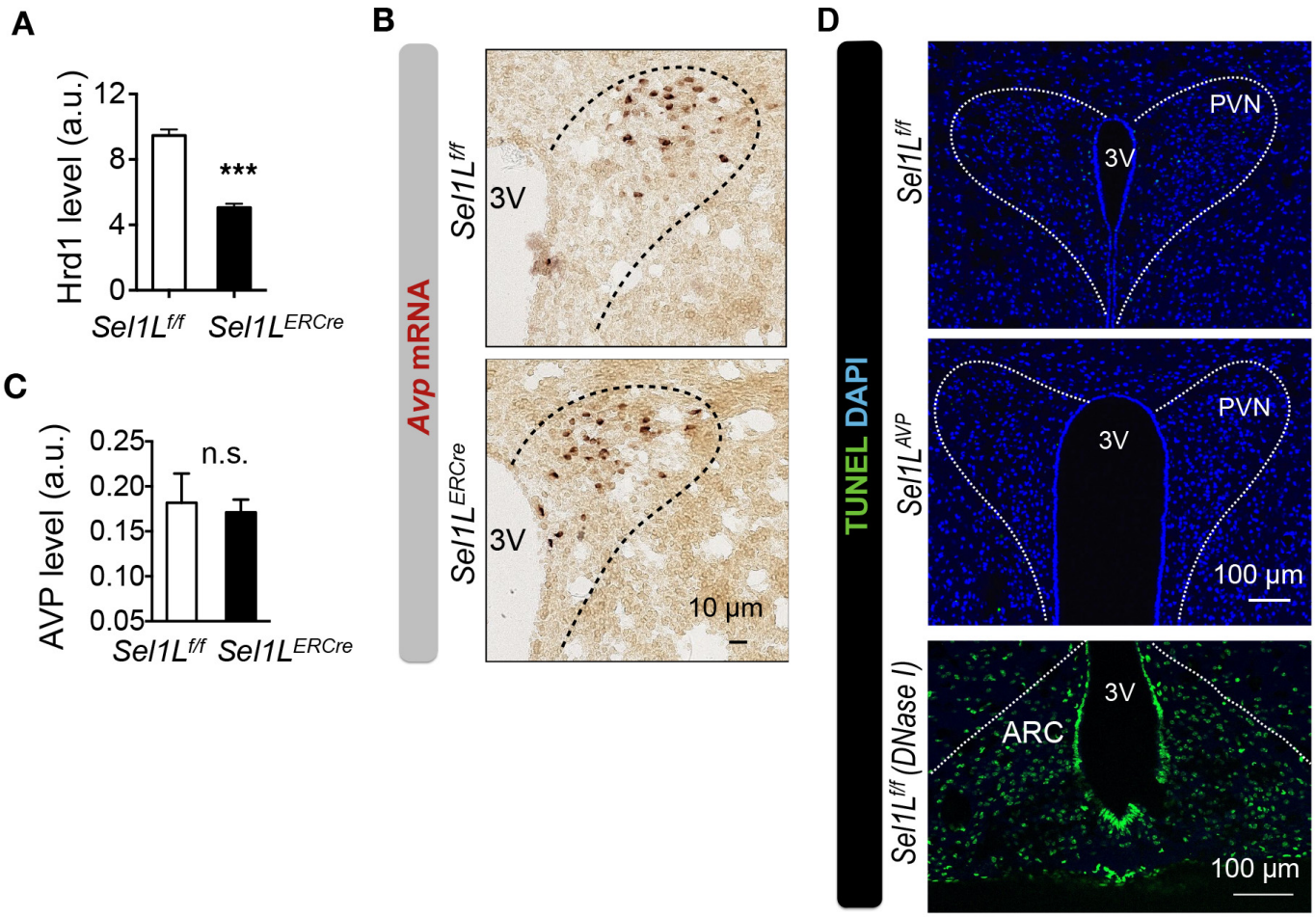


Figure S4. Sel1L deficiency blocks the exit of proAVP from the ER in AVP neurons.
Overexposure of confocal images to demonstrate the lack of AVP-containing vesicles in axons of 1-week-old female *Sel1L*^{AVP} mice compared to *Sel1L*$^{f/f}$ mice. Arrowheads of inset 1 indicate wild-type vesicles transporting AVP along axonal structure. Inset 2 shows the lack of equivalent structure in *Sel1L*^{AVP} mice. n = 3 mice under water *ad libitum* condition.

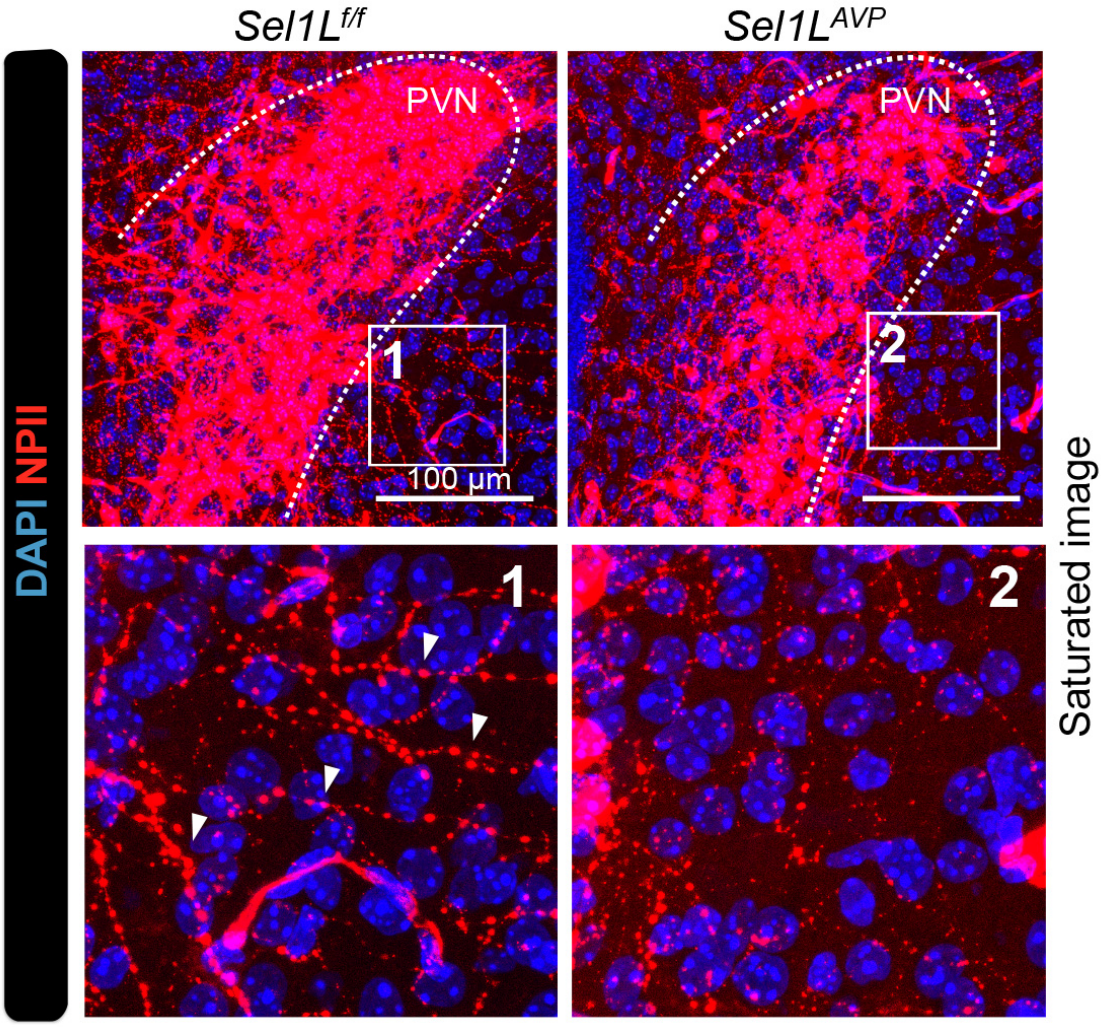


Figure S5. Sel1L deficiency retains proAVP in the ER of N2a cells.
Immunofluorescent staining of proAVP and endogenous BiP in WT **(A)** and Sel1L-deficient **(B)** N2a cells expressing WT or G57S mutant proAVP. Images represents at least 2 independent experiments.

