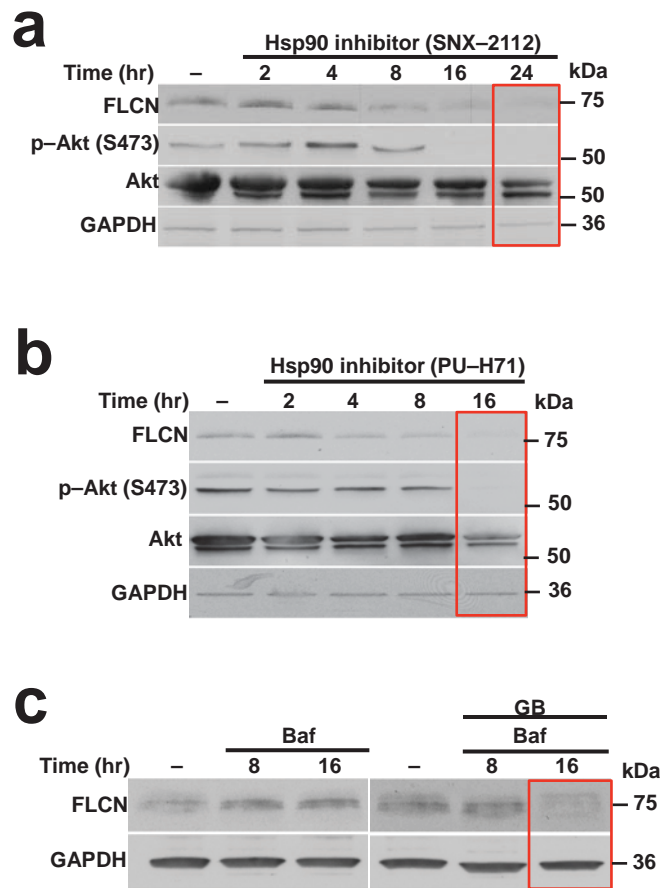


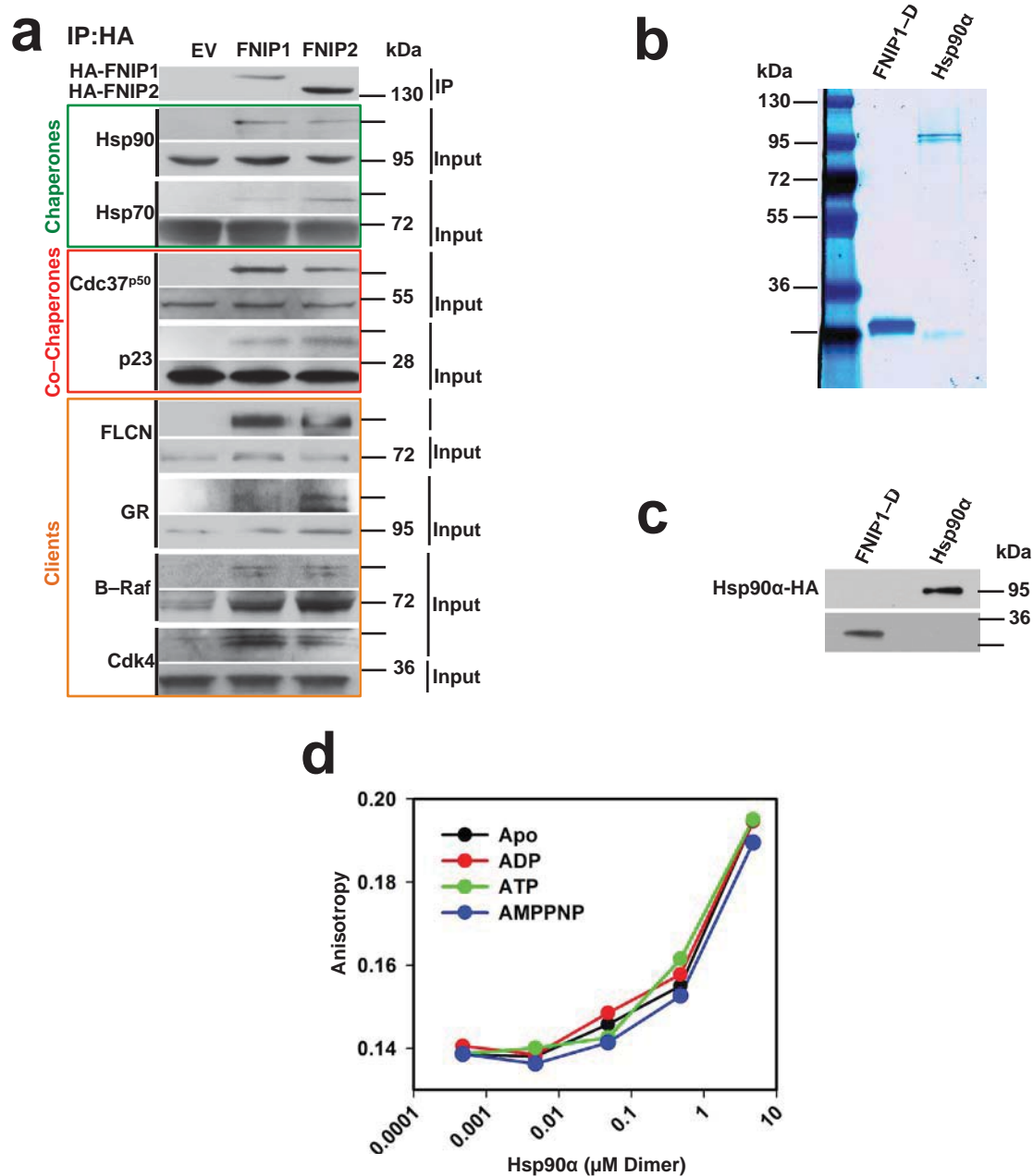
Supplementary Fig. 1



Supplementary Fig. 1. FLCN is a new client of Hsp90. Related to Fig. 1.

- HEK293 cells were treated with 2 μ M of the Hsp90 inhibitor SNX-2112 for the indicated times. FLCN protein was detected by immunoblotting.
- HEK293 cells were treated with 3 μ M of the Hsp90 inhibitor PU-H71 for the indicated times. FLCN was detected by immunoblotting.
- 100nM treatment of HEK293 cells with the lysosome inhibitor bafilomycin (Baf) for 8 or 16 hr in presence or absence of 1 μ M ganetespib (GB).

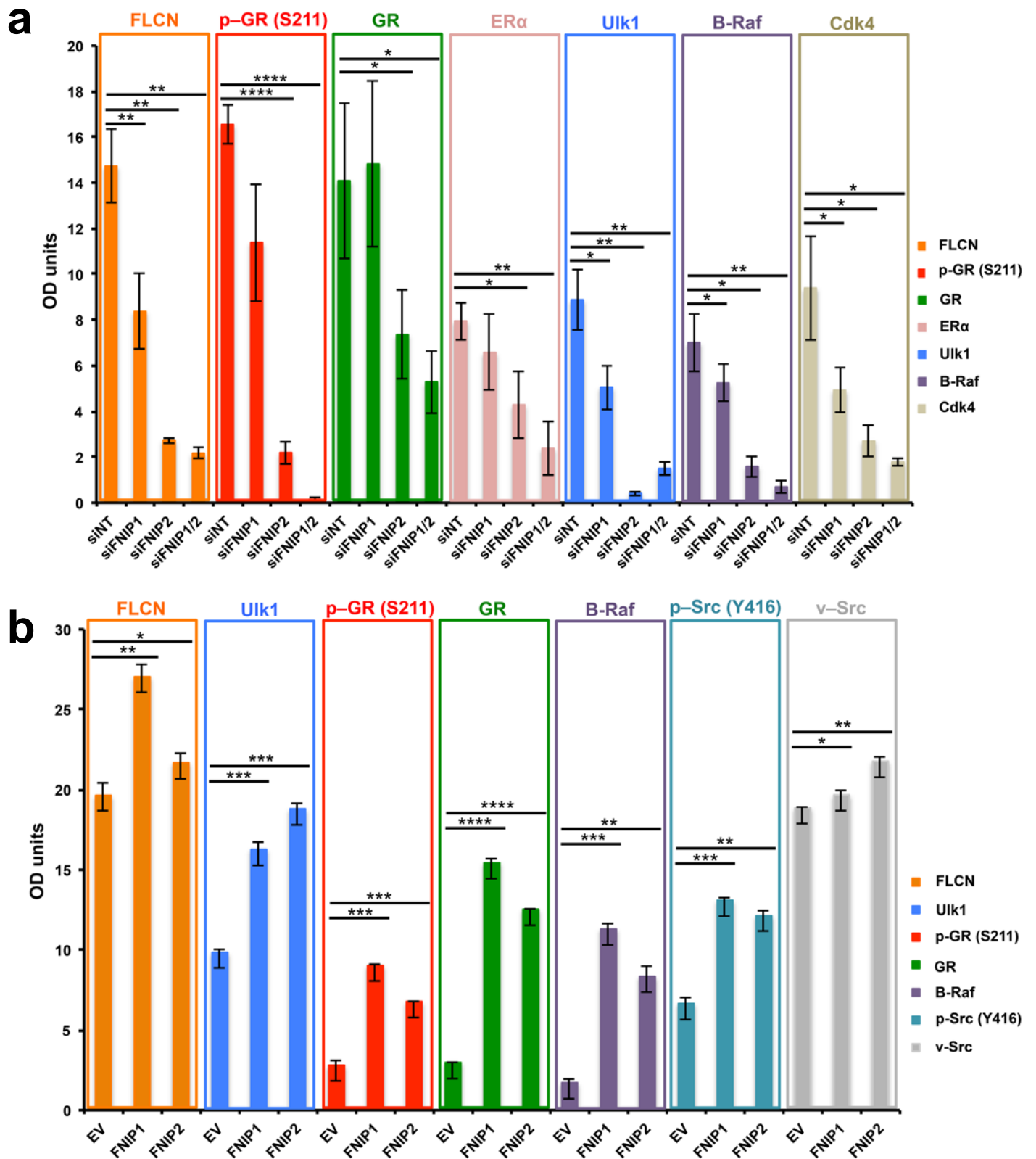
Supplementary Fig. 2



Supplementary Fig. 2. FNIP1 facilitates FLCN binding to the Hsp90 chaperone. Related to Fig. 2-3.

- Empty vector (EV), HA-FNIP1 or HA-FNIP2 constructs transiently expressed and isolated from HEK293 cells. Interaction of FNIP1 and FNIP2 with the molecular chaperones, co-chaperones and indicated client proteins were assessed by immunoblotting.
- 100ng of the purified FNIP1-D-His₆ and 50ng Hsp90α-HA protein samples were resolved on the SDS-PAGE gel and stained with Coomassie stain.
- Western blot analysis of the purified proteins from (A).
- FNIP1-D-His₆:HSP90α-HA binding measured by fluorescence anisotropy in the presence and absence of different adenosine nucleotides.

