SEL1L critically determines the stability of the HRD1-SEL1L ERAD complex to optimize the degradation kinetics of ERAD substrates

Supplemental Data

Supplemental experimental procedures

Northern blotting--Ten μg of total cellular RNA were electrophoresed on a formaldehyde-denaturing gel and stained briefly with ethidium bromide. After extensive washing, RNA was transferred onto a nylon membrane. DNA fragments specific for SEL1L or HRD1 were labeled with a-^{32}P dCTP (PerkinElmer) by unidirectional PCR. The labeled fragments were hybridized in PerfectHyb hybridization buffer (TOYOBO, Japan) for 16 h as described previously (1), and the membrane was exposed to a phosphorimaging plate.

Mass spectrometric analysis--Cells were extracted in a buffer containing 3% digitonin, and immunoprecipitated with affinity-purified anti-SEL1L antibody. Proteins separated by SDS-PAGE were silver-stained and excised. Following trypsin digestion, peptide fragments were analyzed by MALDI-TOF mass spectrometry as described previously (2,3).

Supplemental References

1. Hirao, K., Natsuka, Y., Tamura, T., Wada, I., Morito, D., Natsuka, S., Romero, P., Sleno, B., Tremblay, L. O., Herscovics, A., Nagata, K., and Hosokawa, N. (2006) *J Biol Chem* **281**(14), 9650-9658
2. Jensen, O. N., Podtelejnikov, A., and Mann, M. (1996) *Rapid Commun Mass Spectrom* **10**(11), 1371-1378
3. Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., and Nagata, K. (2008) *J Biol Chem* **283**(30), 20914-20924

Supplemental Figure Legends

Figure S1. Western blotting of HeLa cells treated with siRNA targeted for SEL1L and HRD1.

siRNA treatment and Western blotting were performed as in Figure 1A, except that HeLa

cells were used instead of HEK293 cells. The SEL1L protein was undetectable by Western blotting when HRD1 was silenced. Asterisk indicates the non-specific band detected by the anti-HRD1/synoviolin antibody.

Figure S2. Northern blotting of total cellular RNA from HEK293 cells treated with negative control siRNA (L and M) or siRNA targeted for HRD1 (1 and 3).

Membranes were hybridized with probes specific for HRD1 or SEL1L. Ribosomal RNA (28S and 18S) was stained for loading control. Treatment of cells with siRNA targeted for HRD1 effectively knocked down HRD1 mRNA, but the SEL1L transcript was almost unaffected.

Figure S3. Characterization of proteins associated with the large membrane complex containing HRD1-SEL1L by sucrose density gradient centrifugation.

Fractions of cell extracts treated with negative control or siRNA specific for SEL1L or HRD1 were blotted with antibodies against Herp, Derlin-1, Derlin-2, VIMP, or p97/VCP. Solid bars indicate the fractions that cosedimented with the large complex containing HRD1-SEL1L (Complex I). Dashed bars indicate the second fractions with lower sedimentation rates. Gray bar indicates the broad fractionation of p97. Each protein was consistently distributed in the same fractions, regardless of whether SEL1L or HRD1 was silenced or not.

Figure S4. OS-9 associates with HRD1-SEL1L.

(A) Immunoprecipitates of cells extracted with 3% digitonin using affinity-purified anti-SEL1L antibody were silver-stained (lane 2). By comparing with immunoprecipitates from Protein A-Sepharose beads without antibodies (lane 1), specific bands were excised from the gel for mass spectrometric analysis. Three adjacent bands detected around 100 kDa (bands 1-3) were identified as SEL1L generated by the addition of different numbers of N-glycans. This finding was confirmed by N-glycanase digestion (data not shown). The ratio of HRD1 and OS-9 associated with SEL1L was estimated by quantification of each band using Image J.