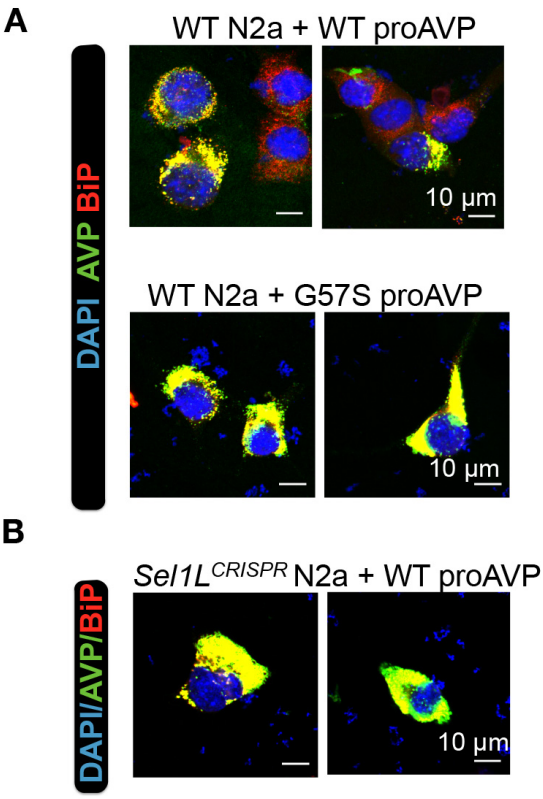
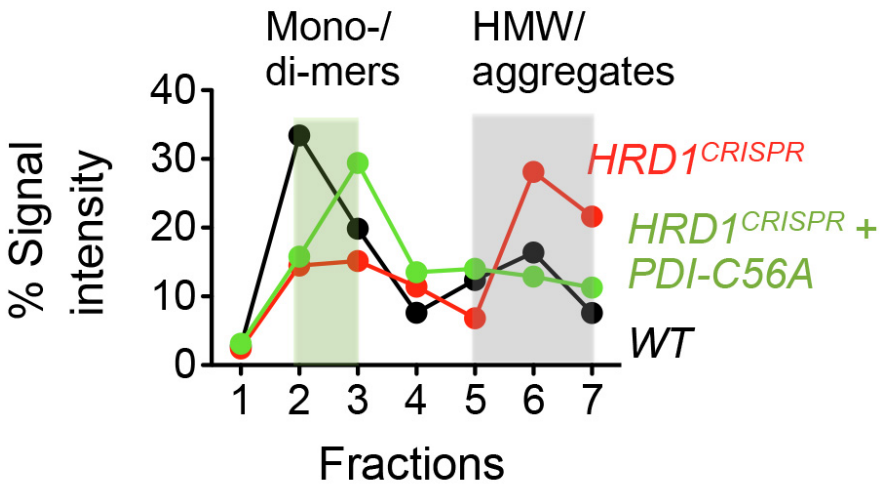


Figure S6. PDI promotes proAVP aggregation in the absence of Sel1L-Hrd1 ERAD.
Quantification of proAVP protein complex abundance (signal intensity in each lane) in HRD1-deficient HEK293T cells with or without PDI-C56A trap-mutant as shown in Fig. 9A.



SUPPLEMENTARY MATERIALS AND METHODS

Water intake and urine output analysis. Water intake analysis was performed in Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments), with 1-day acclimation followed by analysis for 3 consecutive days. For urine output analysis, mice were housed individually in metabolic cages (Techniplast USA Single Mouse Cage, 3600M021) for 24 h with ad libitum access to food and water. Each session was separated by 48 h. Food, water and mouse body weight were measured before and after each session. Urine obtained from metabolic cage collecting vessels or by bladder massage was pipetted into microcentrifuge tubes and stored on ice. Samples were centrifuged for 5 min at 5,000 rpm and either stored at -80°C or tested immediately for osmolality using a freezing point micro-osmometer (Fiske Model 210, Single Sample Micro Osmometer). Urine samples were sent to the Cornell Veterinary Animal Health and Diagnostic Clinic for routine urinalysis, including urine chemistry profile and urine specific gravity measurement.

Water deprivation and salt loading. For water deprivation experiments, mice were deprived of water with ad libitum food access, and sacrificed after 72 h of deprivation with daily monitoring. For salt loading, mice were fed with 2% wt/vol NaCl water with food ad libitum for 7 consecutive days with daily monitoring and then sacrificed immediately. Mice were weighed and examined throughout each experiment for signs of dehydration, which would necessitate immediate end to the experiment.

dDAVP response test. Female, age-matched *Sel1L*$^{f/f}$ and *Sel1L*^{ERcre} mice were injected i.p. with tamoxifen (5 µL/g BM) once a day for three days. Mice at either day 8 or 14 post tamoxifen injection were i.p. injected with 1-deamino-8-D-arginine vasopressin (desmopressin, dDAVP) at 0.4 µg/kg body weight. Mice were injected twice with dDAVP, once at 0 min and again at 30 min respectively. Urine was collected 30 min after the second injection. Urine samples before and post-injection were collected via bladder massage and immediately stored on ice. Samples were aliquoted and stored at -80°C. Samples were thawed on ice and urine osmolality was measured using a freezing point micro-osmometer.

Serum AVP measurement. Collected blood samples were kept at room temp for 10-15 min and then put on ice and were later spun down (3,000 g for 15 min at 4°C) to separate serum. Clear serum supernatant was stored at -80°C. Serum AVP was quantitated using the Arg8-Vasopressin ELISA kit (Enzo Life Sciences, ADI-900-017) per manufacturer protocol. Serum samples were diluted 10-fold prior to the analysis.

Preparation of brain sections. For H&E staining, dissected tissues were directly placed in 4% formaldehyde, stored at 4°C, and later processed by the Cornell or Michigan Histology Core Facility for paraffin embedding, sectioning, and H&E staining on a fee-for-service basis. For immunofluorescence or in-situ hybridization, mice were anesthetized by avertin tribromoethanol (Sigma-Aldrich, T4 840-2) or isoflurane and then euthanized via transcardial fix-perfusion with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, 19210). Brain was then post-fixed in 4% PFA for 2 h at 4°C, dehydrated in 15% sucrose overnight at 4°C, and sectioned on a cryostat (Thermo Fisher Scientific Microm HM 550). Brain sections (30 µm) were stored at DEPC-containing anti-freezing media (50% 0.05M Sodium Phosphate pH7.3, 30% Ethylene Glycol, 20% Glycerol) at −20°C.

