Supplemental Materials

Molecular Biology of the Cell

Dersh et al.

Supplemental Figure 1. Turnover of the non-glycosylated ERAD substrate NS1 is not dependent on GRP94. (A) The indicated HEK293T cell lines (shCtrl, shGRP94, and shGRP94 re-complemented with an shRNA resistant form of GRP94) were transiently transfected with a V5-tagged NS1 and pulse-chased as described in Materials and Methods. NS1 was immunoprecipitated at each time point using anti-V5 Agarose. Values under the respective bands indicate the percentage of NS1 remaining, normalized to the value at time 0. (B) Cells from the pulse chase in A were harvested, lysed, and subjected to SDS PAGE. Western blotting for GRP94 depicts the extent of depletion and re-complementation.

Supplemental Figure 2. OS-9 is not a GRP94 client. (A) NIH3T3 cells were infected with lentivirus expressing shRNAs targeting GRP94 (shGRP94) or a scrambled control (shCtrl). Cells were harvested after 5 days, lysed, and subjected to Western blotting. GRP94 and BiP levels were analyzed simultaneously using the anti-KDEL antibody. GRP94 depletion induced BiP expression, while steady state levels of endogenous OS-9.1 and OS-9.2 were not affected. (B) Pulse-chase assays of S-tagged OS-9 isoforms 1-3 expressed in HEK293T shCtrl or shGRP94 cells. S-OS-9 was isolated at each time point by S-Protein Agarose and the resulting bands quantified by phosphoimaging. Values under the respective bands indicate the percentage of OS-9 remaining, normalized to the value at time 0. (C) Cells from B were analyzed by Western blot to confirm depletion of GRP94.

Supplemental Figure 3. Four alternatively spliced transcripts of OS-9 are detected in cells. RNA from the indicated cell lines was isolated and subject to RT-PCR as described in Materials and Methods. Semi-quantitative PCR was used to amplify OS-9, using the listed primers, which were designed to amplify all predicted isoforms. Band sizes were compared to standard PCR reactions of the respective OS-9-containing expression vectors (OS-9.1, OS-9.2, and OS-9.3), using the same primers.

Supplemental Figure 4. S-OS-9.1 alters the mobility of endogenous GRP94 due to an electrophoresis artifact. (A) A portion of the Western blot in Figure 1B is shown as it was scanned in two channels, depicting the mobility of S-tagged OS-9 isoforms and endogenous GRP94. S-OS-9.1 runs at the exact mobility of endogenous GRP94 and distorts its location in the gel, whereas S-OS-9.2 runs distinctly from GRP94. Yellow indicates co-localization of antibodies from the red and green channels. White arrows indicate the GRP94 species affected by the bait protein S-OS-9.1. (B) Same as A, but for the experiment described in Figure 4E. (C) Same as A, but for the experiment described in Figure 5F.

Supplemental Figure 5. hgGRP94 species are subject to mannose trimming. HEK293T cells expressing S-tagged GRP94 were left untreated or treated with kifunensine (5 µg/mL, 20.5 hours). The shift of hgGRP94 bands relative to mgGRP94 was monitored in cell lysates by immunoblotting with anti-S-tag. The vertical line indicates removal of irrelevant gel lanes from the image.

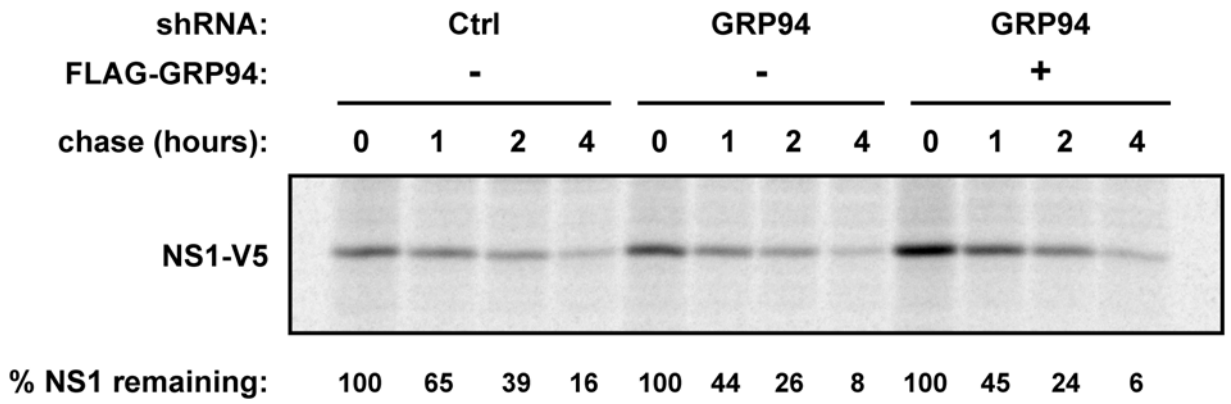
Supplemental Figure 6. Ectopic expression of GRP94 does not induce ER stress. (A) RNA was isolated from HEK293T cells which were untreated, treated with thapsigargin (0.5 µM, 1 hour), or were transiently transfected with either FLAG-GRP94 or a secretory pathway mutant which we are currently characterizing (Sec mut). An XBP-1 splicing assay was conducted as described in Materials and Methods. % spliced, intensity of spliced fragment divided by the sum of the spliced and unspliced bands. Note that while the secretory pathway mutant caused significant XBP-1 splicing, expression of GRP94 did not. (B) HEK293T cells were transiently transfected with increasing amounts of S-GRP94, or left untransfected or treated with tunicamycin (1 µg/mL, 17 hours). Whereas overnight treatment with tunicamycin led to an