(B) Immunoprecipitation of OS-9 and SEL1L from cells treated with negative control siRNA (L & M) or siRNA targeted for OS-9 (OS-9 2 & 3) or SEL1L (SEL1L 2 & 3). Cells were metabolically labeled for 3 h and extracted with 3% digitonin. Open and gray arrowheads

indicate OS-9v1 and v2, respectively. Bracket shows the position of SEL1L, and arrow indicates HRD1. Asterisk denotes the non-specific bands bound to Protein A-Sepharose beads. Proteins immunoprecipitated by antibodies against SEL1L and OS-9 are similar, suggesting the stable association of HRD1-SEL1L and OS-9 in the complex (compare lanes 1, 2 & 7, 8). Specific bands corresponding to OS-9 (v1 & v2) and SEL1L were identified by knockdown of each protein.

(C) Cells were radiolabeled for 3 h, immunoprecipitates of anti-SEL1L antibody were denatured and incubated in a buffer with (+) or without (-) PNGase F for 90 min, and proteins were analyzed by 10% SDS-PAGE. The radioactivity of each protein was determined using a phosphorimager. The ratio of the radioactivity of deglycosylated OS-9v2 (OS-9v2 -CHO) to that of deglycosylated SEL1L (SEL1L-CHO) was estimated to be 0.3 (lane 2). Since SEL1L and OS-9v1 are not well resolved by 10% SDS-PAGE as shown in (B), PNGase F treatment was performed to discriminate between these proteins. Co-immunoprecipitation of HRD1 was detected in cells extracted in a buffer containing 3% digitonin (lane 3) and, after PNGase F digestion, the ratio of the radioactivity of HRD1 to that of deglycosylated SEL1L was estimated to be 1.0 (lane 4).

Figure S5. NHK-QQQ degradation was suppressed by HRD1 silencing.

(A) Pulse-chase experiment of cells transiently expressing NHK-QQQ treated with negative control siRNA or siRNA targeting HRD1.

(B) Quantification of NHK-QQQ in A. Results shown are the mean and corresponding SEM (n = 3).

Figure S6. Coimmunoprecipitation of SEL1L with NHK and NHK-QQQ.

(A) Left panel is same as in Figure 6(A). In the right panel, aliquots of cell extracts from Figure 6A were immunoprecipitated with anti-SEL1L antibody. On lane 13, NHK immunoprecipitated with anti-α1 AT was electrophoresed. The positions of SEL1L and OS-9v2 are shown by the bracket and gray arrowhead, respectively. Endogenous and transfected HRD1 are indicated by the closed and open arrows, respectively. Since the electrophoretic mobility of OS-9v2 is very close to that of HRD1myc, discrimination between these two proteins is difficult under the electrophoretic condition when HRD1myc was cotransfected (lanes 20-25).

(B) Left panel is same as in Figure 6(C). In the right panel, immunoprecipitates of aliquots from

the cell extracts from Figure 6(C) using anti-SEL1L antibody were analyzed. Arrowhead denotes NHK-QQQ. Other notations are the same as in A.

Figure S7. Identification of NHK-QQQ coimmunoprecipitated with SEL1L.

To identify the signal of NHK-QQQ coimmunoprecipitated with SEL1L, cell extracts of 1% NP-40 were transfected with (left panel) or without (right panel) NHK-QQQ. MG132 was added (+) to the medium 4 h prior to pulse labeling. Asterisks indicate the partially degraded fragments of transfected SEL1L.