In-situ hybridization. The experiment was performed as previously described (2). Complementary RNA (cRNA) probes were synthesized from total RNA isolated from mouse PVN region of brain by PCR using primers designed from AVP codon region (5'- ATG CTC GCC AGG ATG CTC AAC-3', 5'- CTT GGC AGA ATC CAC GGA CTC-3').

Immunoreactivity signal quantitation and colocalization analysis. Fluorescent samples were imaged with a Zeiss LSM 710 confocal microscope, and imaging parameters were identical within each set of samples. Kidney H&E and brain in-situ hybridization slides were scanned using an Aperio Scanscope (Leica Biosystems). To quantify immunnostained or hybridized probe signal using ImageJ, individual cells were outlined, and fluorophore intensity was measured as average gray value (intensity/area). Colocalization of

fluorophores was quantitatively assessed by drawing regions around individual cells and computing the Pearson's correlation coefficient by using the ImageJ plugin, Coloc 2 (https://imagej.net/Coloc_2).

TUNEL assay. Brain sections were mounted on gelatin-coated slides, dried at 55°C for 5 min and then permeabilized in 0.5% Triton X-100 at 85°C for 20 min. TUNEL assay was performed using In-Situ Cell Death detection kit (Roche, 11684795910) per manufacture's protocol. Brain section treated with DNase I (ThermoFisher Scientific, EN 0523) for 30 min at 37°C were used as positive controls. Images were obtained by Nikon A1 Confocal Microscope at the Imaging Core of the University of Michigan Medical School.

Cell lines and transfection. HEK293T cells and N2a cells (both from ATCC) were cultured in DMEM (Corning, NY) with 1% Pen/Strip and non-treated or heat inactivated 10% FBS (GIBCO), respectively. Cells were transfected within 24hr after plating with PEI (HEK293T cells) or Lipofectamine 2000 (N2a cells).

Plasmids. Human WT, ΔE47, G57S, cysteine-less (also known as abcd) and abcd-KDEL proAVP mutants were described previously (3). HA and Flag sequences were inserted into the C-terminus (HA, TACCCATACGATGTTCCAGATTACGCT; FLAG, GACTACAAAGACGATGACGACAAG). ER retention KDEL sequence (AAAGATGAACTA) was inserted right before the stop codon. All of these experiments were conducted using quick change mutagenesis with Pfu DNA polymerase (Agilent, CA). Human Hrd1 construct was kindly provided by Dr. Yihong Ye (NIDDK); pcDNA3-HA-Ub was kindly provided Dr. Hideki Nishitoh (University of Miyazaki, Japan). Human WT/C56A mutant PDI-Flag constructs were kindly provided by Dr. Billy Tsai (University of Michigan Medical School, Ann Arbor, MI).

Drug treatment and NP40-based fractionation. Preparation of cell and tissue lysates and Western blot were performed as previously described (4). For cycloheximide (CHX) chase experiment, cells were pretreated with brefeldin A (1 μg/ml) for 1 hour prior to CHX treatment (10 μg/ml) for the indicated times. For denaturing SDS-PAGE, whole-cell lysates were harvested in lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 50 mM Tris HCl [pH 7.5]) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 1 mM DTT, and incubated on ice for 25 min, and further denatured by adding 5X denaturing sample buffer (250 mM Tris HCl pH 6.8, 1% SDS, 50% glycerol, 1.44 M β-mercaptoethanol and 0.05% bromophenyl blue) and boiled for 5 min prior to be separated on a SDS-PAGE gel. For NP-40 soluble and insoluble fraction analysis, cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 0.5% NP40, 150 mM NaCl, 5 mM $MgCl_2$) supplied with protease inhibitor (Sigma) and 10 mM N-ethylmaleimide on ice for 20 min. After centrifugation, the supernatant (NP-40S) was mixed with reducing or non-reducing sample buffer. The pellet (NP-40P) was washed with lysis buffer, and then resuspended in 1X denaturing sample buffer, sonicated for 10 seconds, and heated at 95ºC for 30 min. NP-40S and NP-40P fractions were then loaded onto SDS-PAGE for Western blot analysis. For reducing SDS-PAGE analysis, NP-40S was mixed with 5X non-reducing sample buffer (250 mM Tris HCl pH 6.8, 1% SDS, 50% glycerol, 1.44 M β-mercaptoethanol and 0.05% bromophenyl blue). For non-reducing SDS-PAGE, lysates were prepared in 5X non-denaturing sample buffer (250 mM Tris HCl pH 6.8, 1% SDS, 50% glycerol and 0.05% bromophenyl blue) and incubate at 55ºC for 20 min prior to be separated on a SDS-PAGE gel.

Immunoprecipitation. Cells were lysed in IP lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.5, protease inhibitor and protein phosphatase inhibitor cocktails, and 10 mM N-ethymaleimide) supplemented with 1% NP-40. A total of 2 mg protein lysates was incubated with antibody-conjugated agarose beads overnight at 4°C with gentle rocking (anti-HA agarose beads, Sigma A2095; anti-FLAG agarose beads, Sigma A2220). Immunocomplexes were washed (137 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 7.5, 10% glycerol) and eluted under denaturing or non-denaturing sample buffer as described below.