increase in BiP levels and phosphorylation of PERK (slower gel mobility), increasing expression of GRP94 had no effect. Additionally, the maximal amount of DNA used for transfection caused less than a doubling of the endogenous GRP94 level. endog mgGRP94, endogenous mono-glycosylated GRP94; exog mgGRP94, exogenous mono-glycosylated S-GRP94; exog hgGRP94, exogenous hyper-glycosylated S-GRP94; non-phos PERK, non-phosphorylated PERK.

Supplemental Figure 7. Kifunensine does not inhibit formation of the OS-9/GRP94 complex. (A) S-OS-9.2 was transiently expressed in HEK293T cells and left untreated or treated with kifunensine (5 µg/mL) or thapsigargin (0.3 µM) for 16 hours. Co-precipitation of endogenous GRP94 was monitored via immunoblot analysis after S-Protein Agarose affinity purification of OS-9. 14-3-3, cytosolic control to ensure stringency of AP. A higher contrast image of the lysate samples shows the formation of hgGRP94 species. (B) HA-tagged NHK was transiently expressed in HEK293T cells, which were left untreated or exposed to kifunensine (5 µg/mL, 18 hours). NHK was then affinity purified with anti-HA Agarose to observe co-IP of endogenous OS-9 by Western blot analysis. h.c.*, heavy chain of the anti-HA antibody.

Supplemental Figure 8. Location of GRP94 glycosylation acceptor sites. An existing crystal structure of GRP94 (PDB, 2O1U) is shown with a portion of the C-terminus removed for visibility. AMP-PNP residing in the ATP-binding pocket is shown in stick form. The asparagines of the potential NXS/T glycan acceptor sites are shown in stick form and magenta; one amino acid N- and C-terminal of each asparagine is also highlighted. Note: N41 is not present in this crystal structure.

A


shRNA:
Ctrl
GRP94
GRP94
FLAG-GRP94:
-
-
+
chase (hours):
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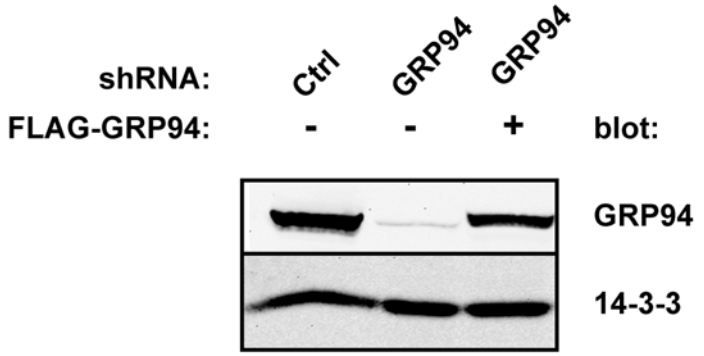
NS1-V5
% NS1 remaining:
100 65 39 16 100 44 26 8 100 45 24 6