HeLa cells

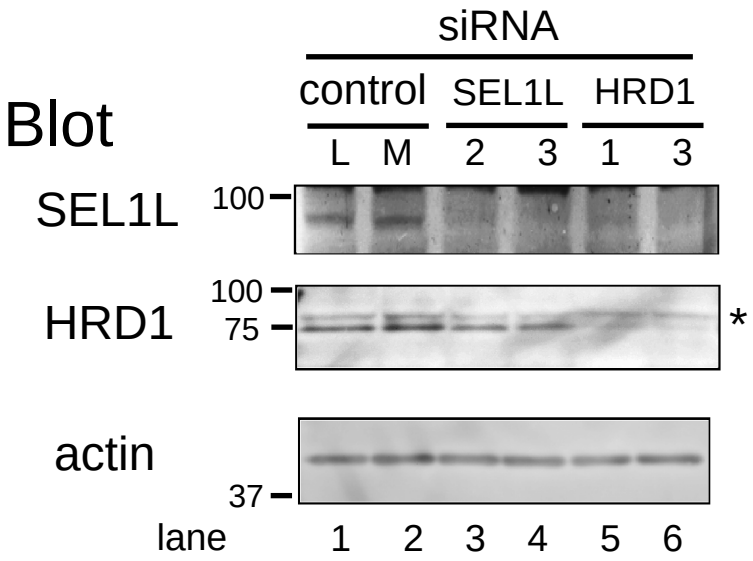


Figure S1

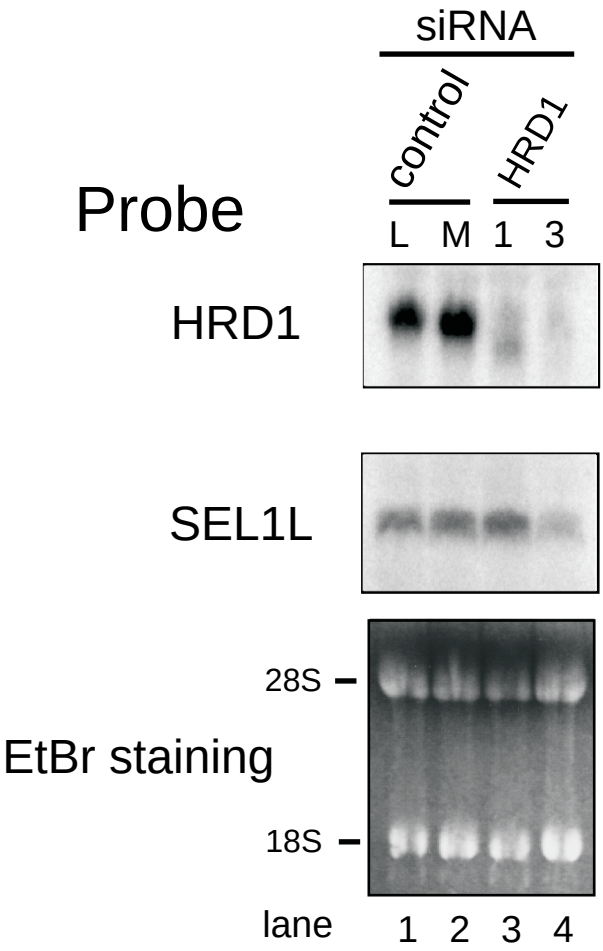

siRNA
control
HRD1
Probe
L M 1 3
HRD1
SEL1L
28S
EtBr staining
18S
lane
1 2 3 4

Figure S2

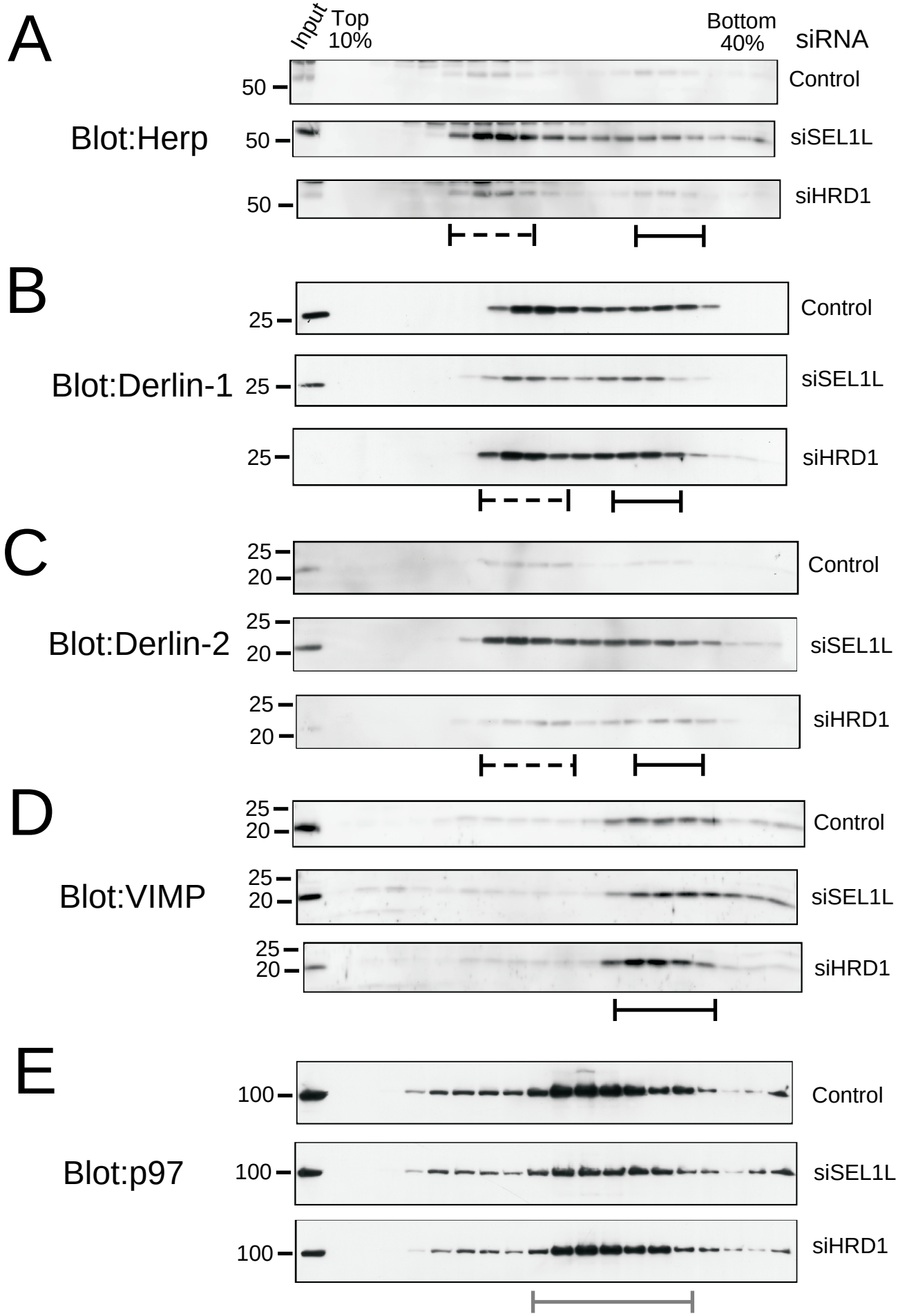

A
Input
Top 10%
Bottom 40%
siRNA
Blot:Herp
50
Control
50
siSEL1L
50
siHRD1
B
Blot:Derlin-1
25
Control
25
siSEL1L
25
siHRD1
C
Blot:Derlin-2
25
20
Control
25
20
siSEL1L
25
20
siHRD1
D
Blot:VIMP
25
20
Control
25
20
siSEL1L
25
20
siHRD1
E
Blot:p97
100
Control
100
siSEL1L
100
siHRD1