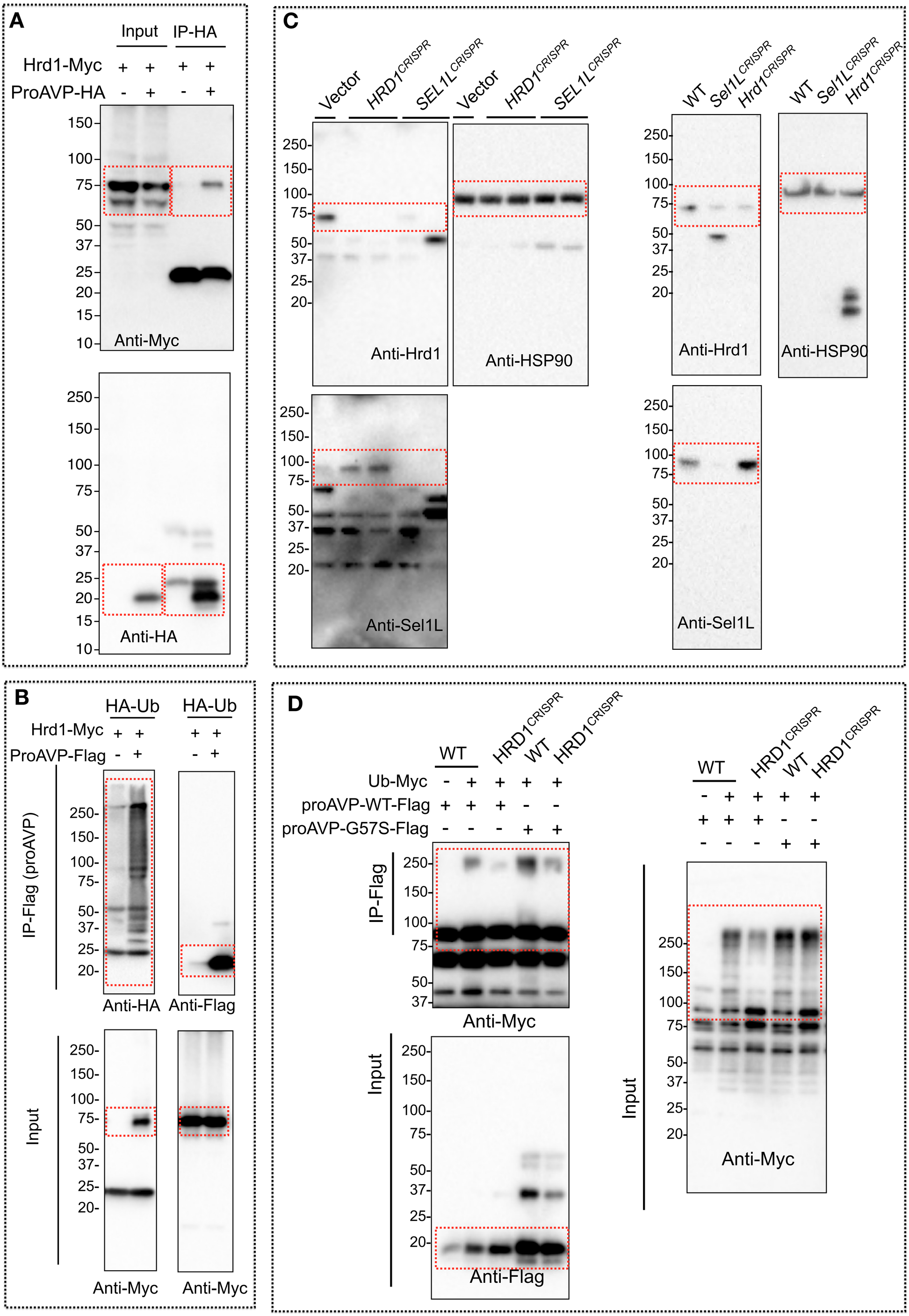
Pulse labeling and pulse-chase analysis. HEK293T cells were transfected with different constructs were cultured with cysteine and methionine-free medium (Invitrogen 21013024) containing 10 mM HEPES, pH 7.4 at 37℃ for 30 min and pulse labeled with 100 μCi/ml [^{35}S]-cysteine and methionine (EasyTag, PerkinElmer) at 37℃ for 30 min. In the pulse-chase assay, following 30 min pulse, cells were cultured in chase medium (regular media supplemented with 10 mM HEPES, 5 mM cysteine, 5 mM methionine) for the

indicated times. Cells were then washed with ice-cold HBSS buffer (Gibco, Life Technologies) and snap-frozen in liquid nitrogen. Cells were lysed by NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0) supplemented with 100X proteinase inhibitor cocktail (Sigma), 1 mM PMSF (Sigma), and 10 mM N-ethylmaleimide, followed by immunoprecipitation as described above. Denaturing samples were prepared by adding 2X denaturing sampling buffer into the immunoprecipitated beads followed by 10 min boiling at 95℃, while non-denaturing samples were prepared by adding 2X non-denaturing sampling buffer into the immunoprecipitated beads followed by incubation for 10 min at room temperature. Insoluble fractions of cell lysates were washed with lysis buffer, resuspended in 1/5 original volume of lysis buffer with 5 mM DTT and boil for 30min, followed by adding 4/5 volume of original lysis buffer and subjected to IP as described above. Immunoprecipitates were separated on a 4-15% gradient SDS-PAGE gel (BioRad), then incubated with the enhancer buffer (1.5 M sodium salicylate (Sigma) in 30% (v/v) methanol in H_2O) for 10 min. The gel was then dried at 80℃ for 2 h using a gel drier (Model 583, Bio-Rad) and subjected to autoradiography with X-film (Laboratory Product Sales).

Sucrose gradient sedimentation analysis Transfected HEK293T cells were harvested and lysed in 1% NP40 lysis buffer as described in immunoprecipitation without any reducing reagents. Extracts were centrifuged through 3.7 ml of 5%– 25% sucrose gradients (prepared in 150 mM NaCl, 1 mM EDTA, 50 mM Tris HCl pH 7.5 and protease inhibitors) prepared freshly by progressively layering high- to low-density sucrose fractions in 5% increments in polyallomer tubes of 11×3×60 mm (Beckman Coulter, Brea, CA, USA). Extracts were centrifuged at 58,000 rpm for 14.5 hr at 4°C using an SW 60 Ti rotor (Beckman Coulter). Each 4.2 ml gradient was divided evenly into 6 fractions (700 µl each). The fraction 7 was the pellet resuspended with 700 µl lysis buffer by pipetting and votexing. Fractions were subsequently subjected to Western blot analyses under denaturing or non-denaturing conditions.

References:

1. Yang, B., Zhao, D., Qian, L., and Verkman, A.S. 2006. Mouse model of inducible nephrogenic diabetes insipidus produced by floxed aquaporin-2 gene deletion. Am J Physiol Renal Physiol 291:F465-472.
2. Montag-Sallaz, M., and Montag, D. 2003. Learning-induced arg 3.1/arc mRNA expression in the mouse brain. Learn Mem 10:99-107.
3. Birk, J., Friberg, M.A., Prescianotto-Baschong, C., Spiess, M., and Rutishauser, J. 2009. Dominant pro-vasopressin mutants that cause diabetes insipidus form disulfide-linked fibrillar aggregates in the endoplasmic reticulum. J Cell Sci 122:3994-4002.
4. Sha, H., He, Y., Chen, H., Wang, C., Zenno, A., Shi, H., Yang, X., Zhang, X., and Qi, L. 2009. The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis. Cell Metab. 9:556-564.