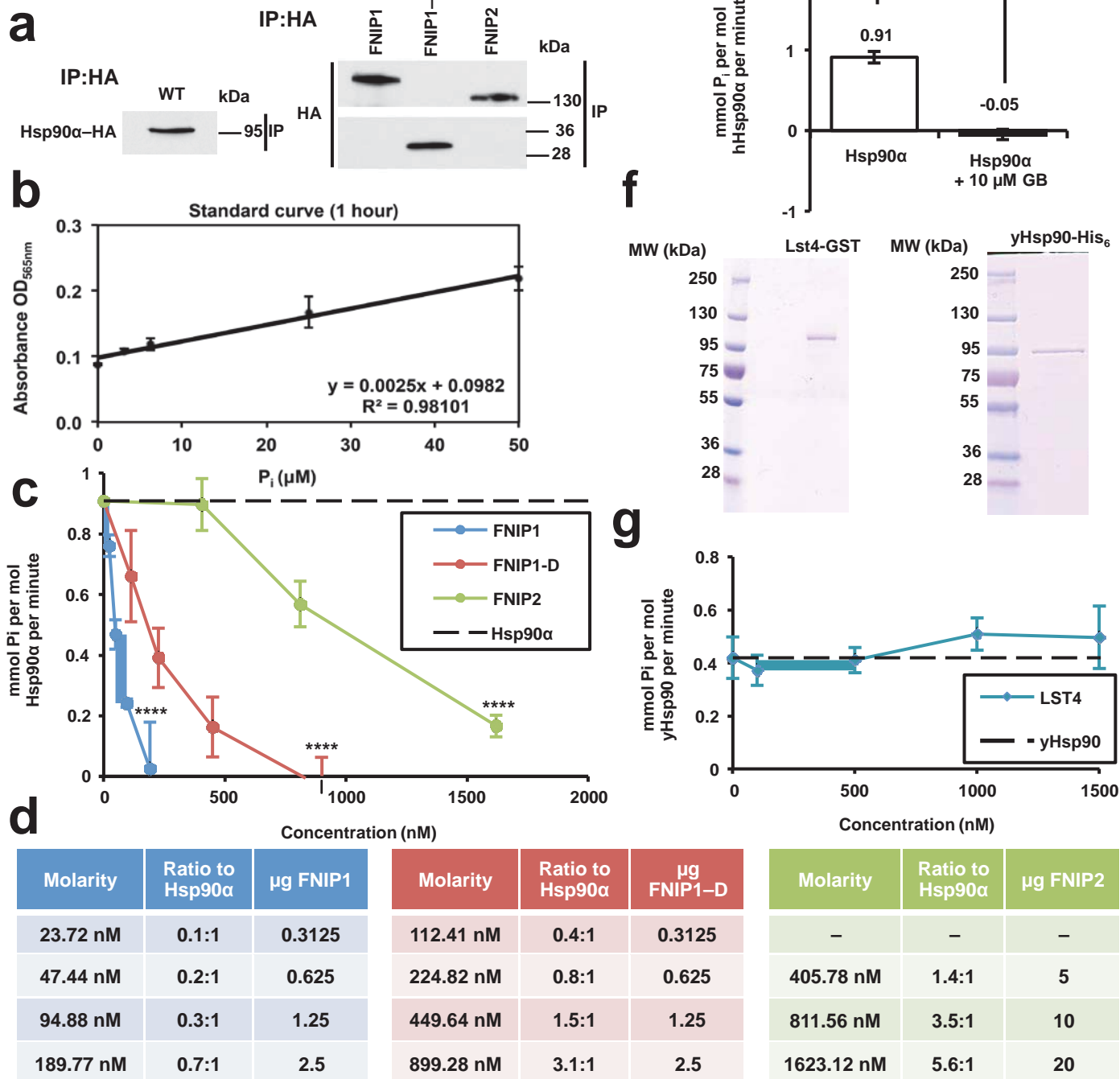
Supplementary Fig. 3



Supplementary Fig. 3 . FNIP co-chaperones facilitate chaperoning of the clients. Related to Fig. 4.

- a) Effect of siRNA knockdown of *FNIP1* and *FNIP2* on Hsp90 clients. Stability and activity of the indicated Hsp90 clients were assessed by immunoblotting. Densitometry (optical density, OD) of the Western blot in Fig. 3a are represented as mean \pm S.D. (* $p < 0.05$, ** $p < 0.005$, **** $p < 0.00001$).
- b) Transient overexpression of cMyc-tagged *FNIP1* or *FNIP2* in HEK293 cells and their impact on levels of Hsp90 clients was assessed by immunoblotting in Fig. 3b. Densitometry (OD) of the Western blot in Fig. 3a are represented as mean \pm S.D. (* $p < 0.05$, ** $p < 0.005$, **** $p < 0.00005$, ***** $p < 0.00001$).

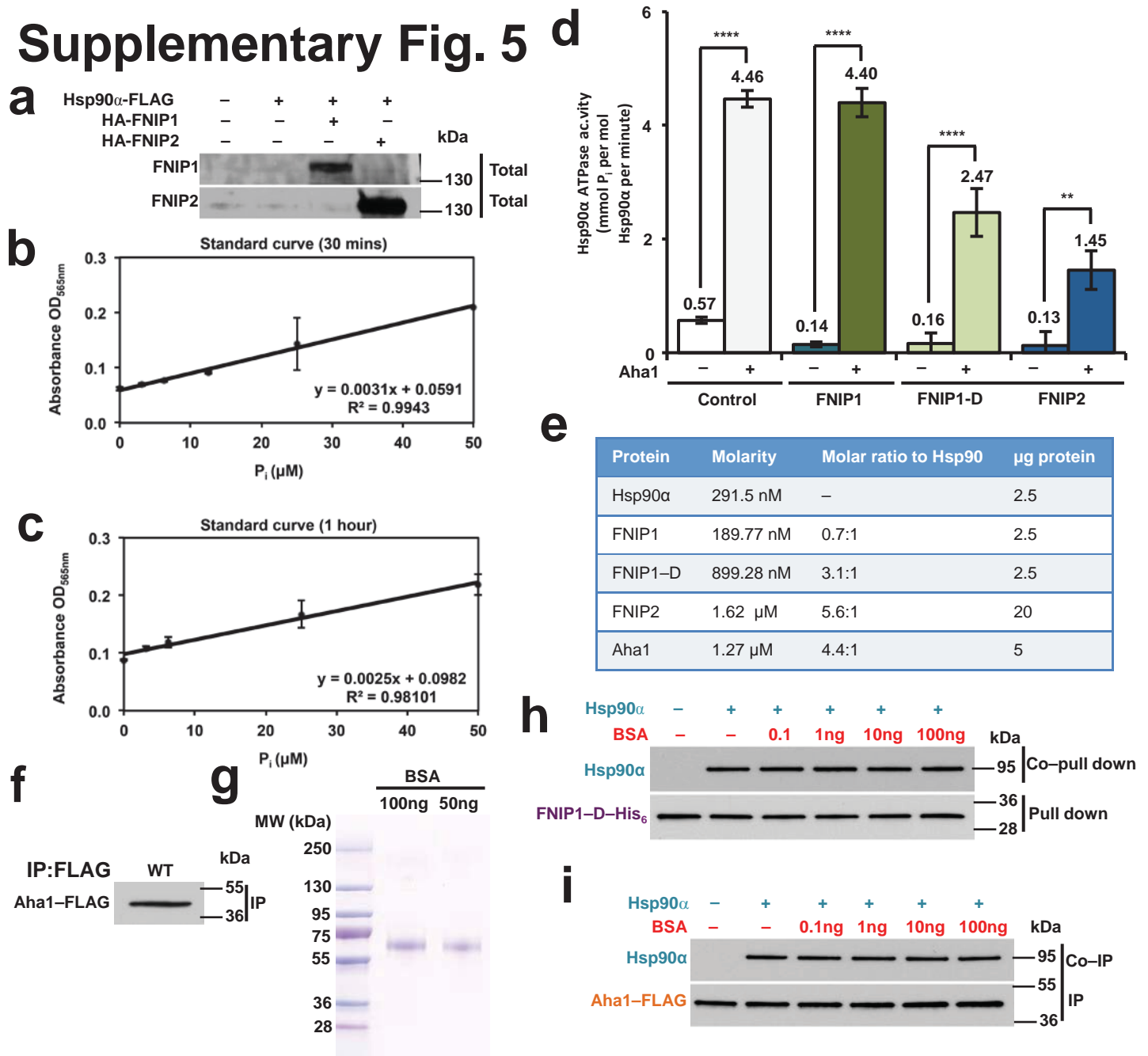
Supplementary Fig. 4



Supplementary Fig. 4. FNIP co-chaperones inhibit Hsp90 chaperone cycle and facilitate chaperoning of the clients. Related to Fig. 4-5.

- PC3 cells were transiently transfected with Hsp90α-HA and HEK293 cells were transiently transfected with HA-FNIP1, HA-FNIP1-D, or HA-FNIP2 constructs. Hsp90α-HA, HA-FNIP1, HA-FNIP1-D, and HA-FNIP2 were immunoprecipitated, competed with the relevant peptides and proteins were detected by immunoblotting.
- Inorganic phosphate (P_i) standard curve at 1 hr. The x-axis shows μM of P_i per assay and the y-axis shows absorbance at 565nm. Mean \pm S.D. from values obtained in three independent experiments.
- ATPase activity of Hsp90α-HA with or without HA-FNIP1, HA-FNIP1-D, or HA-FNIP2 expressed in mmol P_i per mol Hsp90α per minute. Mean \pm S.D. from values obtained in three independent experiments with $***p < 0.0005$.
- Molarity of the proteins in the ATPase assay, ratio to Hsp90, and total quantity of protein added.
- ATPase activity of Hsp90α-HA with or without 10 μM GB expressed in mmol P_i per mol Hsp90α per minute. Mean \pm S.D. from values obtained in three independent experiments with $****p < 0.0001$.
- 50ng of Lst4-GST and yHsp90-His6 were resolved on the SDS-PAGE gel and stained with Coomassie stain.
- In vitro* ATPase activity of the yHsp90-His₆ with indicated amounts Lst4-GST. All the data represent mean \pm S.D.

Supplementary Fig. 5



Supplementary Fig. 5. FNIPs compete with the Aha1 co-chaperone for binding to Hsp90. Related to Fig. 6.

- Hsp90 α with and without HA-FNIP1 and HA-FNIP2 proteins from Figure 4B. Western blotting validates overexpression of FNIPs compared to endogenous levels of FNIP1 and FNIP2.
- Inorganic phosphate (Pi) standard curve at 30 min and
- 1 hour. The x-axis shows μ M of Pi per assay and the y-axis shows absorbance at 565nm. Mean \pm S.D. from values obtained in three independent experiments.
- Inhibition of Hsp90 α -HA ATPase activity 30 min after the addition of HA-FNIP1 or HA-FNIP2 and subsequent stimulation by Aha1-FLAG after an additional 30 min expressed in mmol Pi per mol Hsp90 α per min. Mean \pm S.D. from three independent experiments with **p < 0.005, ****p < 0.0001.
- Molarity of the protein in the ATPase assay, ratio to Hsp90, and total quantity of protein added are listed.
- HEK293 cells were transiently transfected with Aha1-FLAG. Following immunoprecipitation, protein was competed with peptide and detected by immunoblotting with anti-FLAG antibody.
- Indicated amounts of BSA were resolved on the SDS-PAGE gel and stained with Coomassie stain.
- FNIP1-D-His₆ was attached to Ni-NTA agarose and then incubated with Hsp90 α . Ni-NTA agarose was then washed and incubated with the indicated amounts of BSA.
- Aha1-FLAG attached to anti-FLAG M2 affinity gel was incubated with Hsp90 α initially and then washed and incubated with indicated amounts of BSA.

Supplementary Fig. 6. Full original immunoblots and gel images.