B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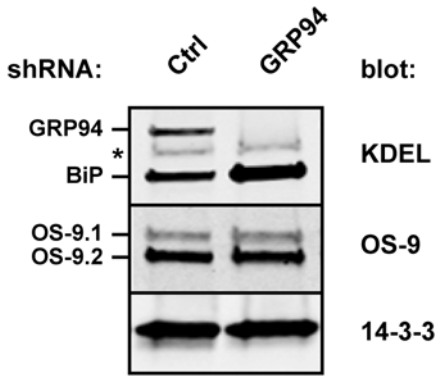
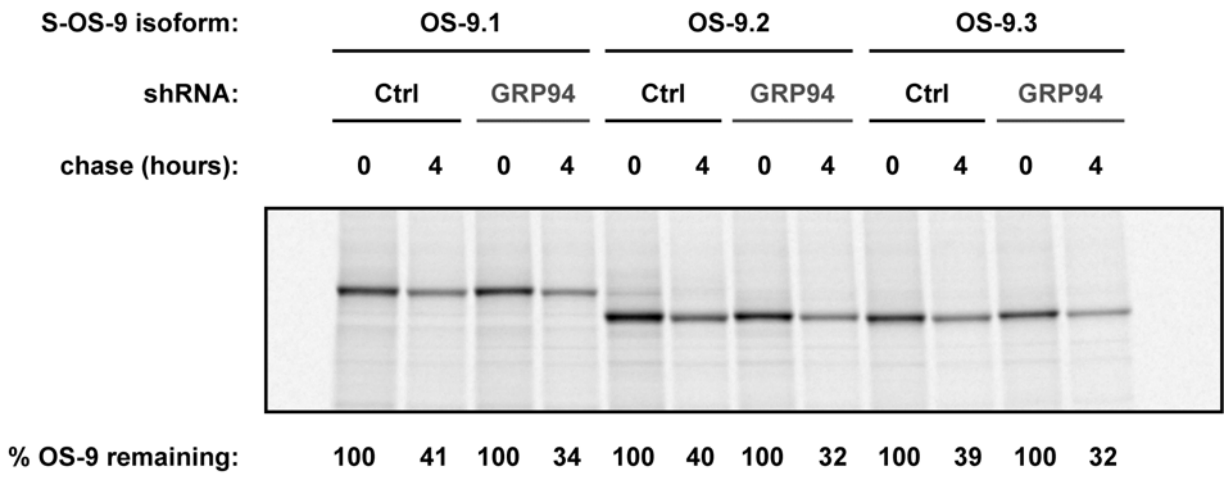
Ctrl
GRP94
GRP94
FLAG-GRP94:
-
-
+
blot:
GRP94
14-3-3

A


shRNA:
Ctrl
GRP94
blot:
GRP94
*
BiP
KDEL
OS-9.1
OS-9.2
OS-9
14-3-3

B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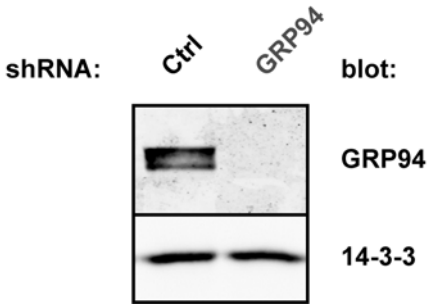
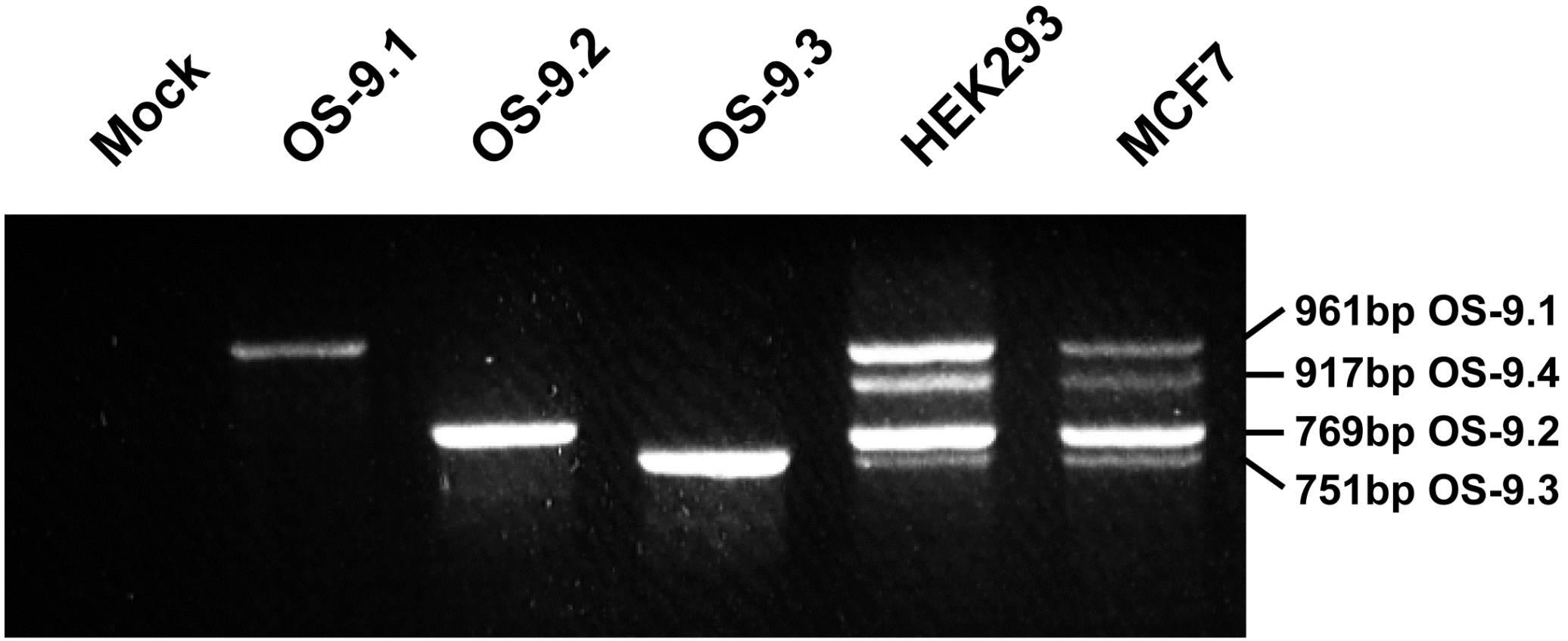
OS-9.1
OS-9.2
OS-9.3
shRNA:
Ctrl
GRP94
Ctrl
GRP94
Ctrl
GRP94
chase (hours):
0 4 0 4 0 4 0 4 0 4 0 4
% OS-9 remaining:
100 41 100 34 100 40 100 32 100 39 100 32