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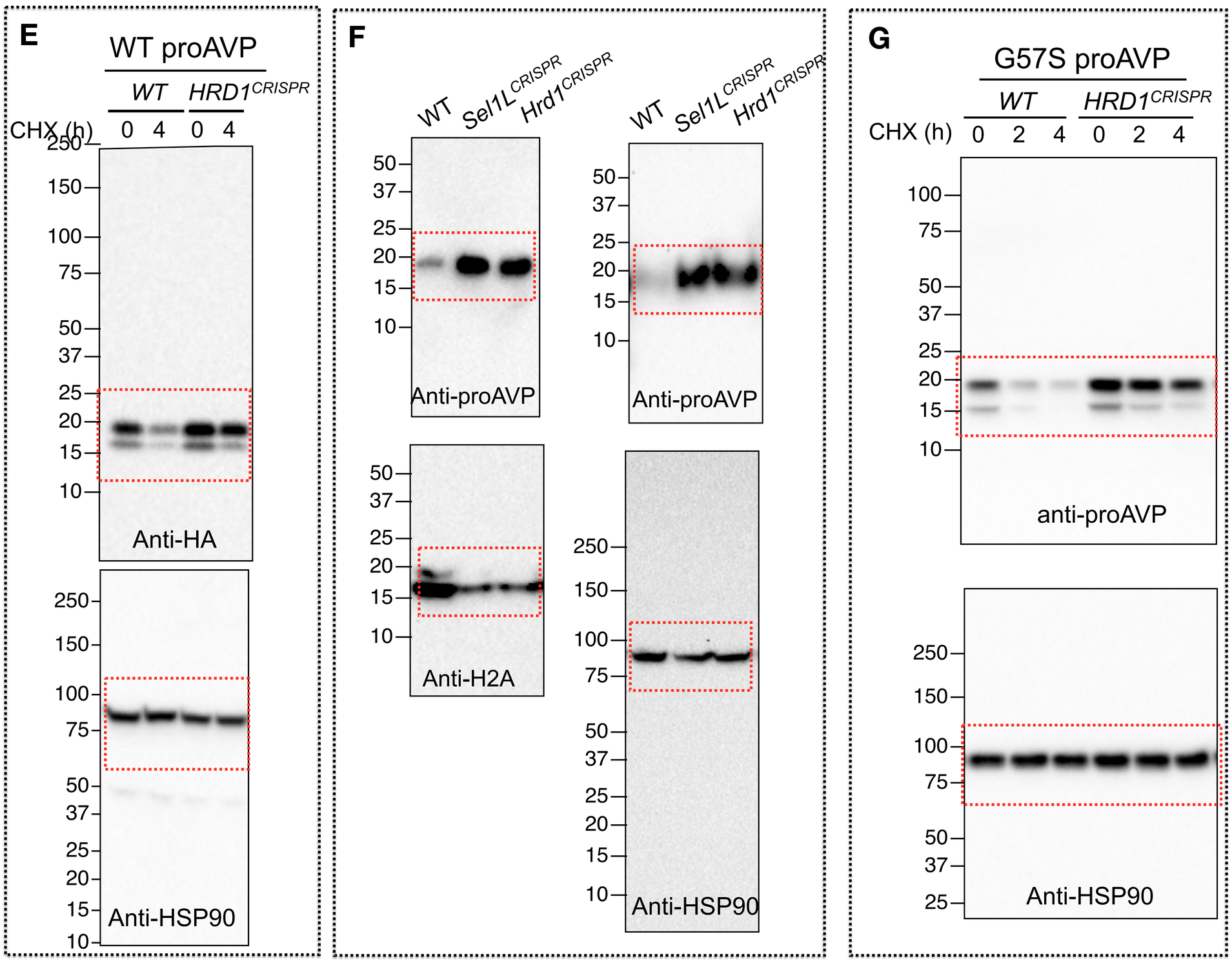