Figure 1b

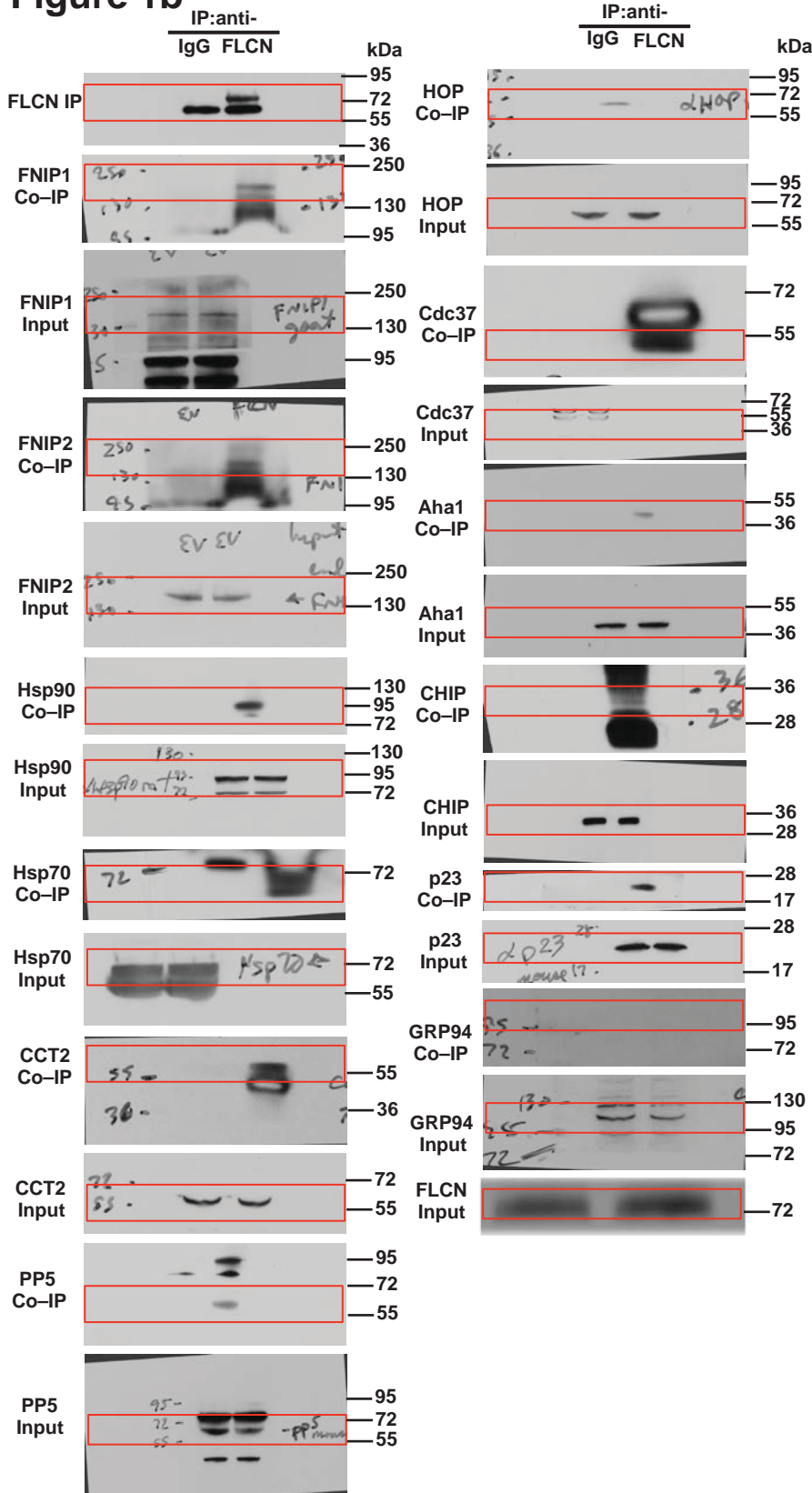


Figure 1c

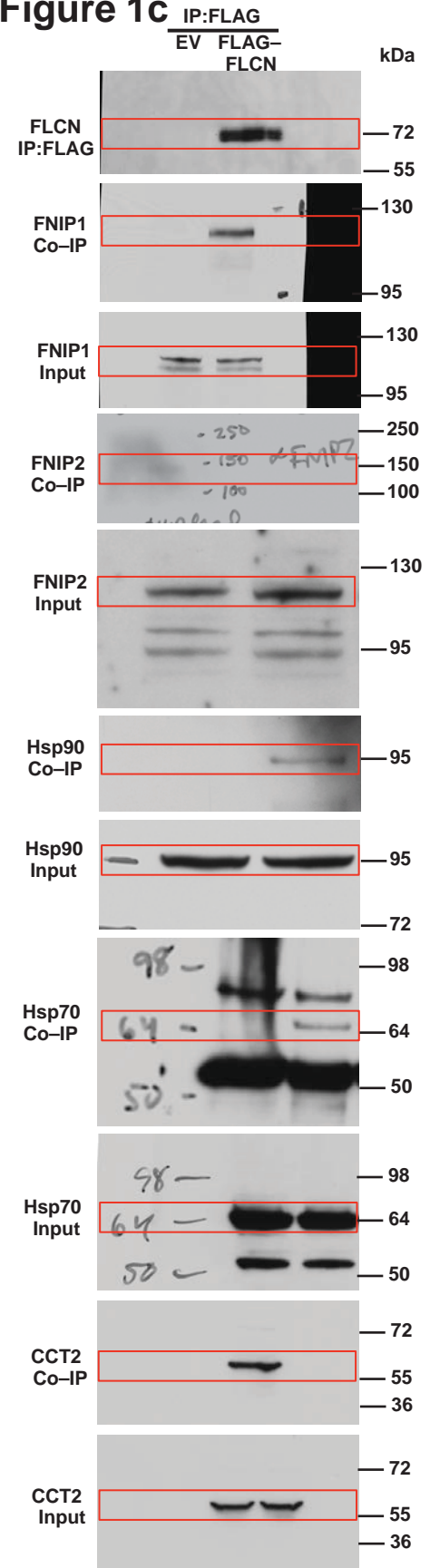


Figure 1c continued

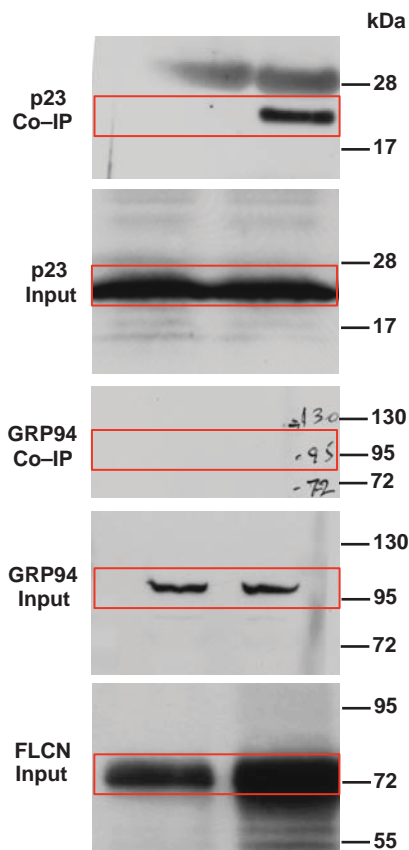
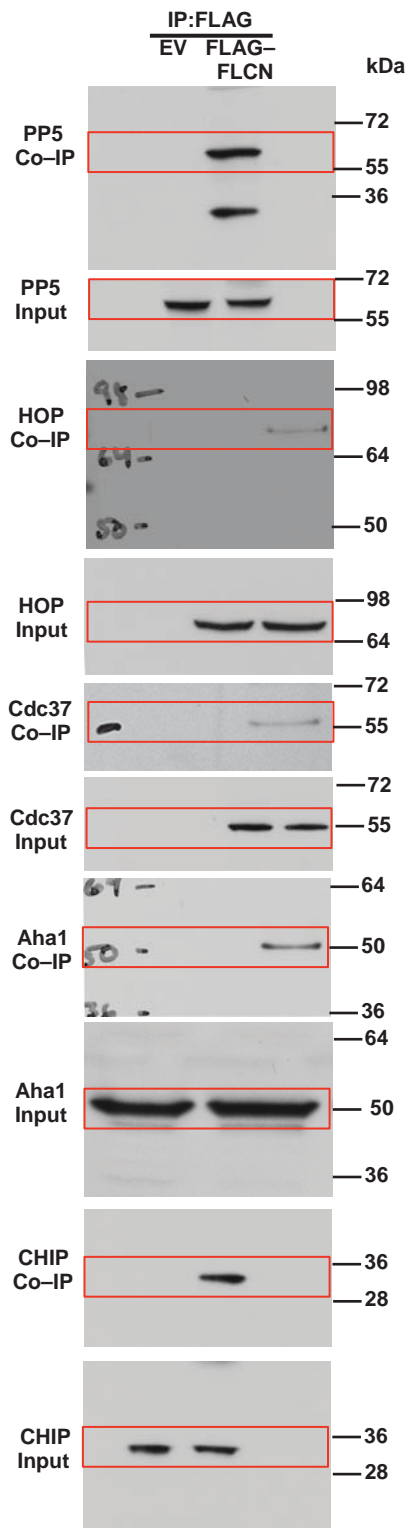