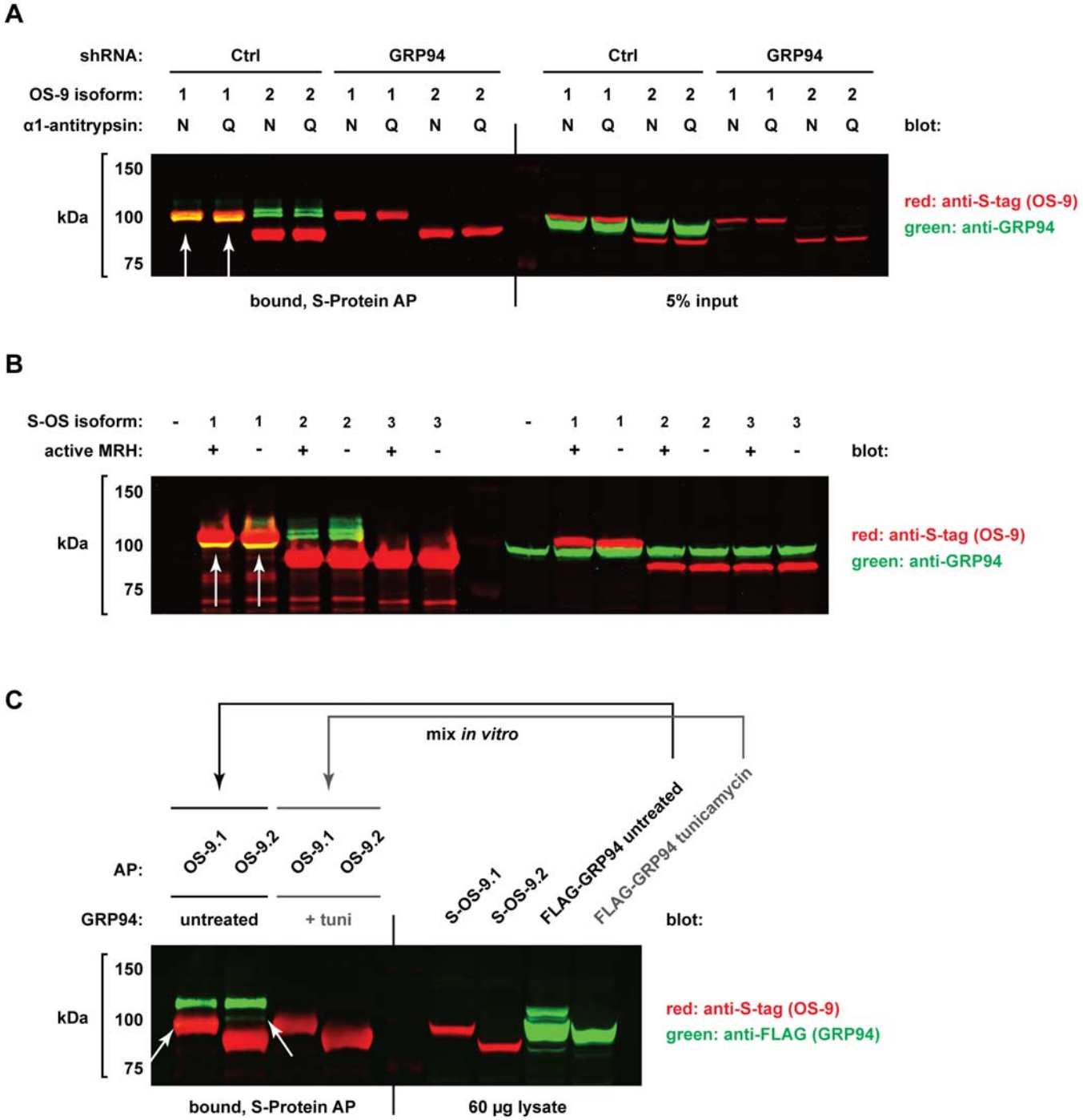
C

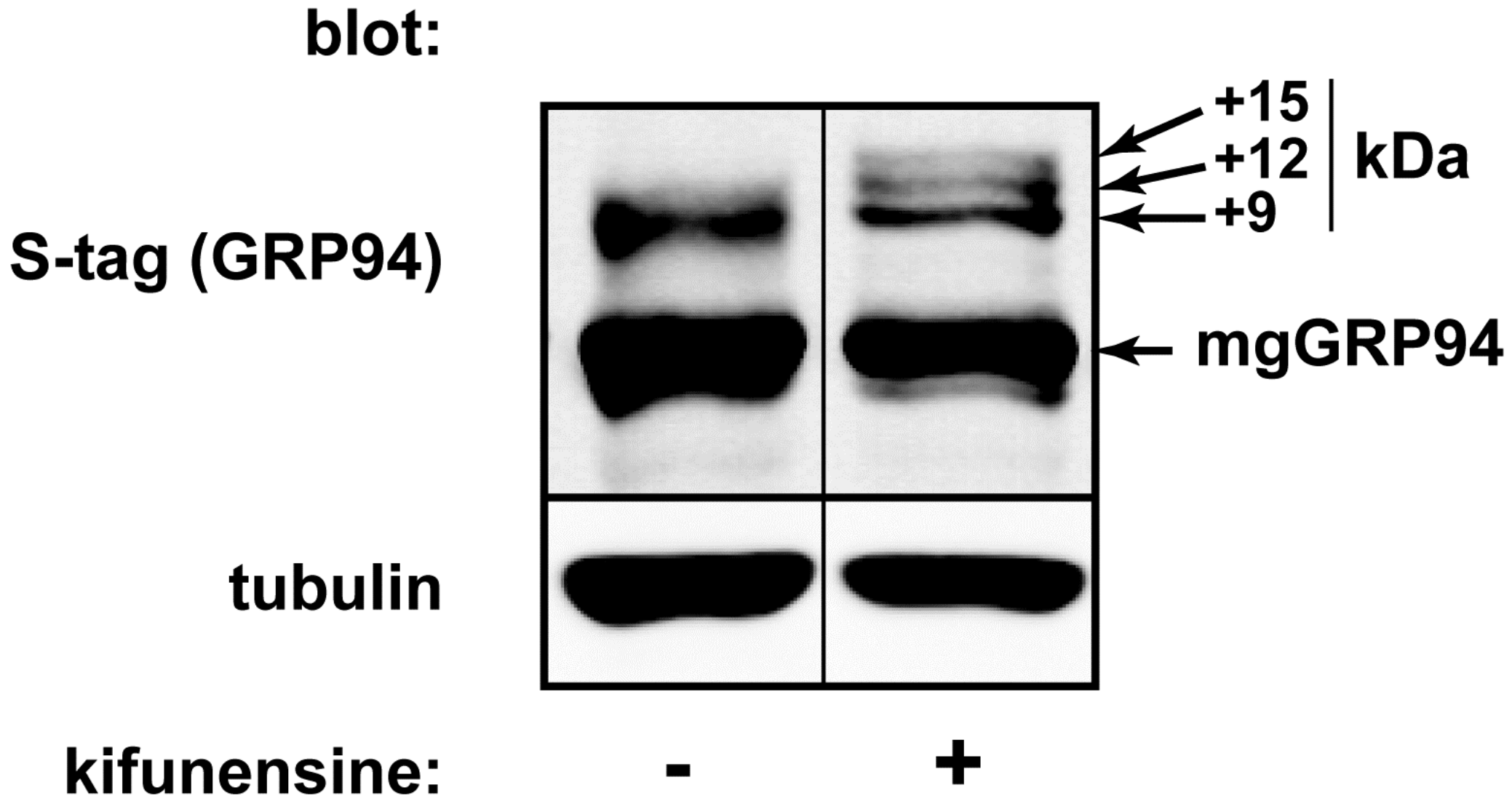

shRNA:
Ctrl
GRP94
blot:
GRP94
14-3-3

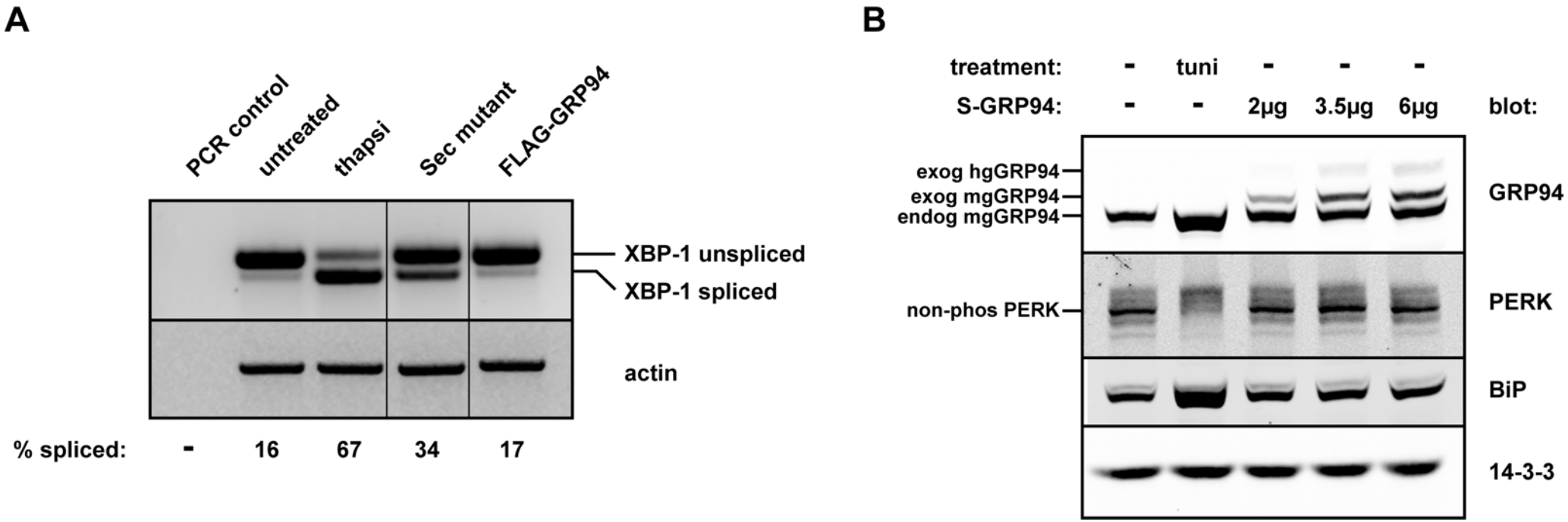


5’-AGCCCGACCAAGGATGATACAGTAAG-3’ (forward)

5’-AGTCAGCCAACGTGCACCCTC-3’ (reverse)


A
shRNA:
Ctrl
GRP94
Ctrl
GRP94
OS-9 isoform:
1 1 2 2 1 1 2 2 1 1 2 2 1 1 2 2
α1-antitrypsin:
N Q N Q N Q N Q N Q N Q N Q N Q
blot:
kDa
150
100
75
red: anti-S-tag (OS-9)
green: anti-GRP94
bound, S-Protein AP
5% input
B
S-OS isoform:
- 1 1 2 2 3 3 - 1 1 2 2 3 3
active MRH:
+ - + - + - + - + - + -
blot:
kDa
150
100
75
red: anti-S-tag (OS-9)
green: anti-GRP94
C
mix in vitro
AP:
OS-9.1
OS-9.2
OS-9.1
OS-9.2
GRP94:
untreated
+ tuni
S-OS-9.1
S-OS-9.2
FLAG-GRP94 untreated
FLAG-GRP94 tunicamycin
blot:
kDa
150
100
75
red: anti-S-tag (OS-9)
green: anti-FLAG (GRP94)
bound, S-Protein AP
60 µg lysate