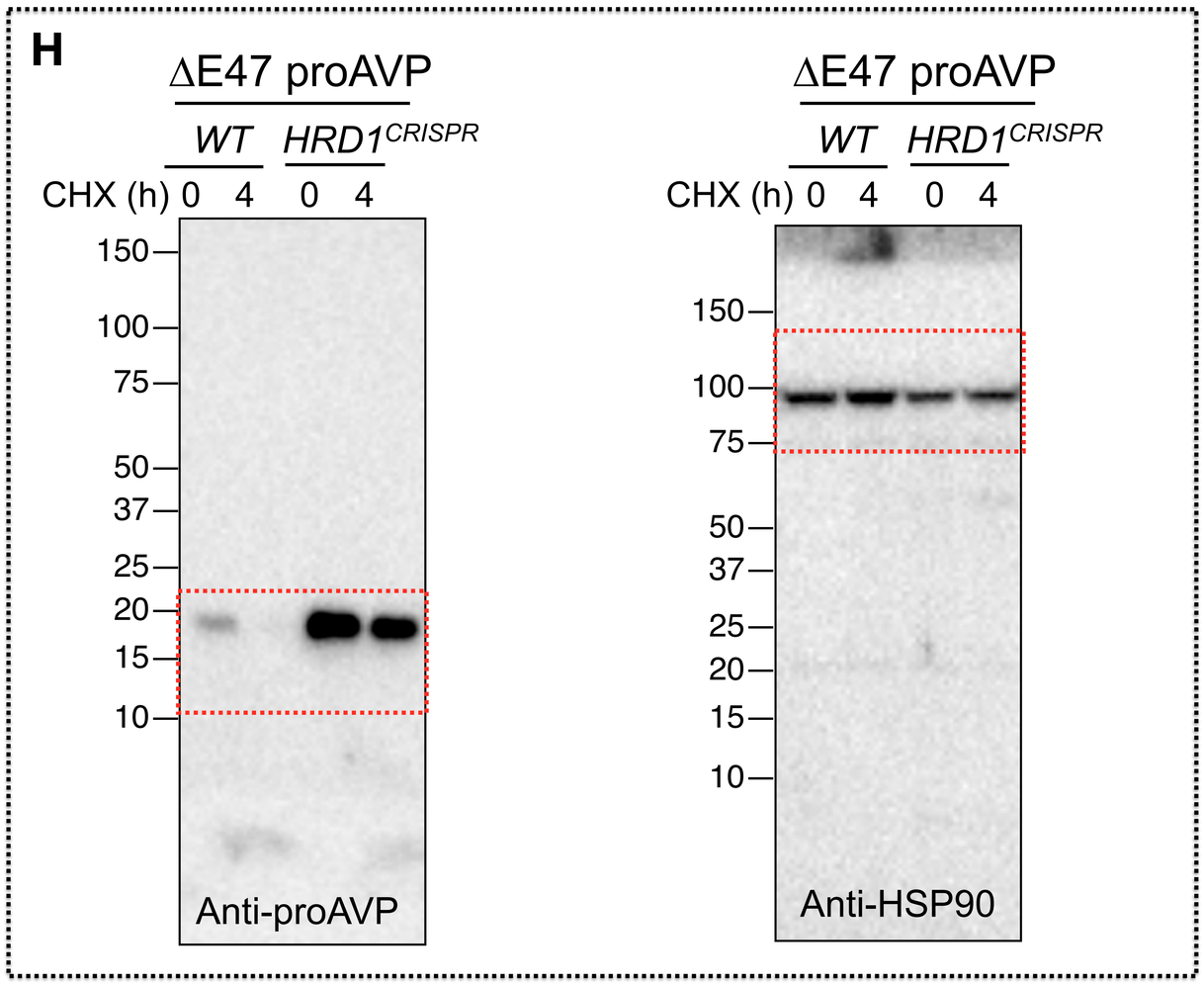

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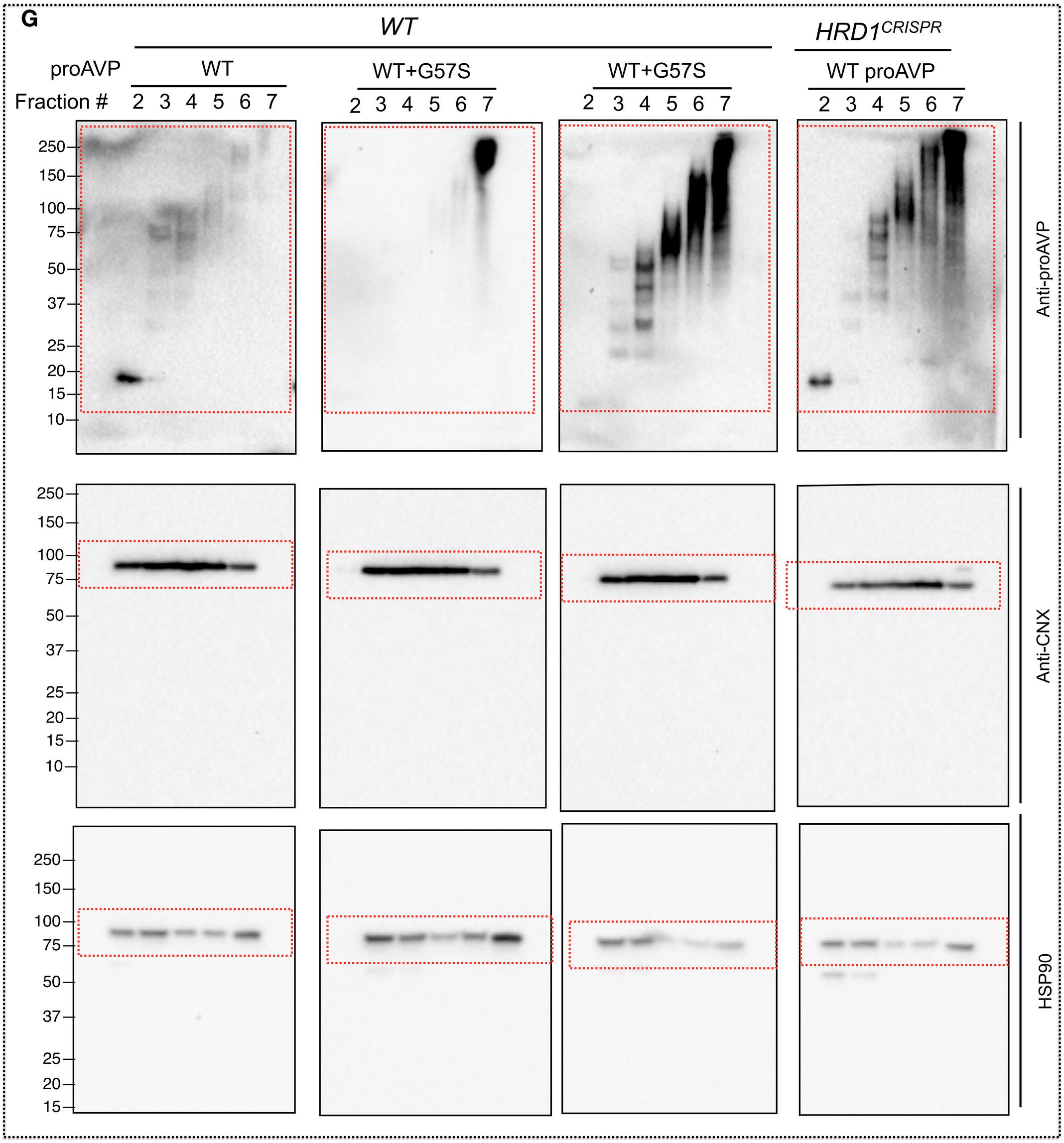

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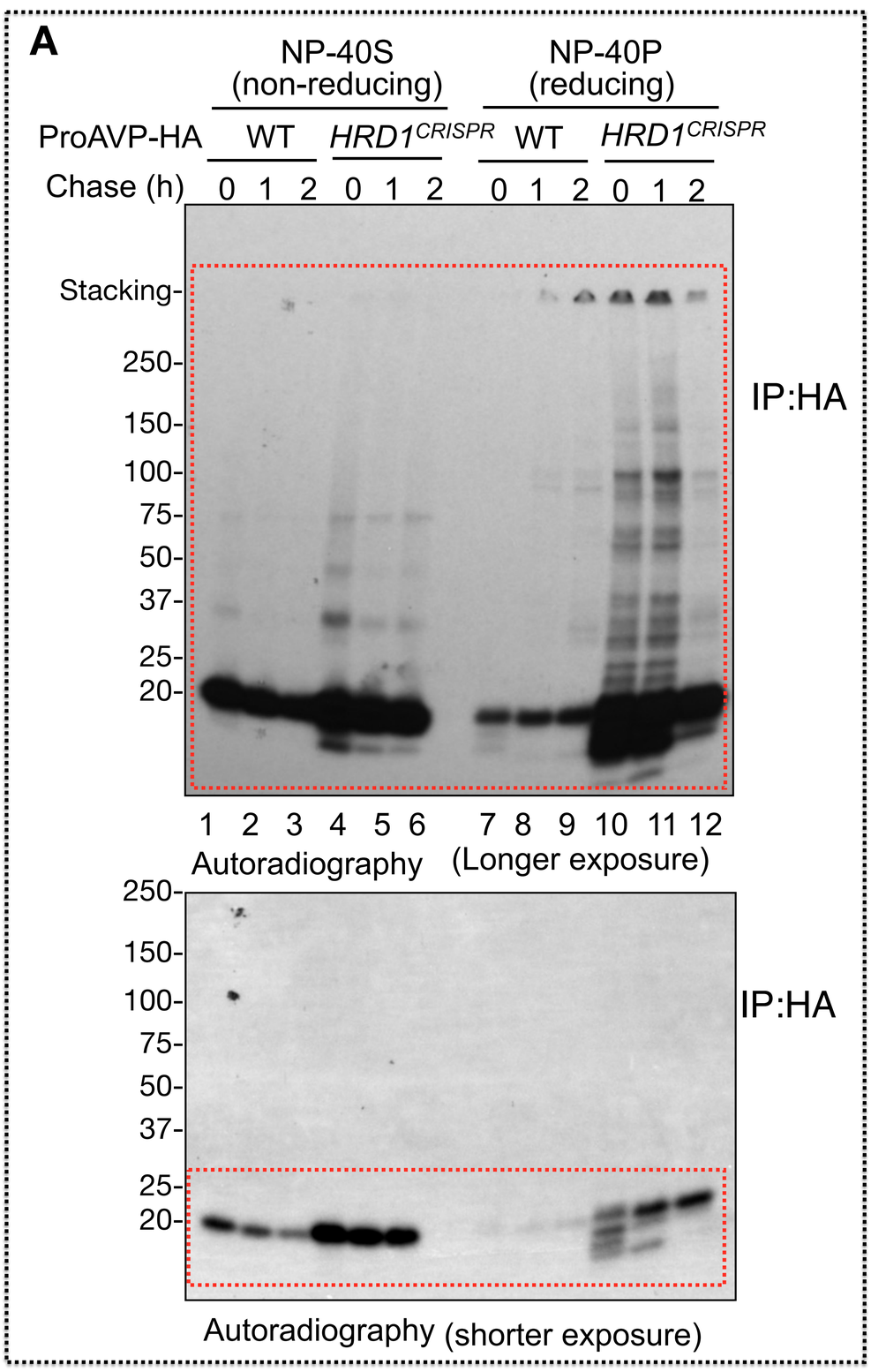