Figure S3

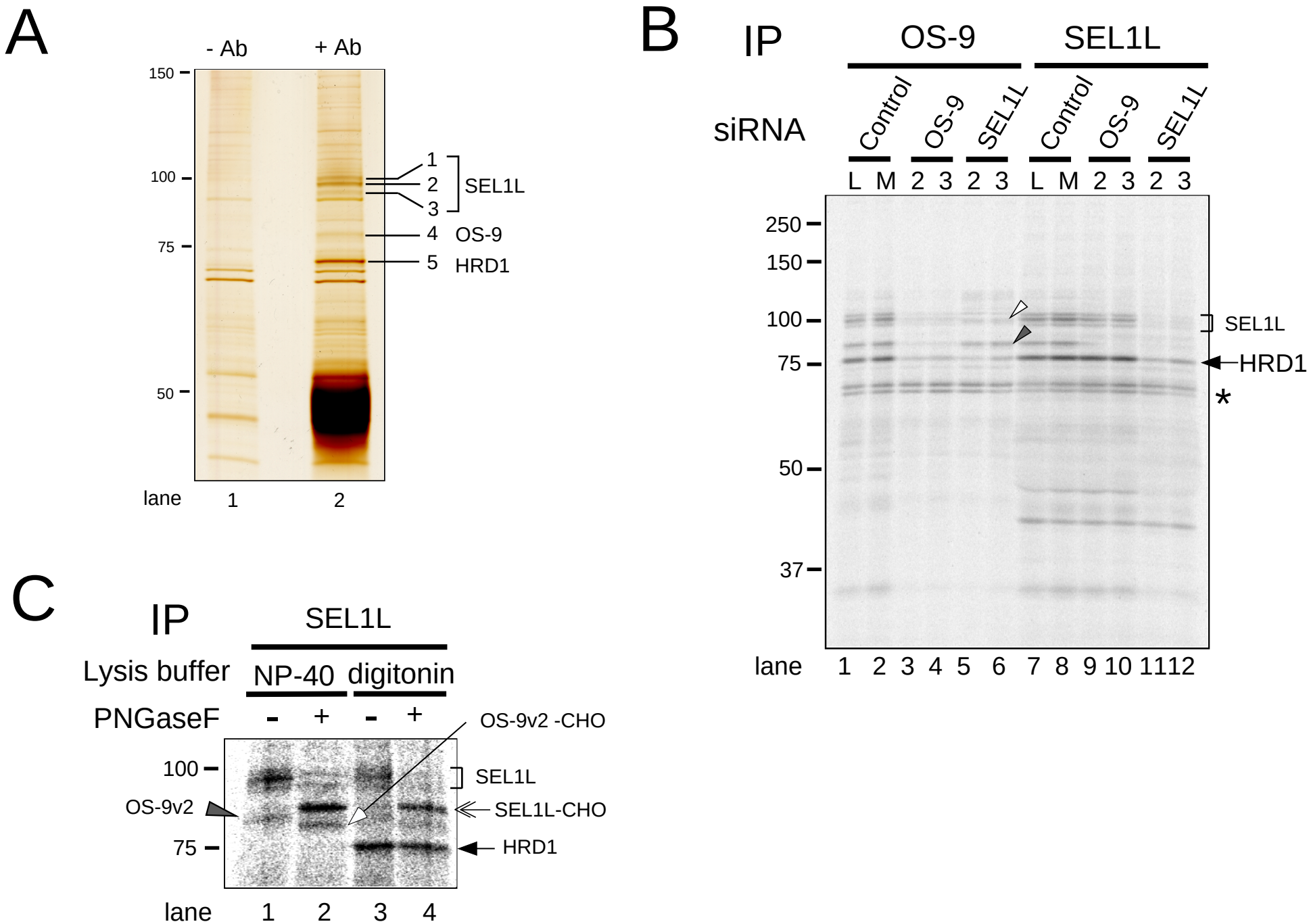
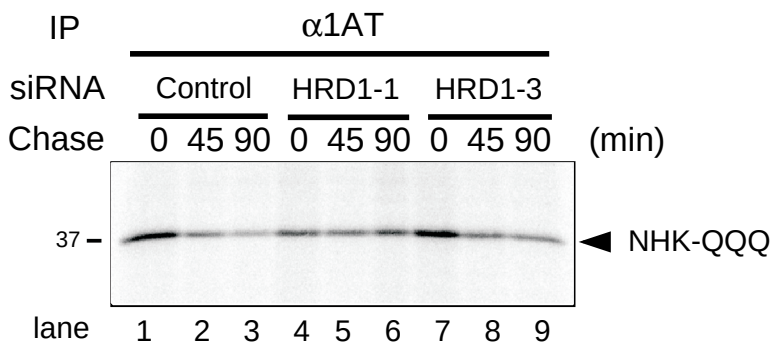