blot:
S-tag (GRP94)
tubulin
kifunensine:
-
+
+15
+12
+9
kDa
mgGRP94


A
PCR control
untreated
thapsi
Sec mutant
FLAG-GRP94
XBP-1 unspliced
XBP-1 spliced
actin
% spliced:
-
16
67
34
17
B
treatment:
-
tuni
-
-
-
S-GRP94:
-
-
2µg
3.5µg
6µg
blot:
exog hgGRP94
exog mgGRP94
endog mgGRP94
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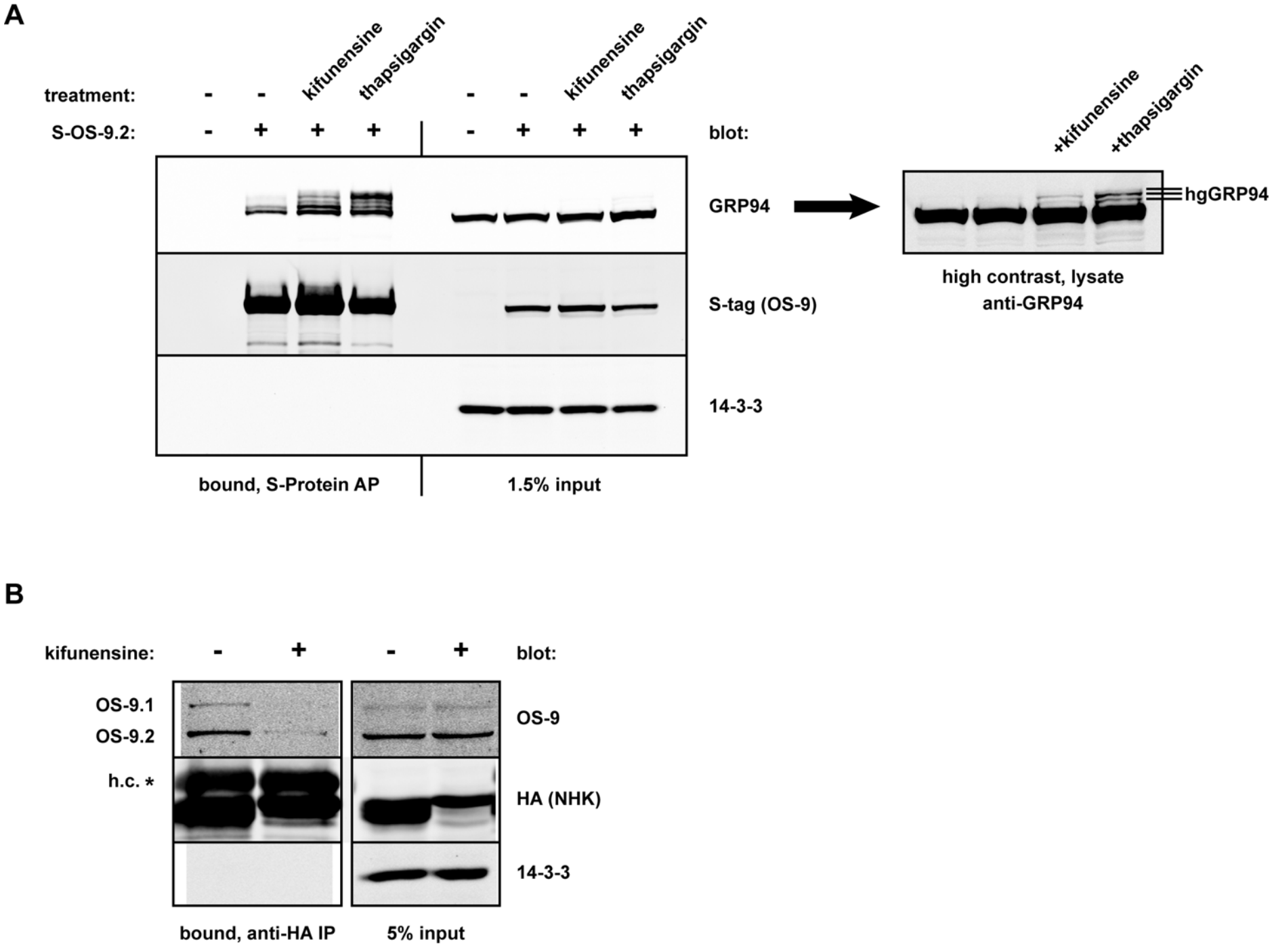