Figure 1d

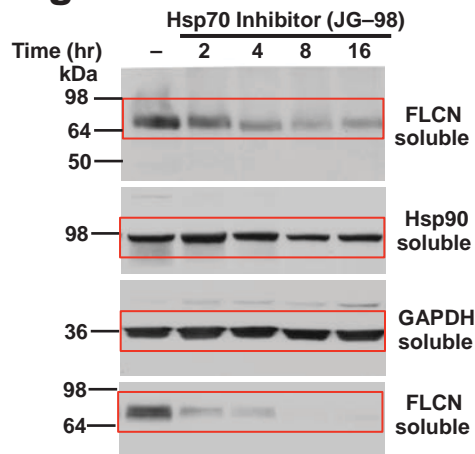


Figure 1e

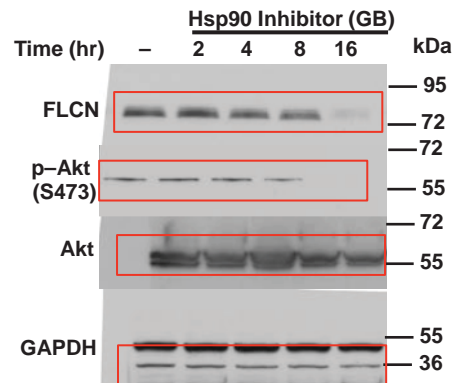


Figure 1f

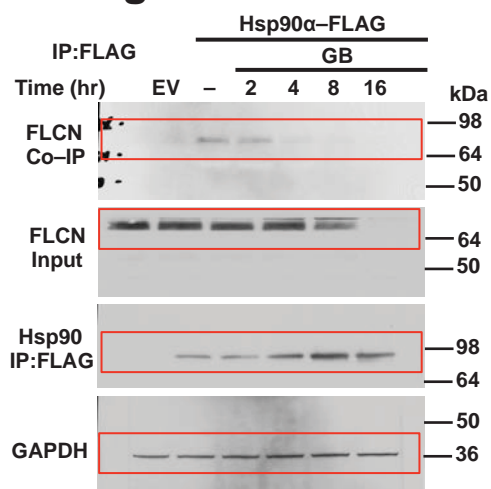


Figure 1g

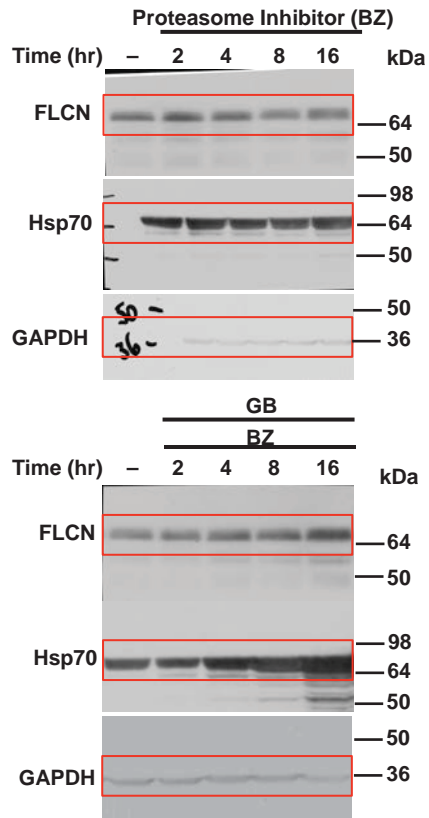


Figure 1h

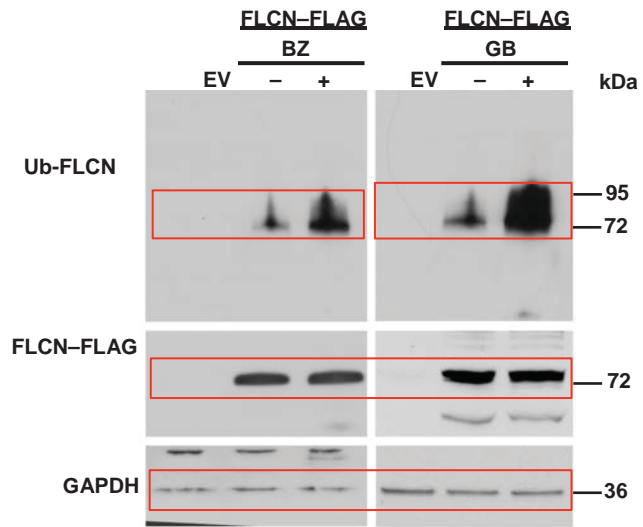


Figure 2a

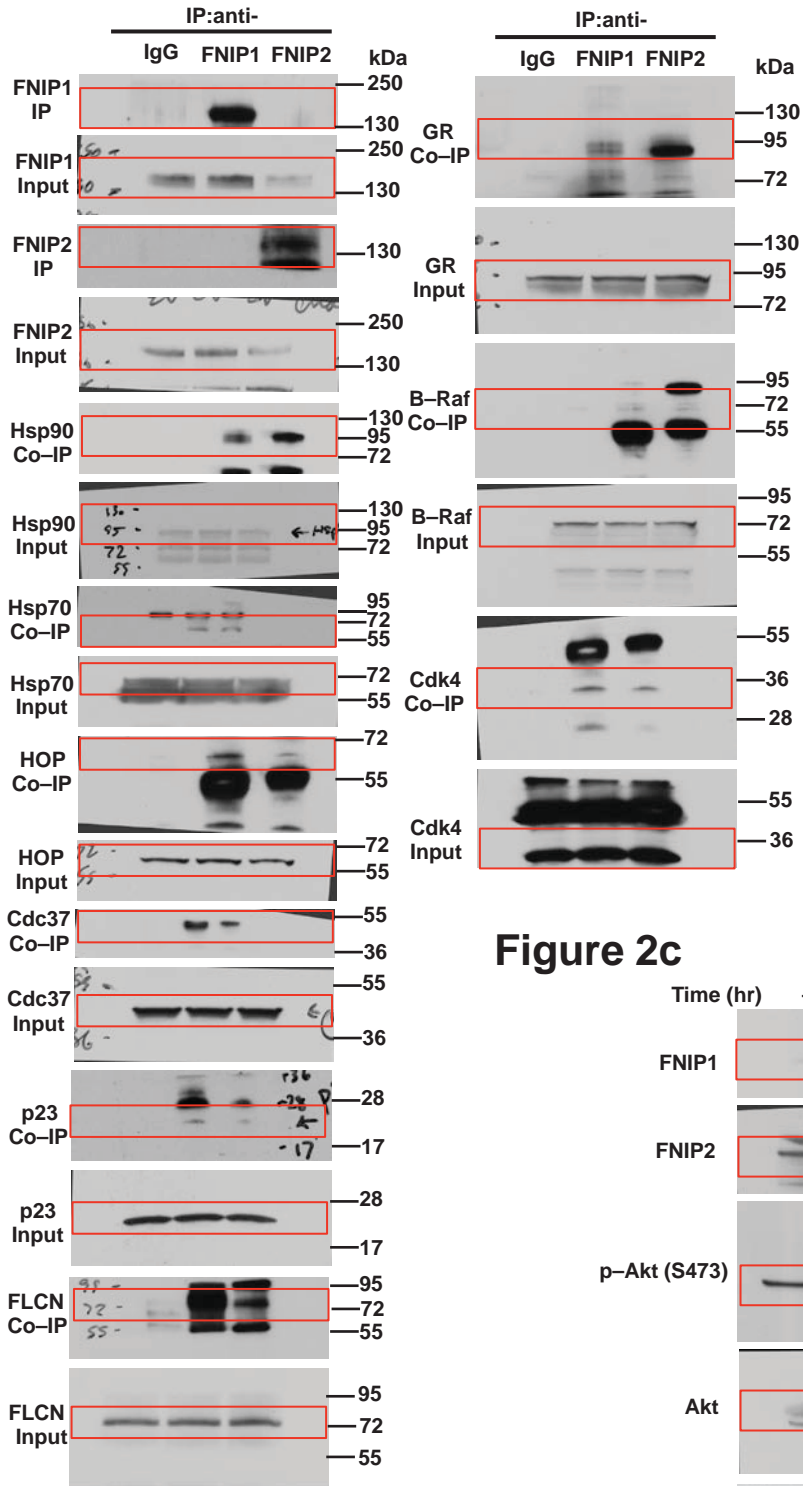


Figure 2b

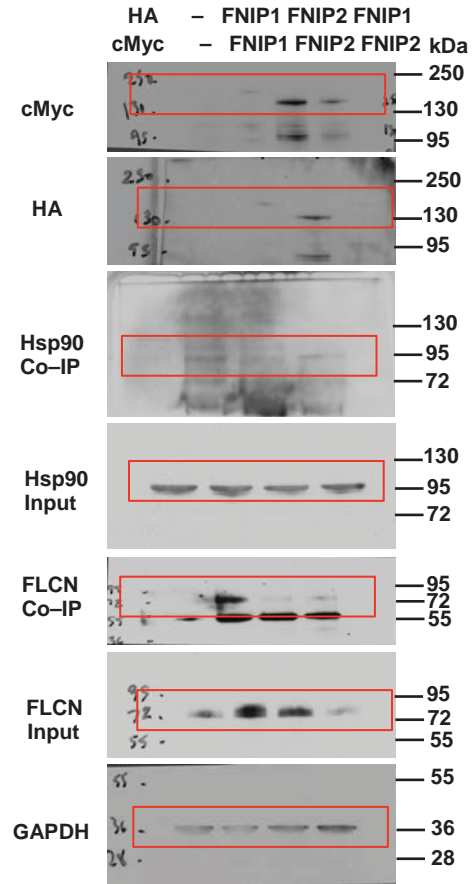


Figure 2c

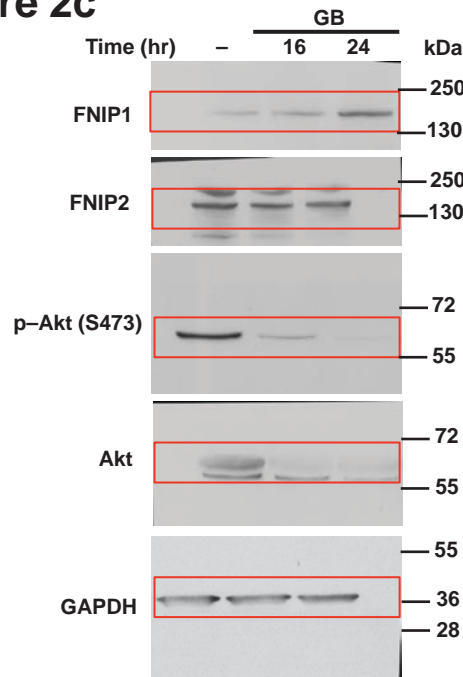


Figure 2d

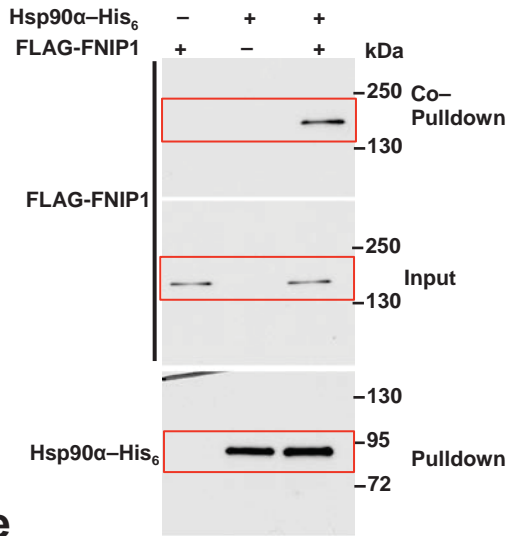


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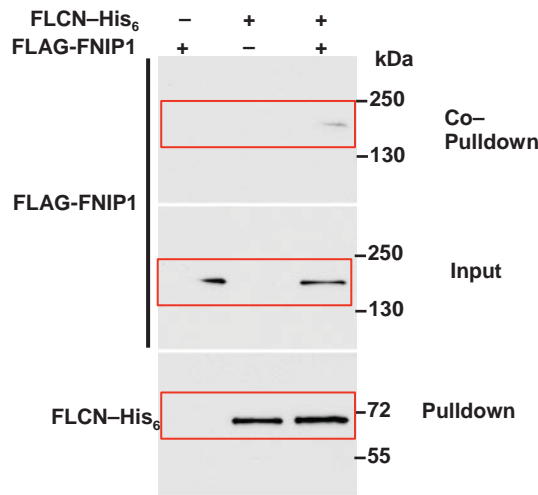