A
- Ab
+ Ab
150
100
75
50
1
2
3
SEL1L
4
OS-9
5
HRD1
lane
1
2
B
IP
OS-9
SEL1L
siRNA
Control
OS-9
SEL1L
Control
OS-9
SEL1L
L M 2 3 2 3 L M 2 3 2 3
250
150
100
75
50
37
SEL1L
HRD1
*
lane
1 2 3 4 5 6 7 8 9 10 11 12
C
IP
SEL1L
Lysis buffer
NP-40
digitonin
PNGaseF
- + - +
OS-9v2 -CHO
100
OS-9v2
75
SEL1L
SEL1L-CHO
HRD1
lane
1 2 3 4

Figure S4

A


IP
α1AT
siRNA
Control
HRD1-1
HRD1-3
Chase
0 45 90 0 45 90 0 45 90
(min)
37
NHK-QQQ
lane
1 2 3 4 5 6 7 8 9

B

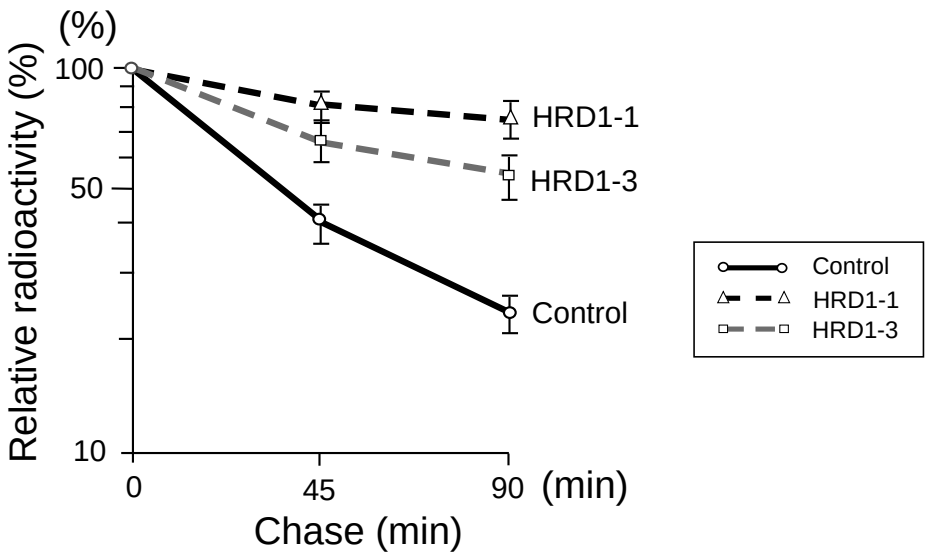

(%)
100
50
10
Relative radioactivity (%)
HRD1-1
HRD1-3
Control
0
45
90
(min)
Chase (min)
Control
HRD1-1
HRD1-3