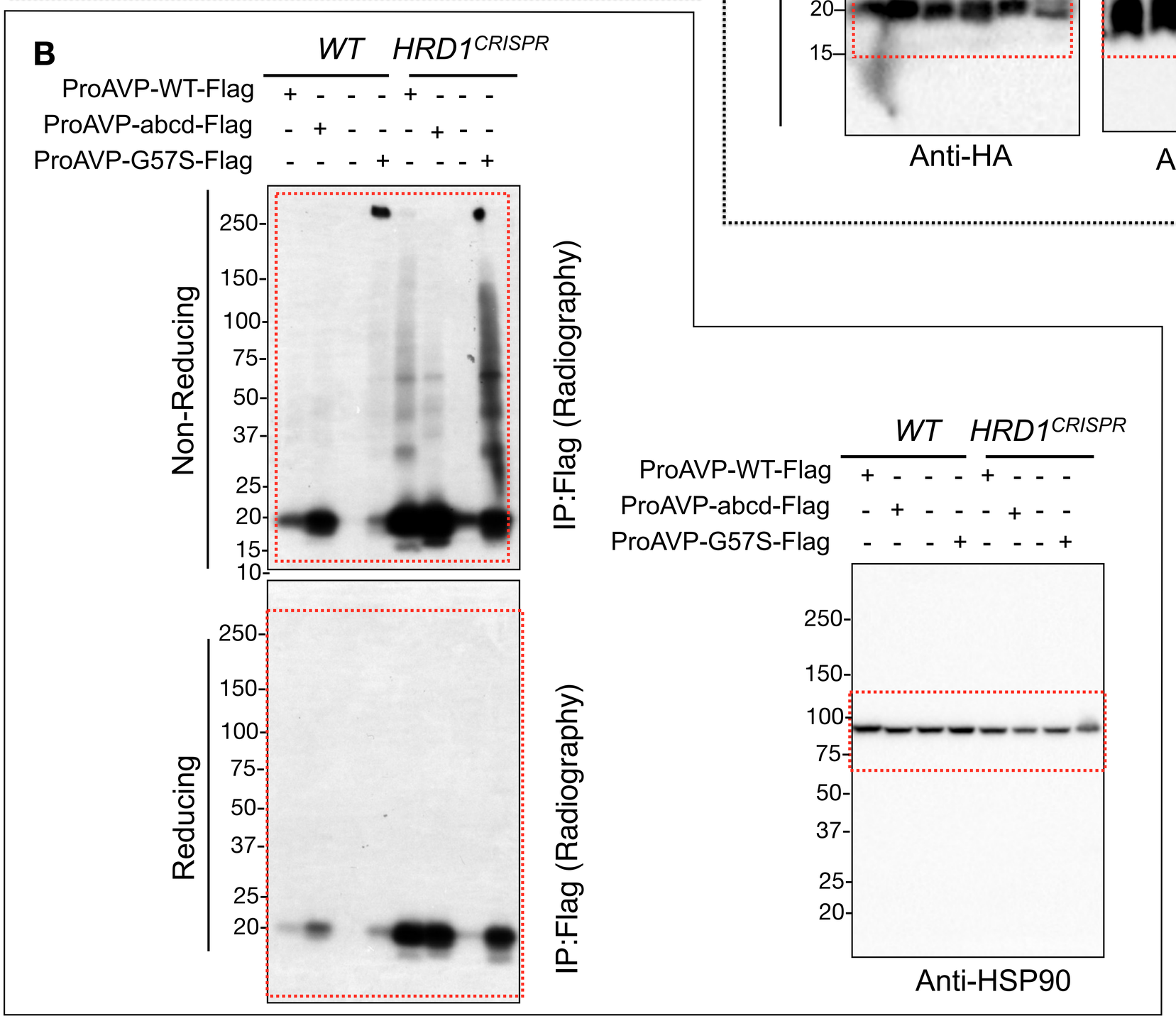

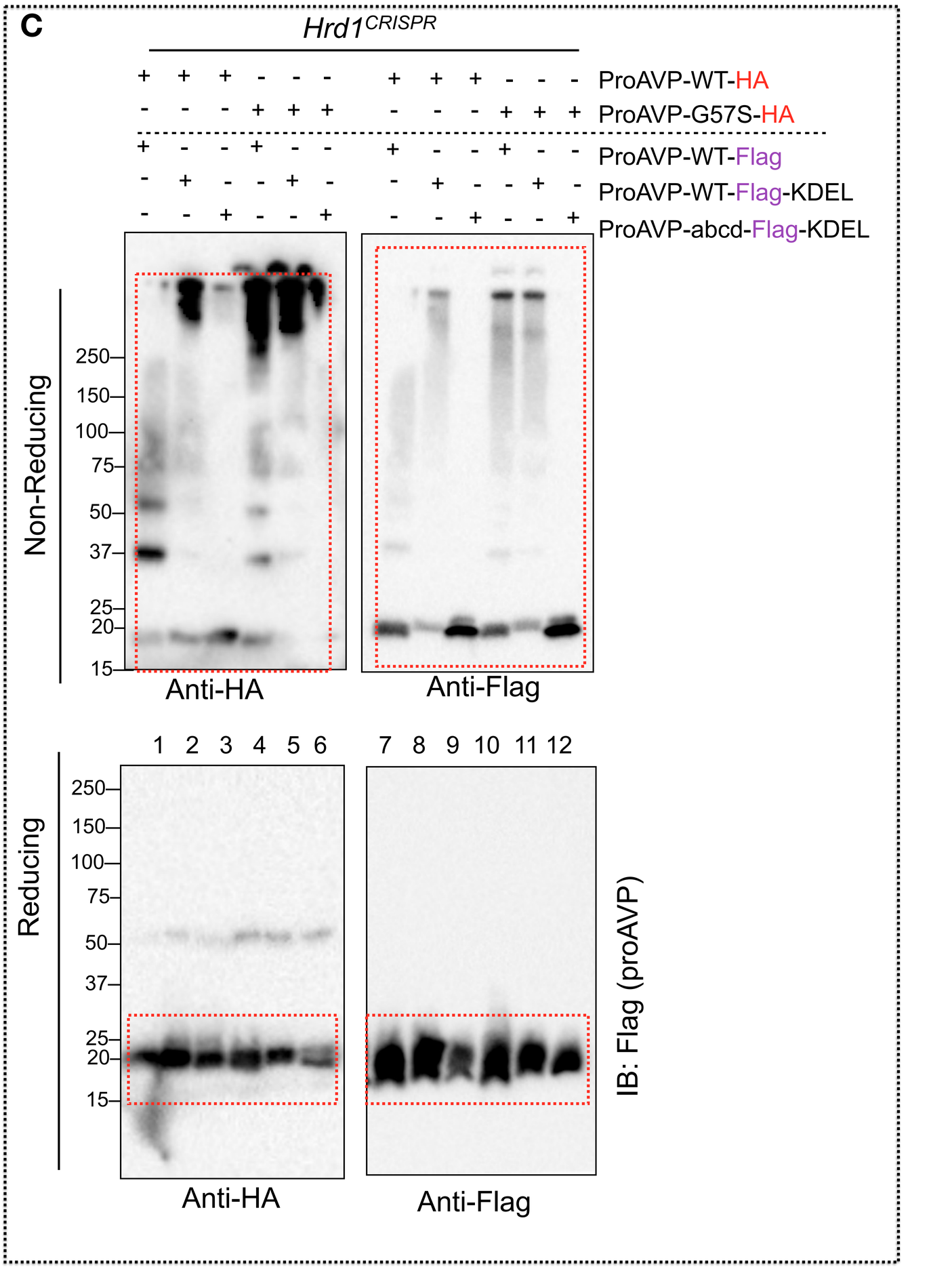

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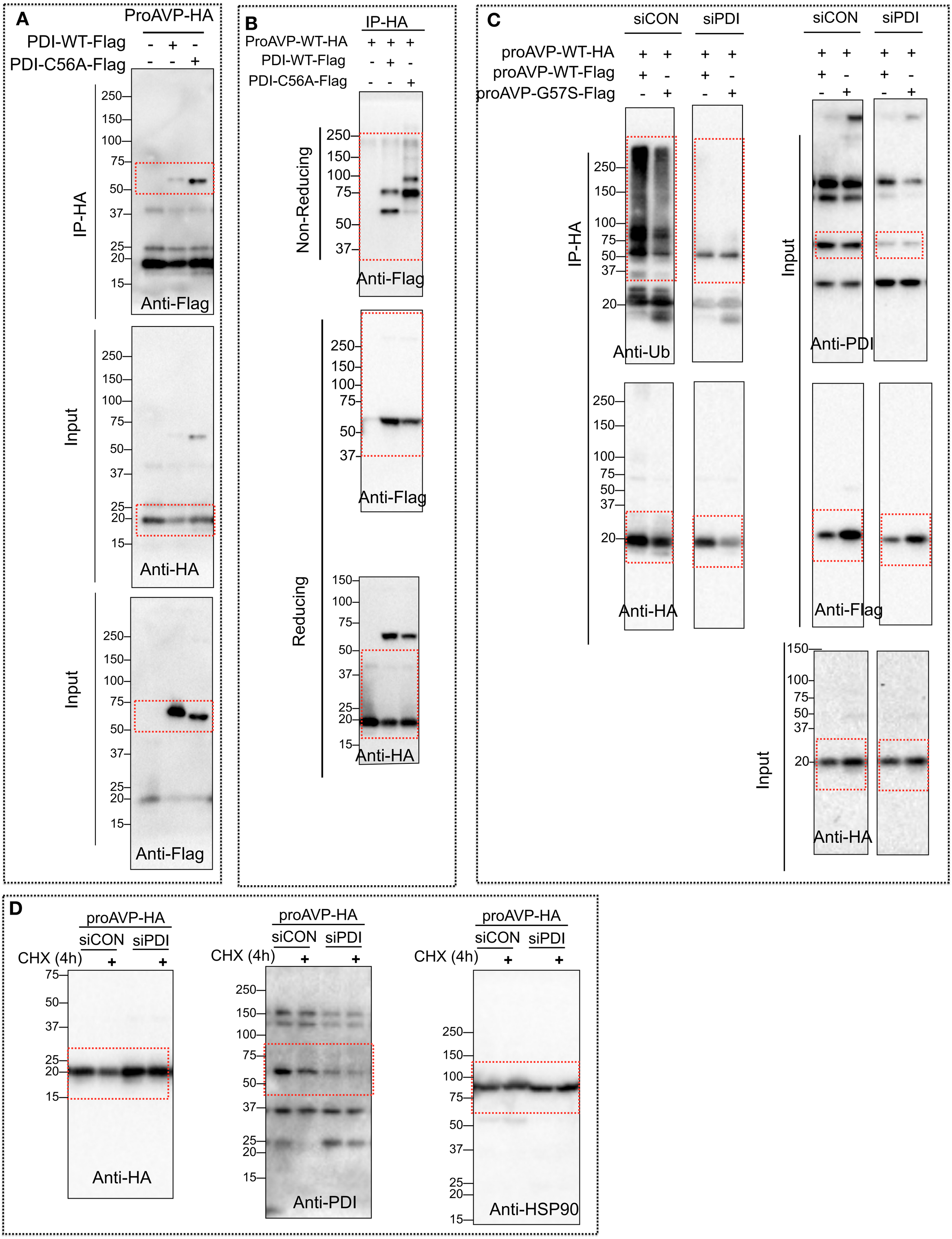