Figure 2f

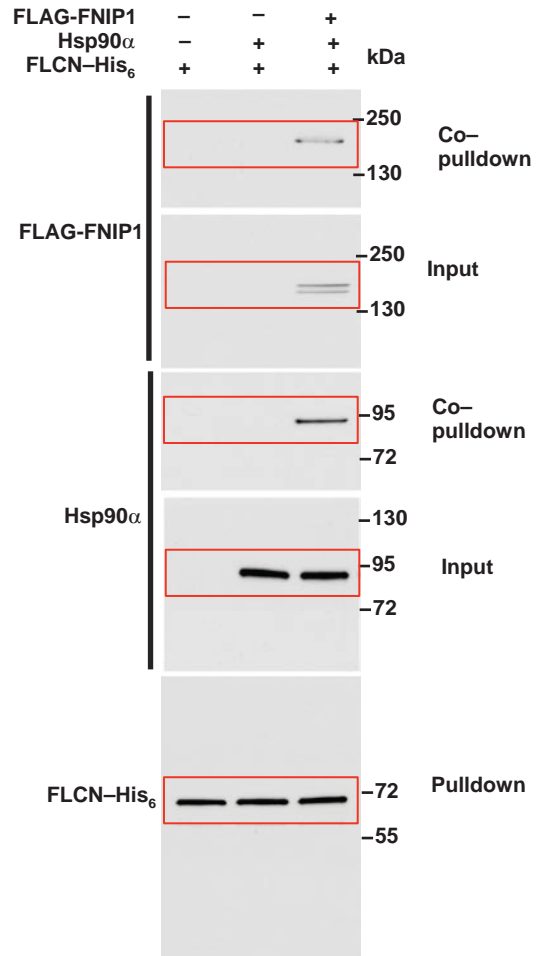


Figure 3a

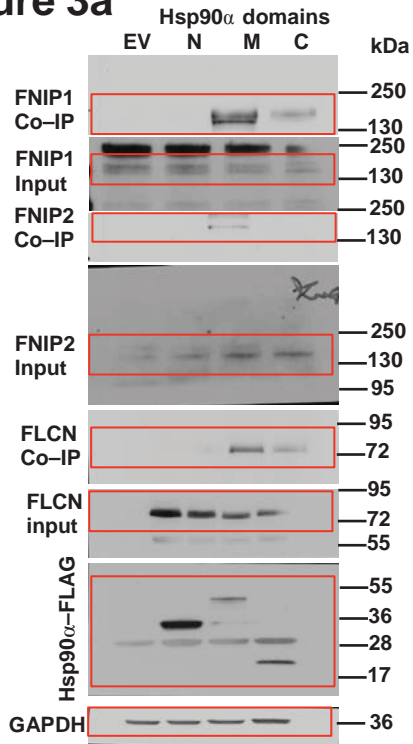


Figure 3b

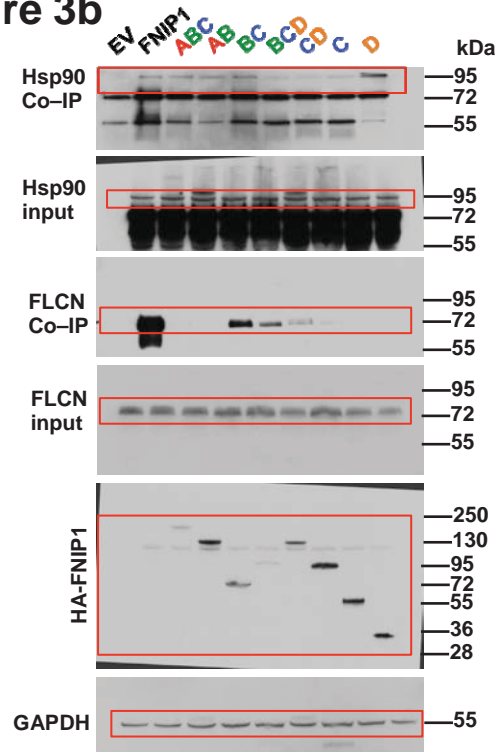


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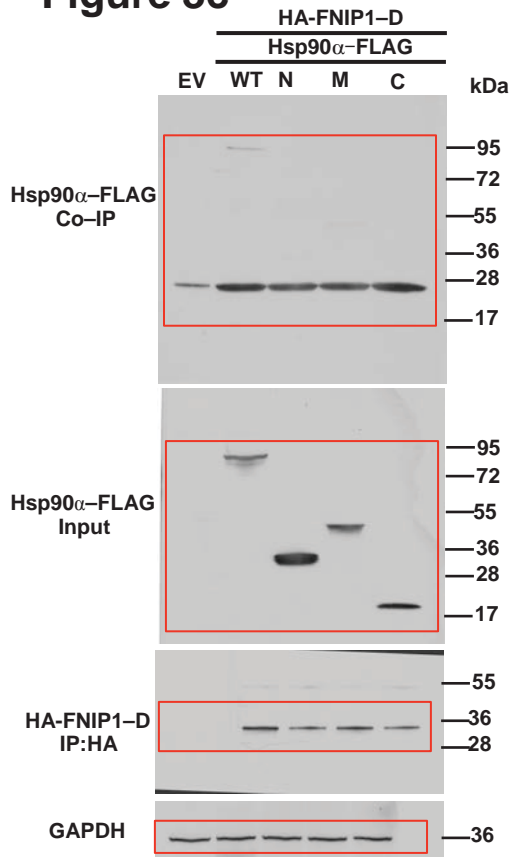