Figure S5

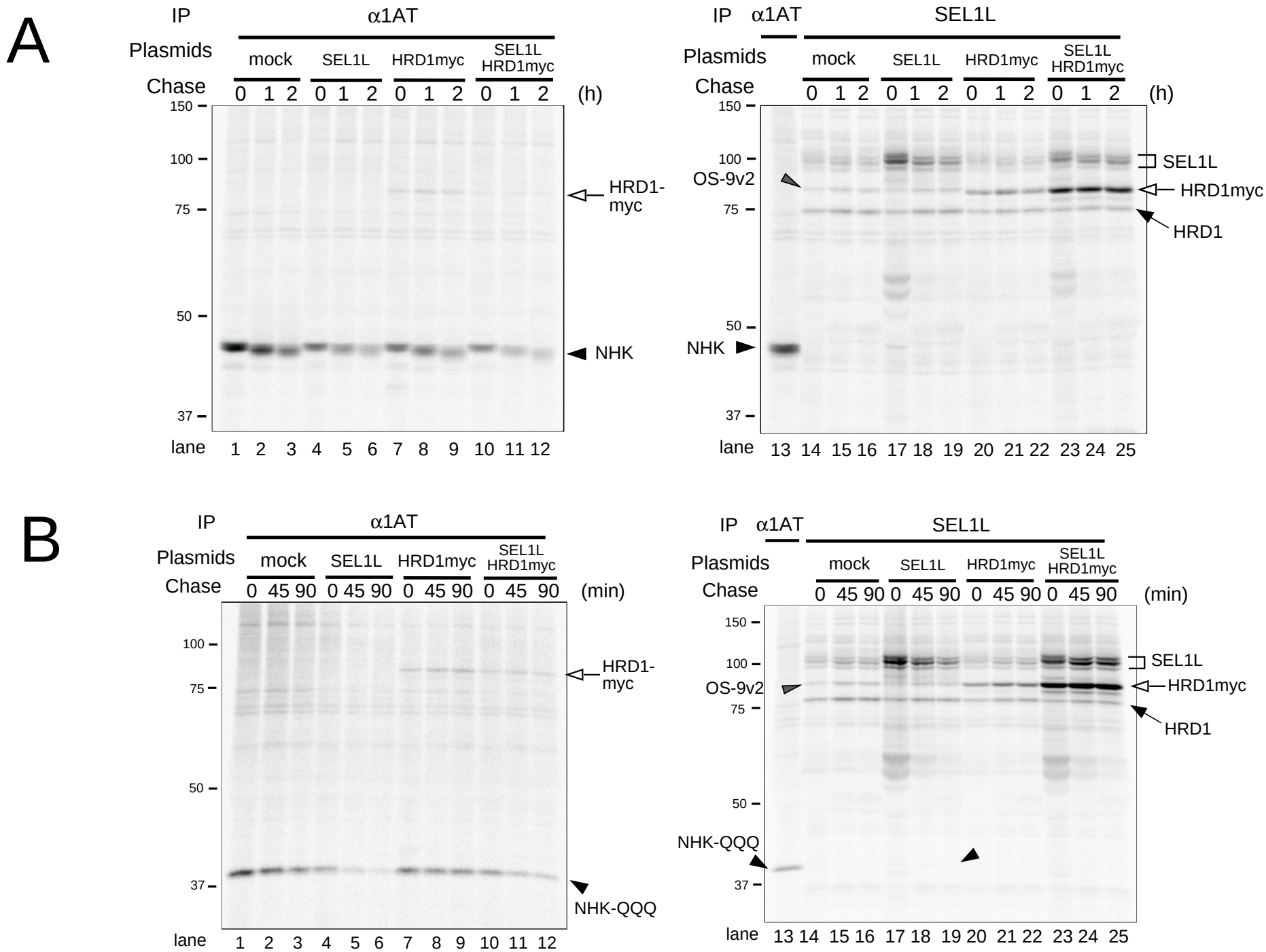

A
IP
α1AT
Plasmids
mock
SEL1L
HRD1myc
SEL1L HRD1myc
Chase
0 1 2 0 1 2 0 1 2 0 1 2
(h)
150
100
75
50
37
HRD1-myc
NHK
lane
1 2 3 4 5 6 7 8 9 10 11 12
IP
α1AT
SEL1L
Plasmids
mock
SEL1L
HRD1myc
SEL1L HRD1myc
Chase
0 1 2 0 1 2 0 1 2 0 1 2
(h)
150
100
OS-9v2
75
50
NHK
37
SEL1L
HRD1myc
HRD1
lane
13 14 15 16 17 18 19 20 21 22 23 24 25
B
IP
α1AT
Plasmids
mock
SEL1L
HRD1myc
SEL1L HRD1myc
Chase
0 45 90 0 45 90 0 45 90 0 45 90
(min)
100
75
50
37
HRD1-myc
NHK-QQQ
lane
1 2 3 4 5 6 7 8 9 10 11 12
IP
α1AT
SEL1L
Plasmids
mock
SEL1L
HRD1myc
SEL1L HRD1myc
Chase
0 45 90 0 45 90 0 45 90 0 45 90
(min)
150
100
OS-9v2
75
50
NHK-QQQ
37
SEL1L
HRD1myc
HRD1
lane
13 14 15 16 17 18 19 20 21 22 23 24 25