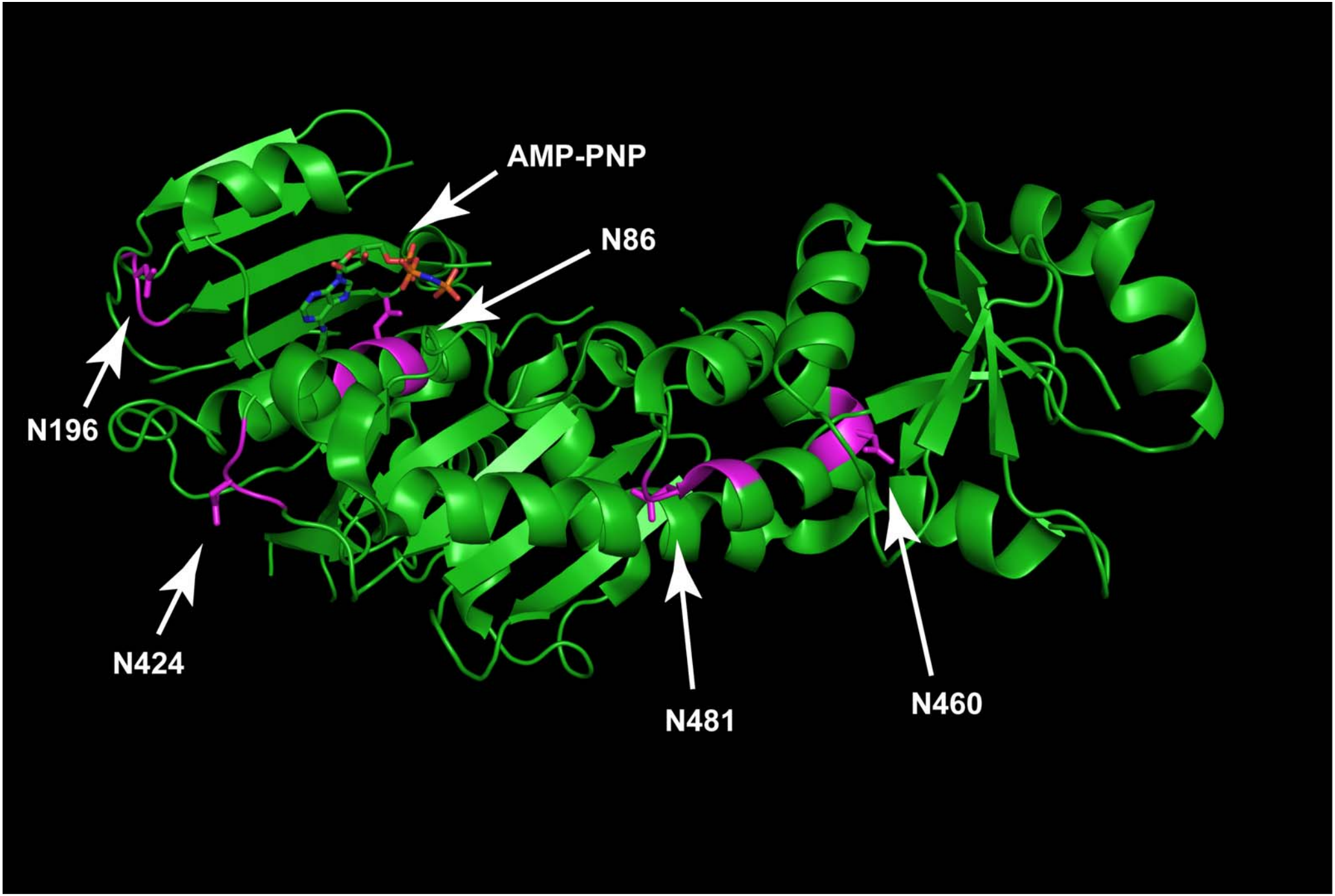
non-phos PERK
PERK
BiP
14-3-3


A
treatment:
S-OS-9.2:
kifunensine
thapsigargin
blot:
GRP94
S-tag (OS-9)
14-3-3
bound, S-Protein AP
1.5% input
+kifunensine
+thapsigargin
hgGRP94
high contrast, lysate
anti-GRP94
B
kifunensine:
blot:
OS-9.1
OS-9.2
h.c. *
OS-9
HA (NHK)
14-3-3
bound, anti-HA IP
5% input


AMP-PNP
N86
N196
N424
N481
N460