Figure 4a

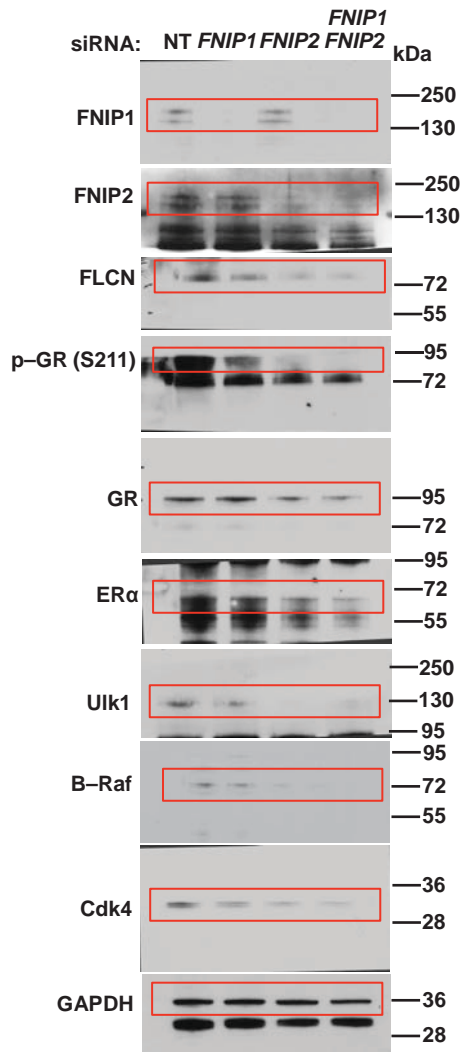


Figure 4b

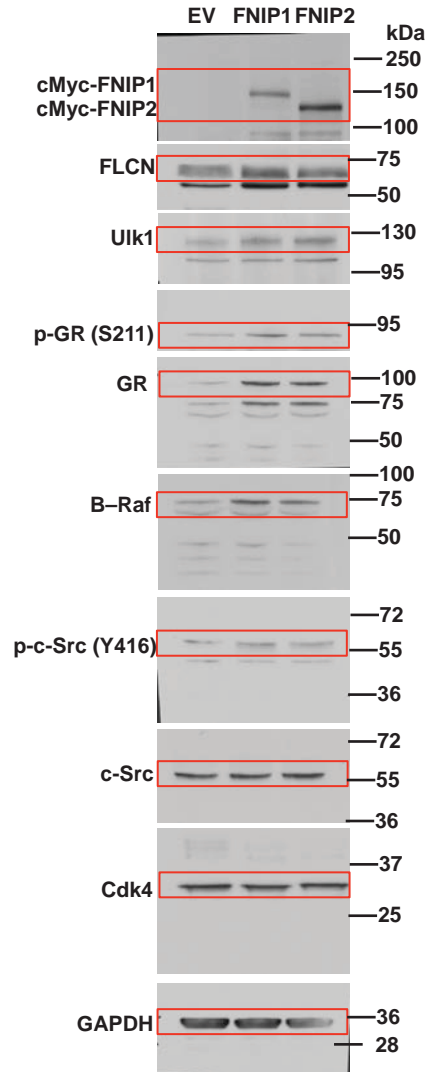


Figure 4c

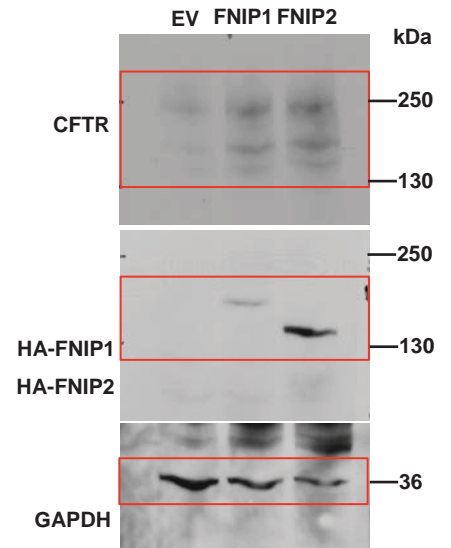


Figure 5a

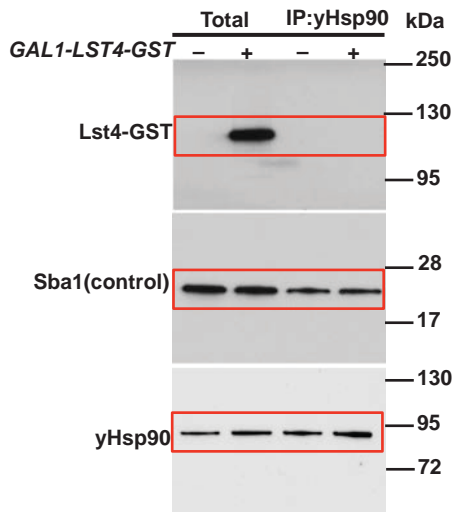


Figure 5d

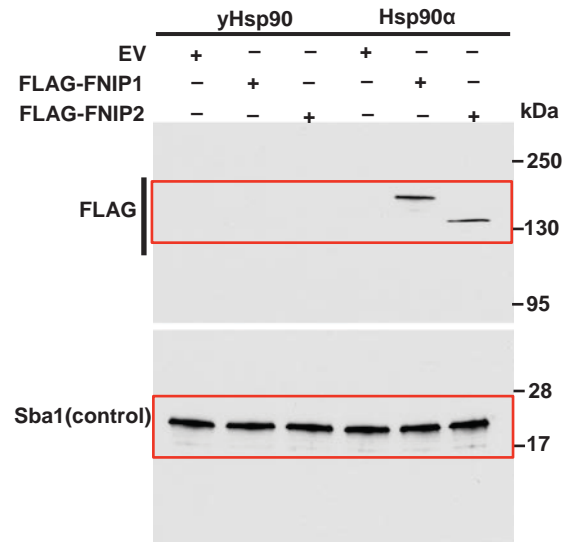


Figure 6a

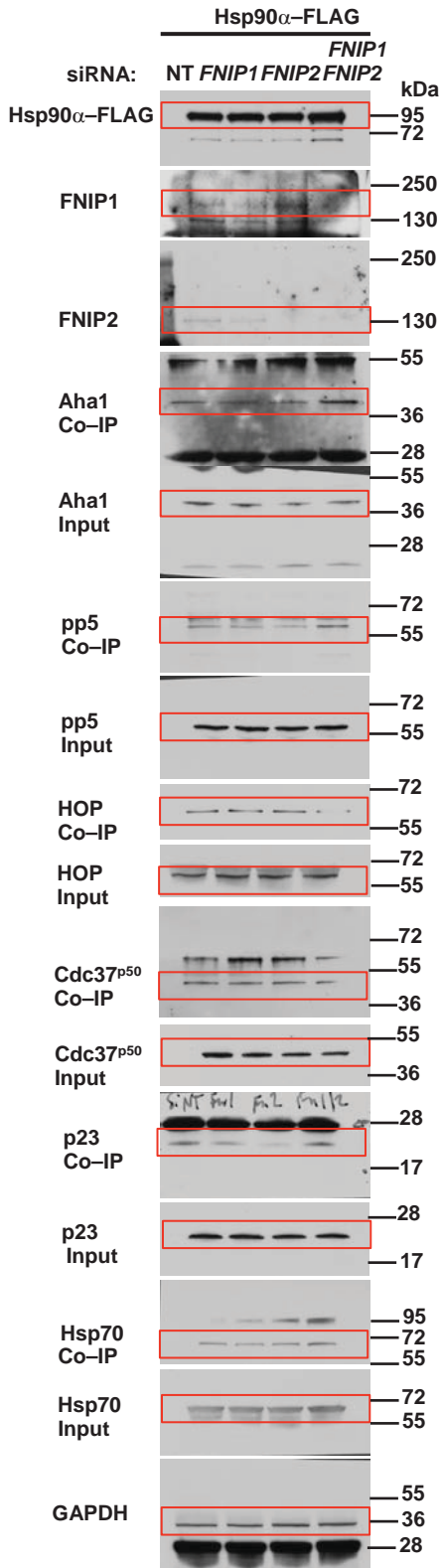


Figure 6b

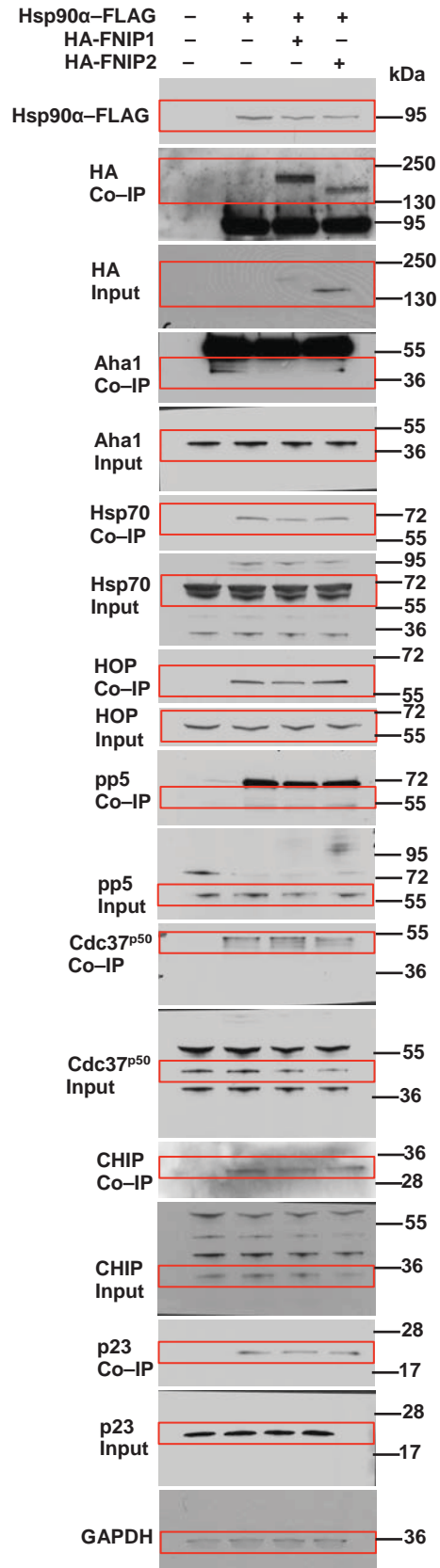


Figure 6d

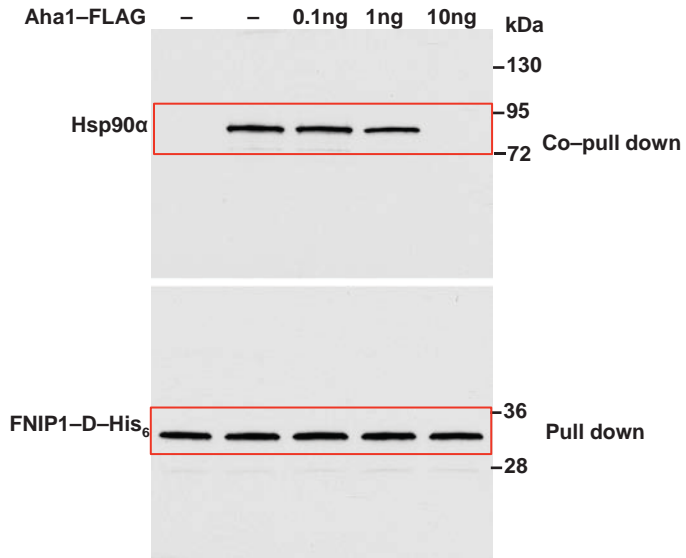


Figure 6e

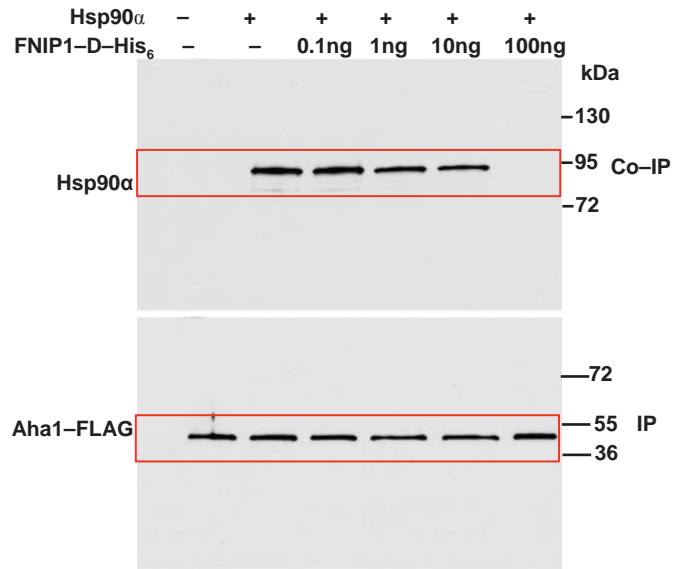