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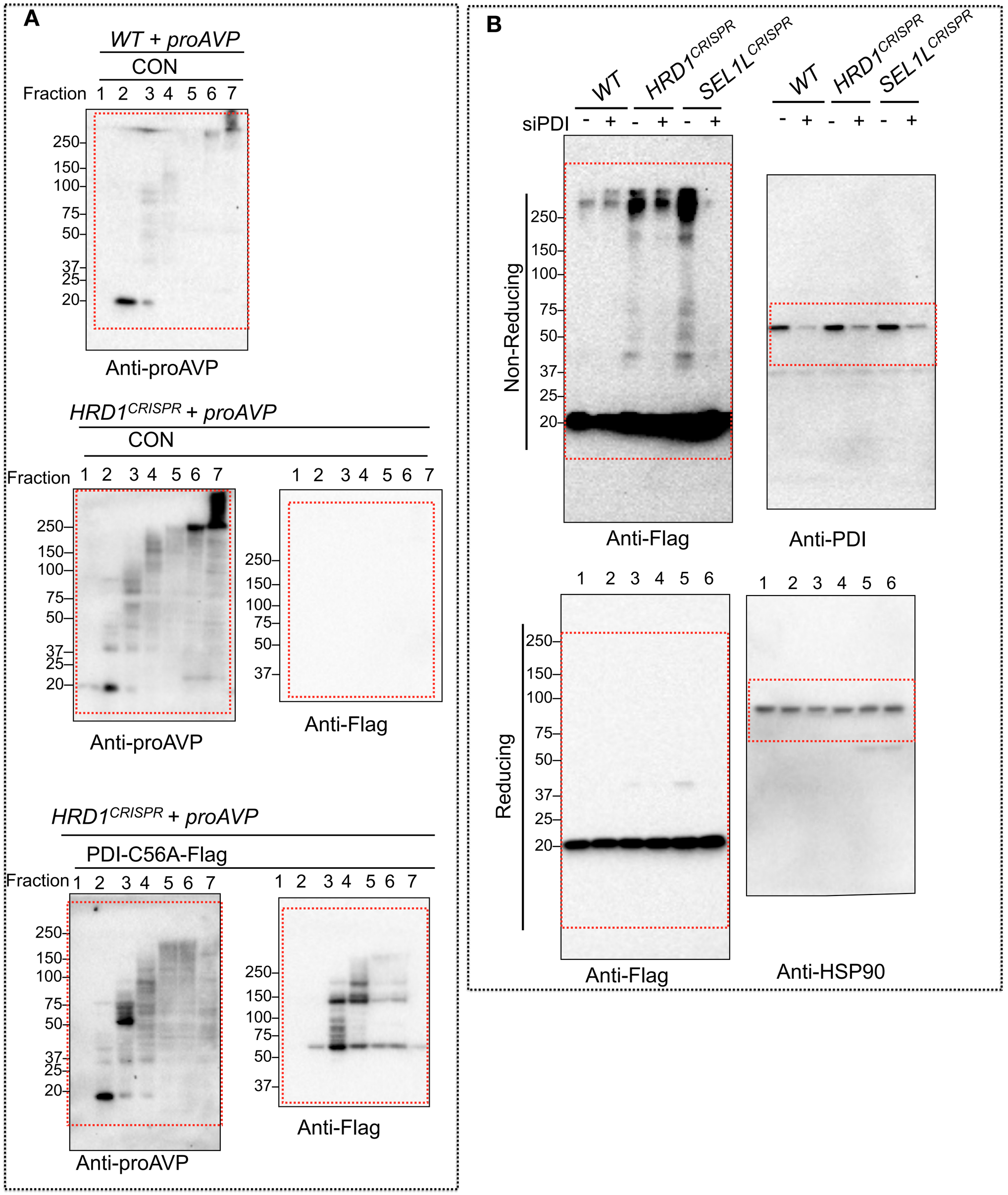

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