Figure S6

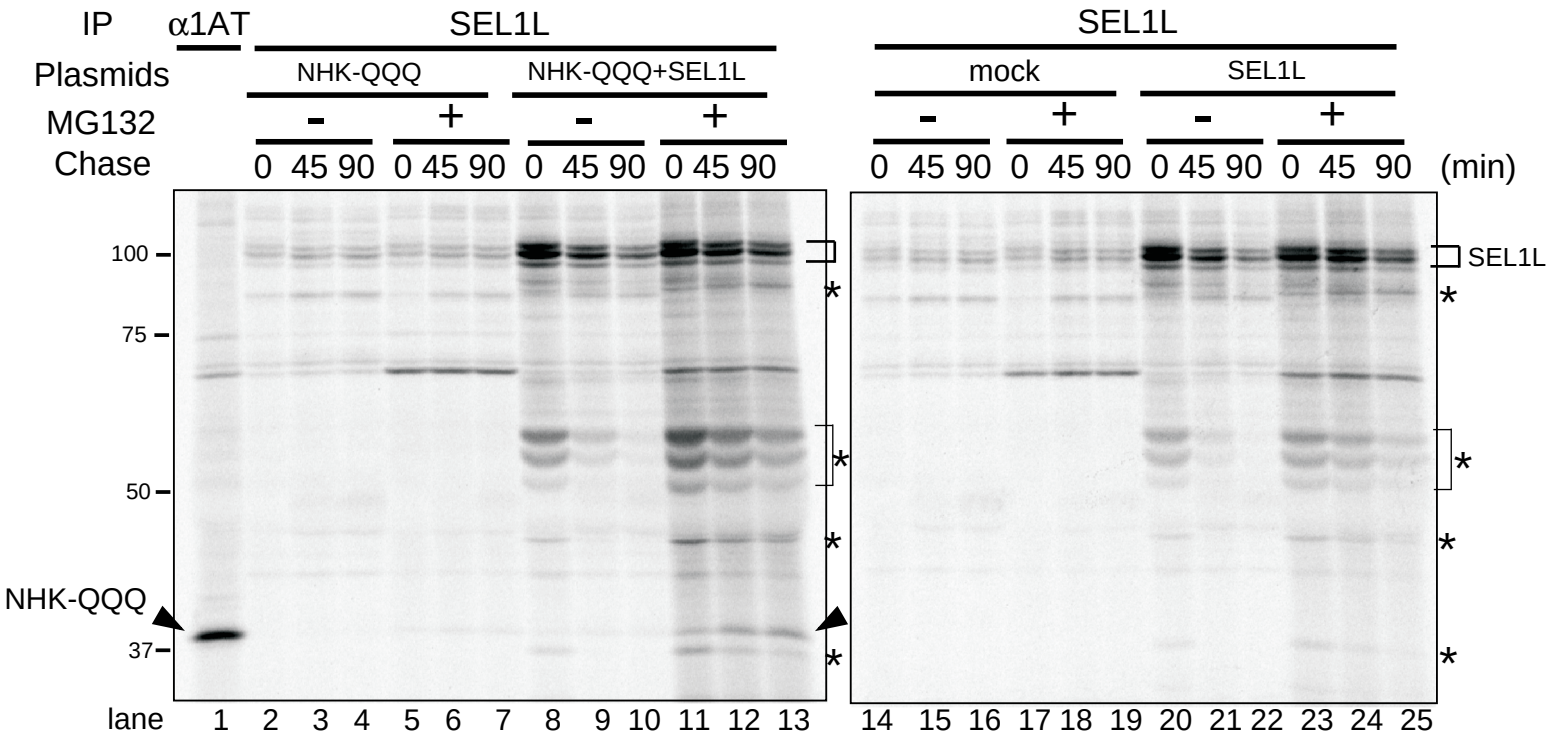

IP
α1AT
SEL1L
SEL1L
Plasmids
NHK-QQQ
NHK-QQQ+SEL1L
mock
SEL1L
MG132
- + - +
- + - +
Chase
0 45 90 0 45 90 0 45 90 0 45 90
0 45 90 0 45 90 0 45 90 0 45 90 (min)
100
75
50
37
NHK-QQQ
SEL1L
*
lane
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25

Figure S7