Figure 7b

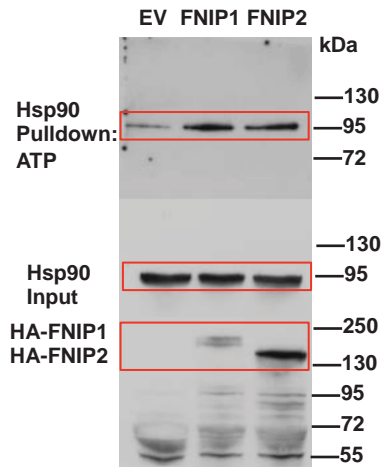


Figure 7d

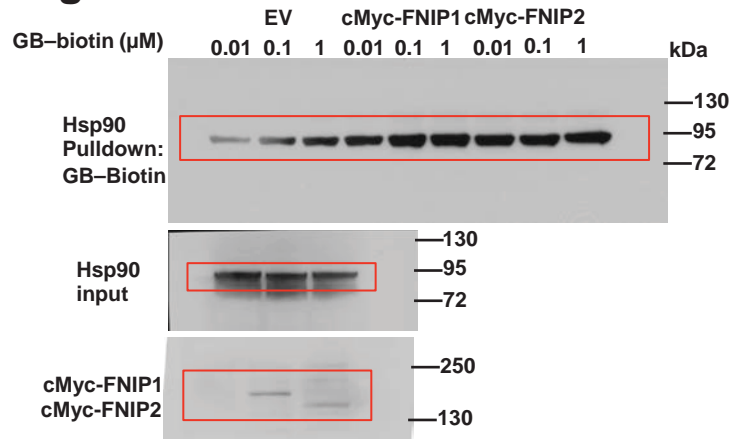


Figure 7f



Figure 7g

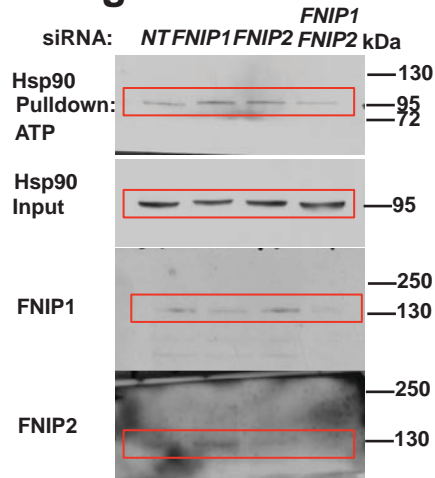


Figure 7h

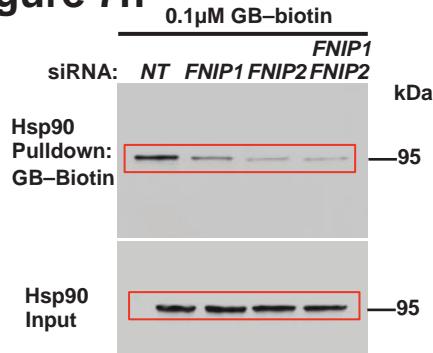


Figure 8a

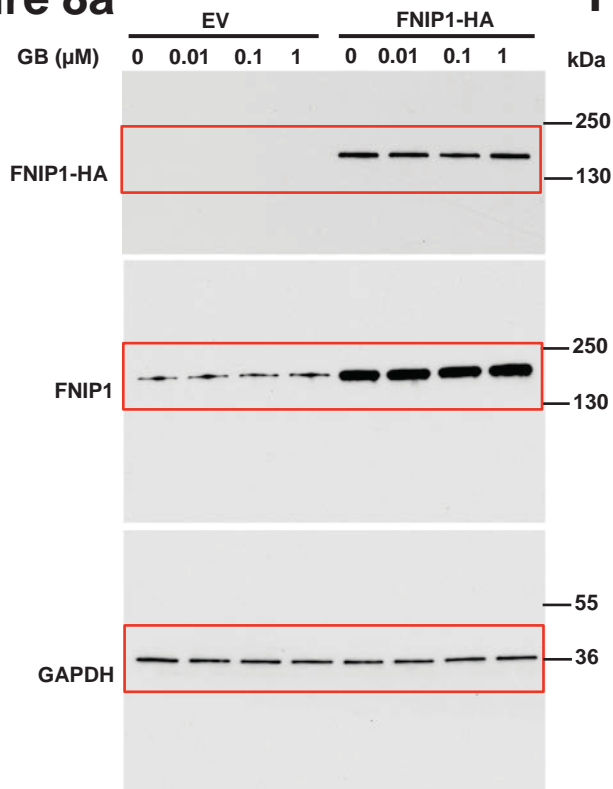


Figure 8b

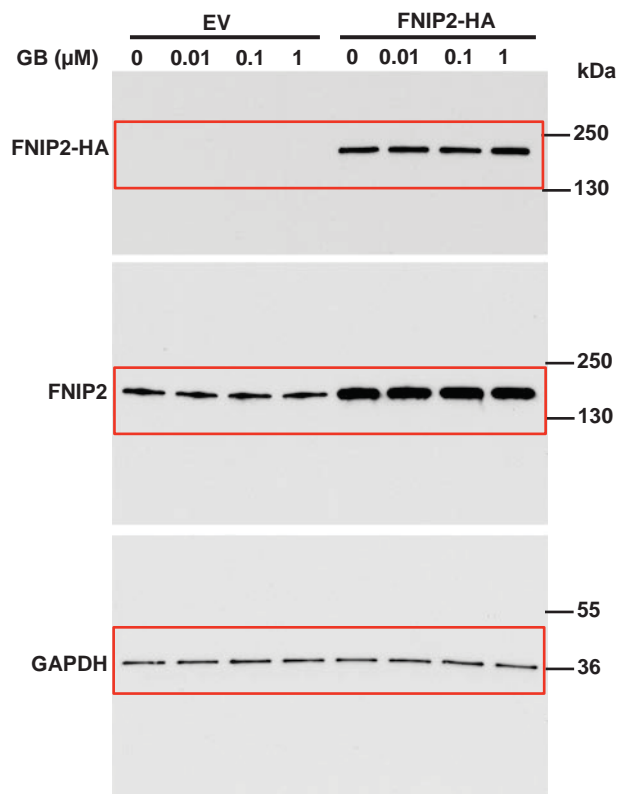


Figure 8c

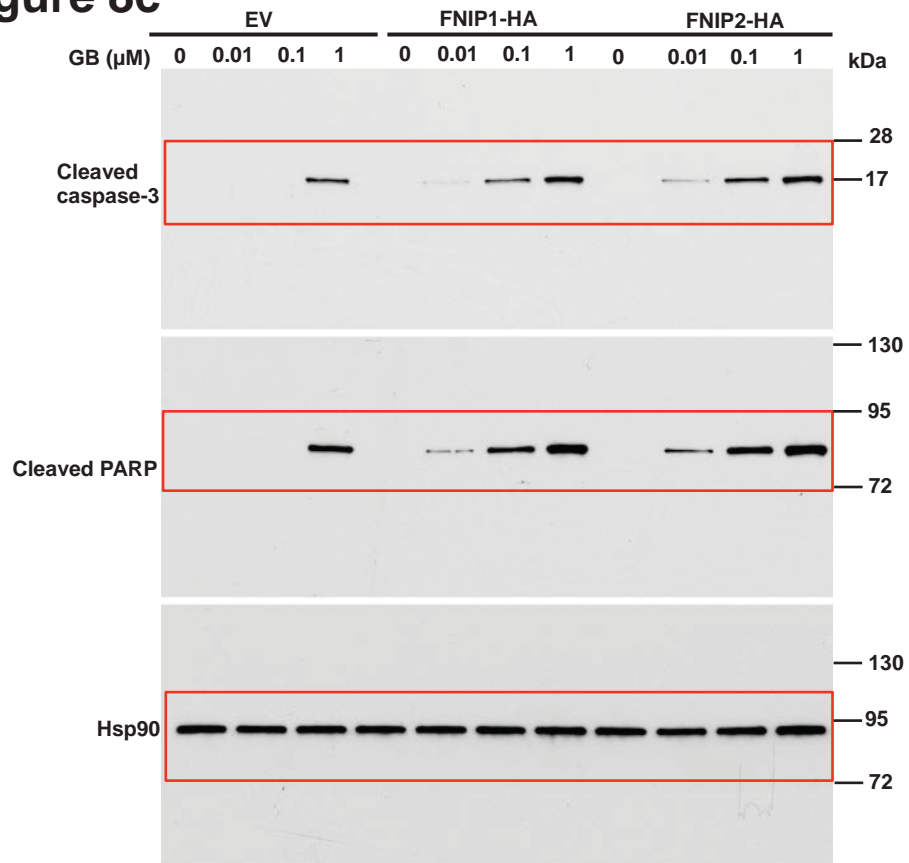


Figure 8d

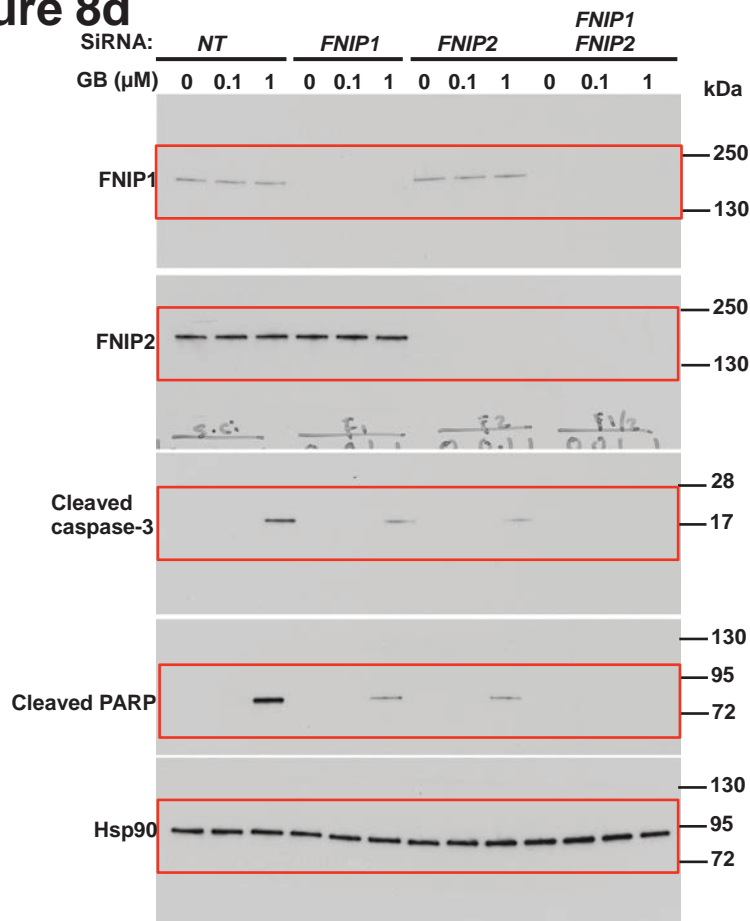


Figure 9a

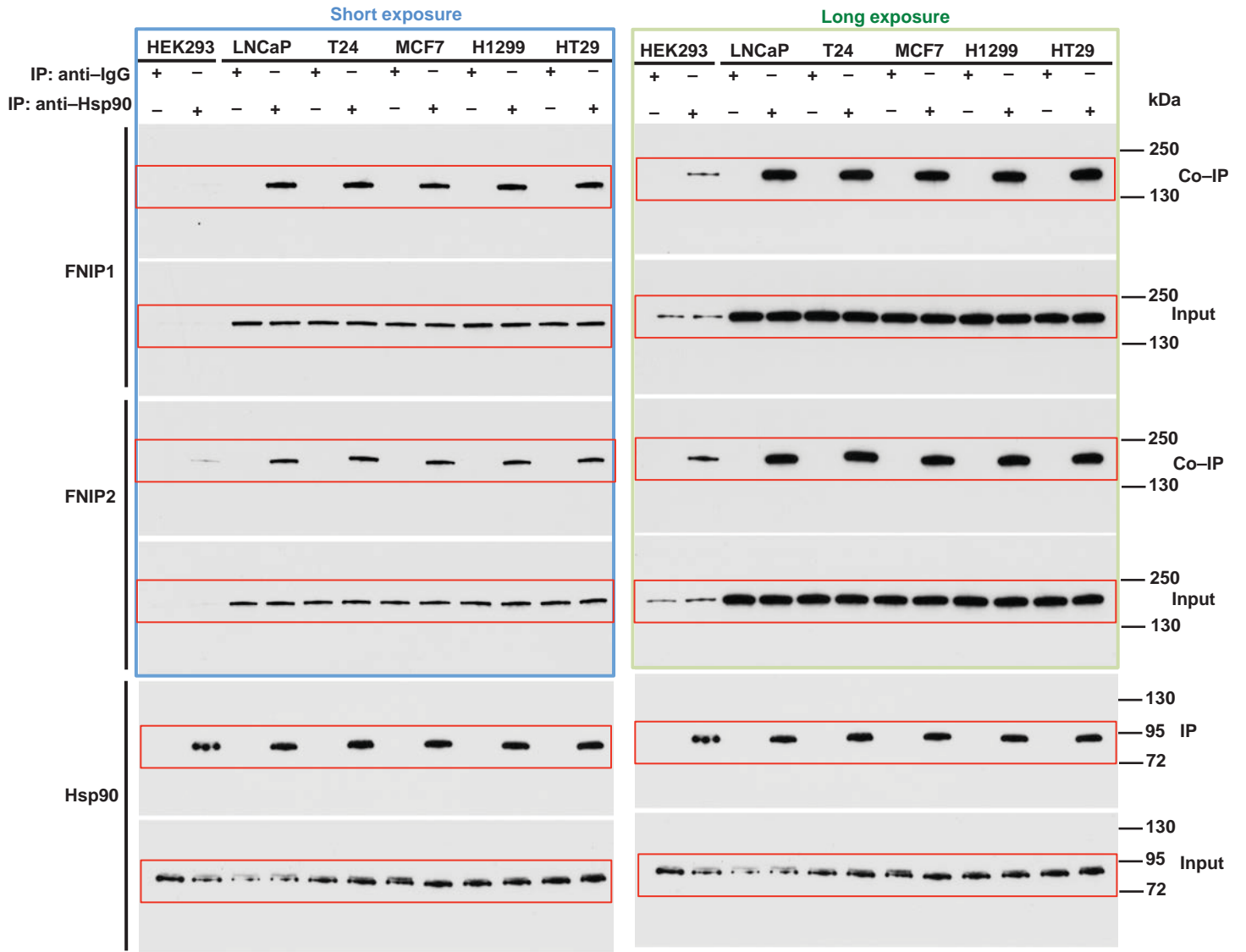


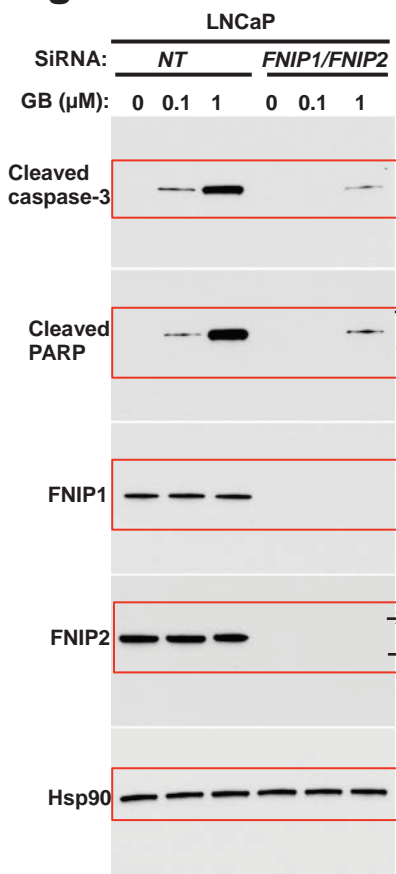
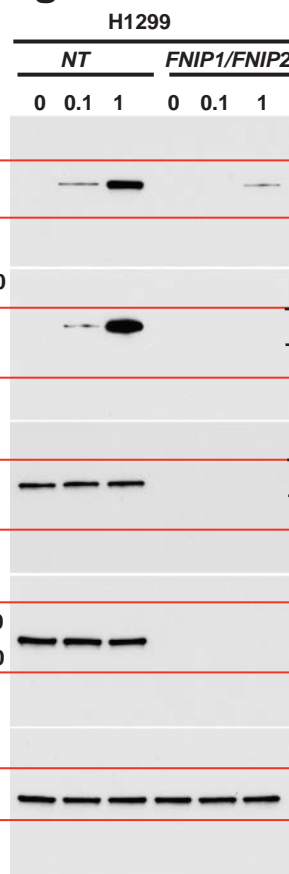
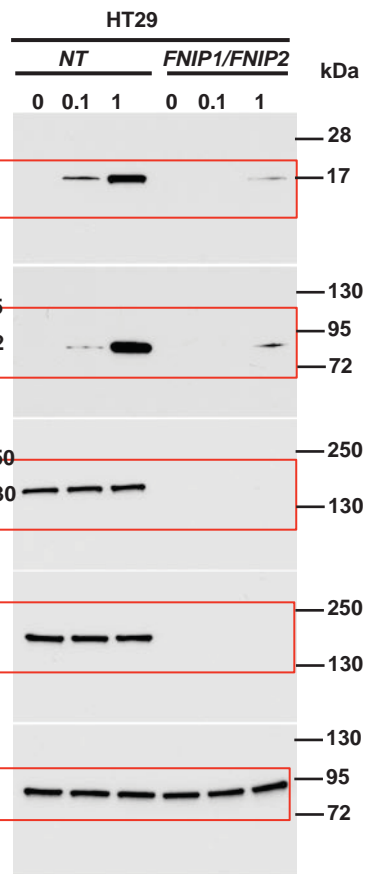
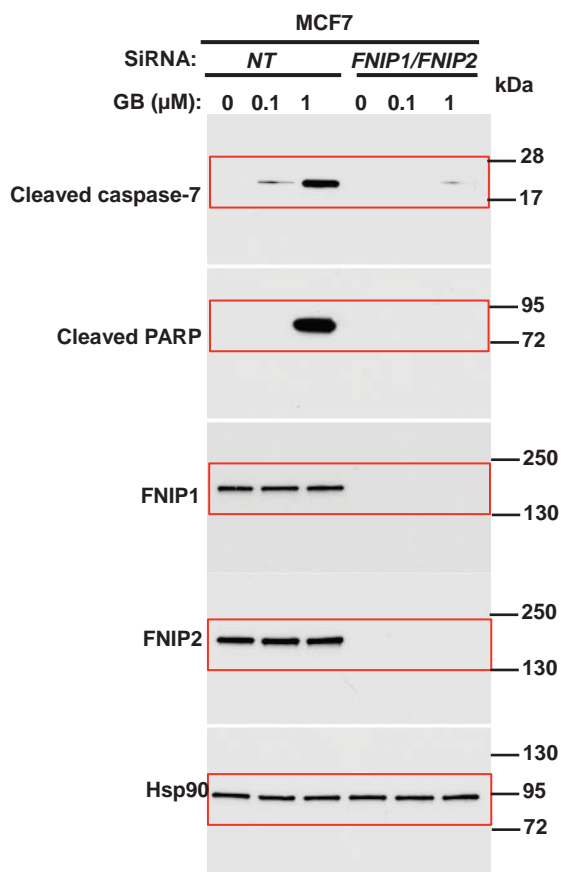
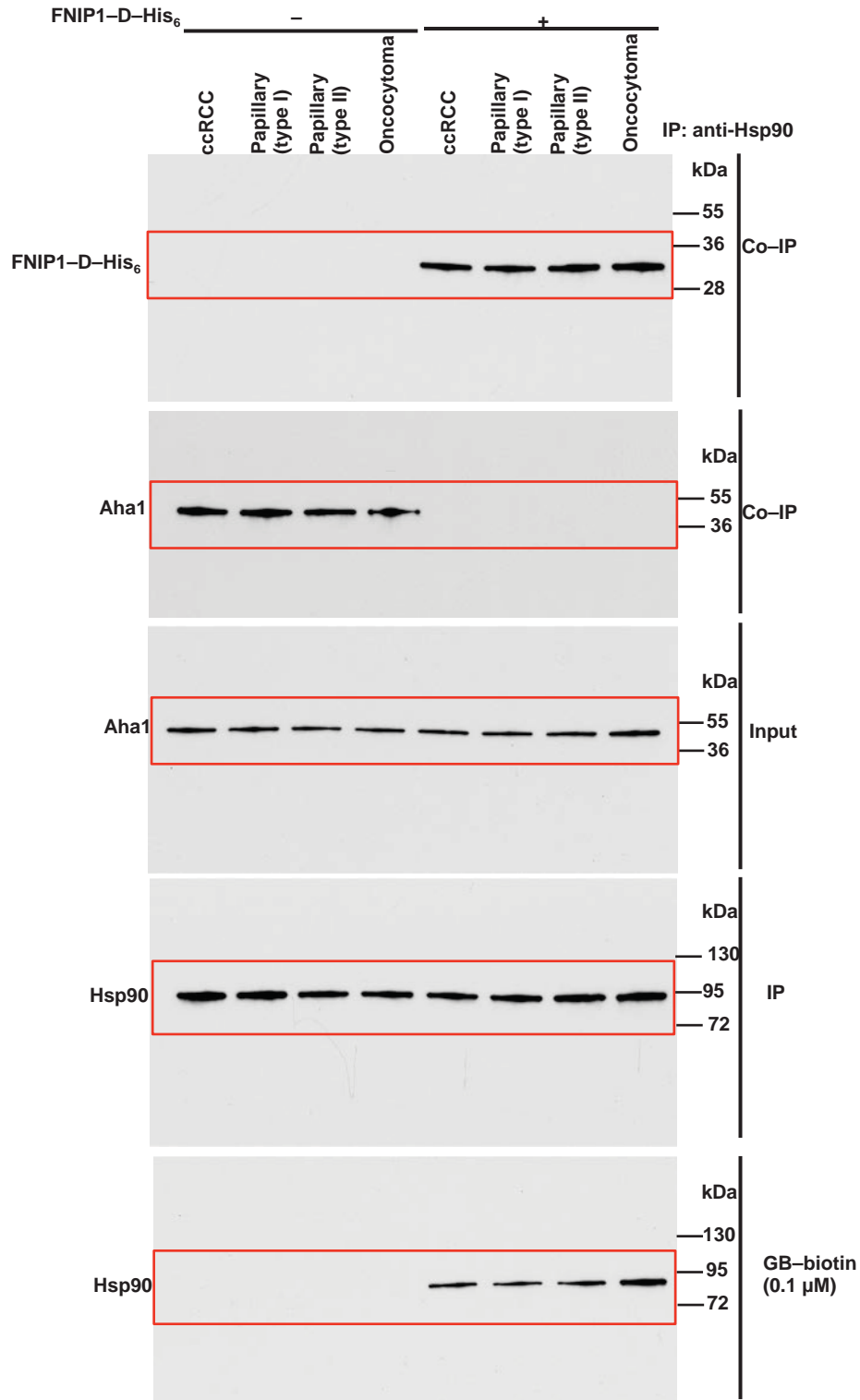
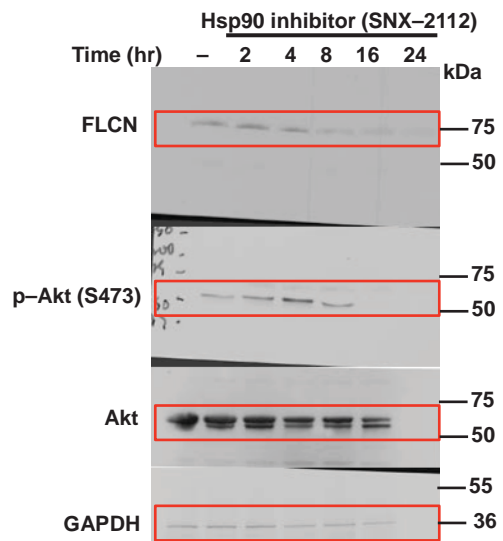
Figure 9b**Figure 9c****Figure 9d****Figure 9e****Figure 9f**

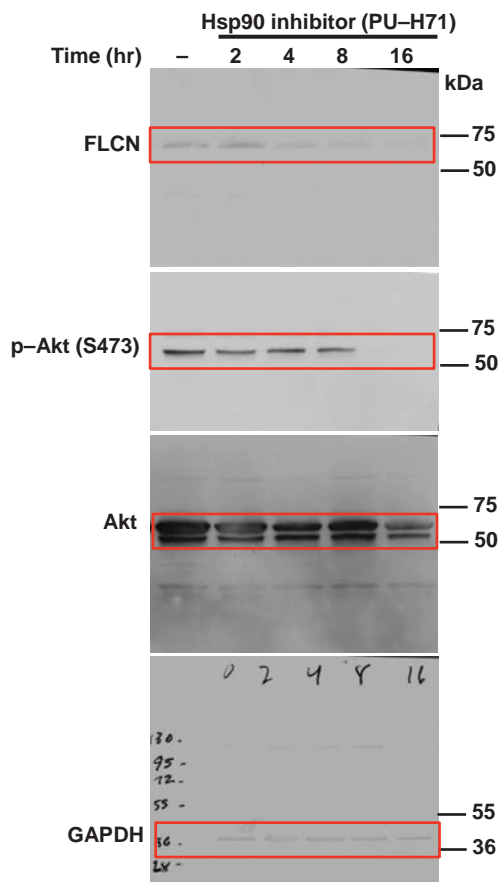
Figure 10f



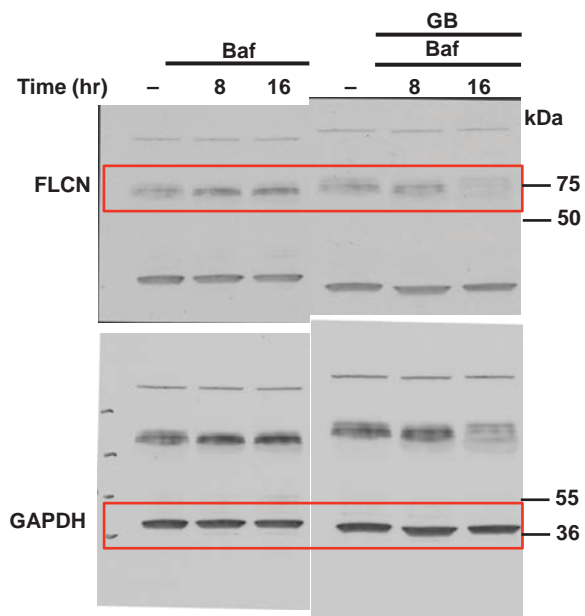
Supp. Figure 1a



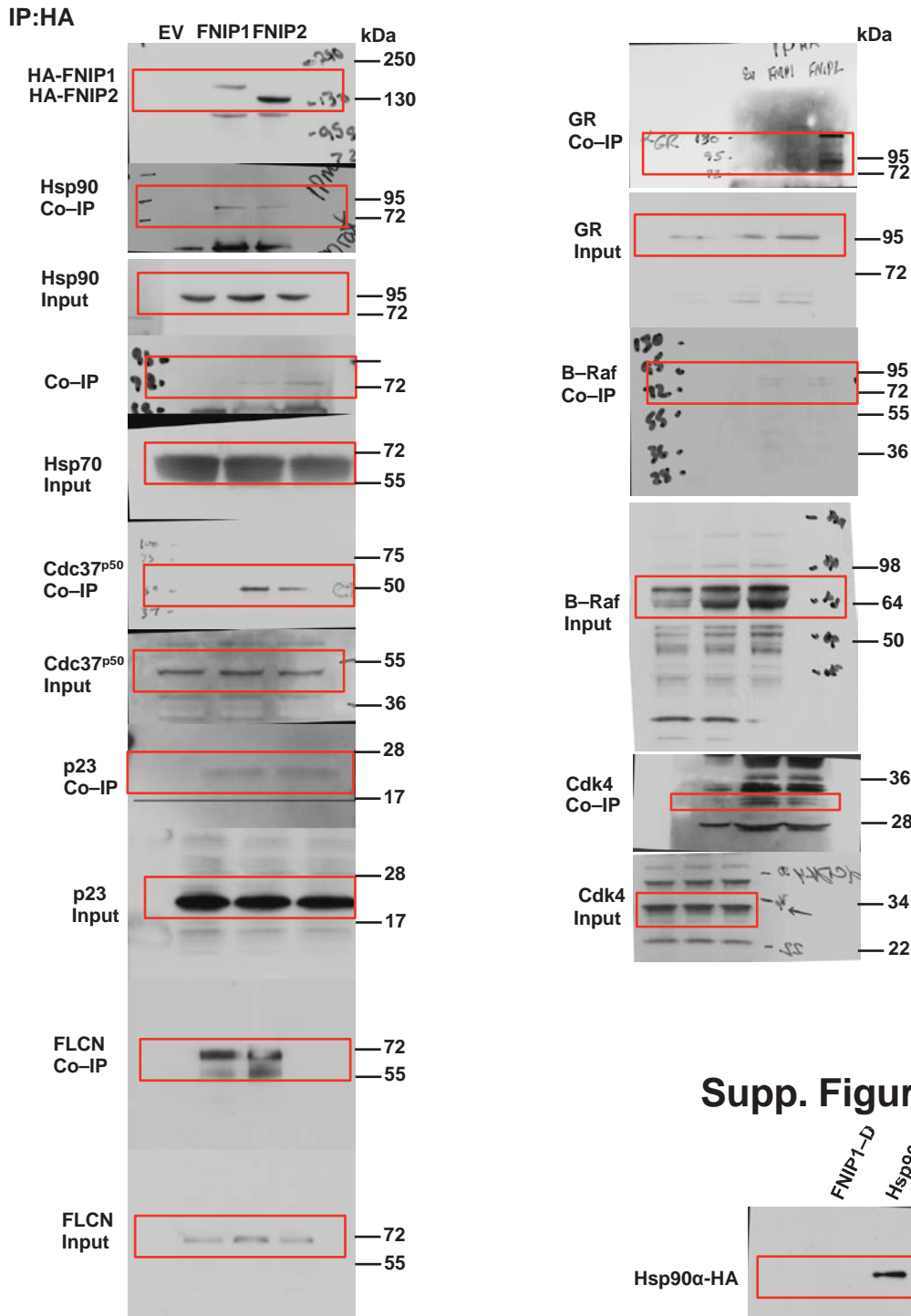
Supp. Figure 1b



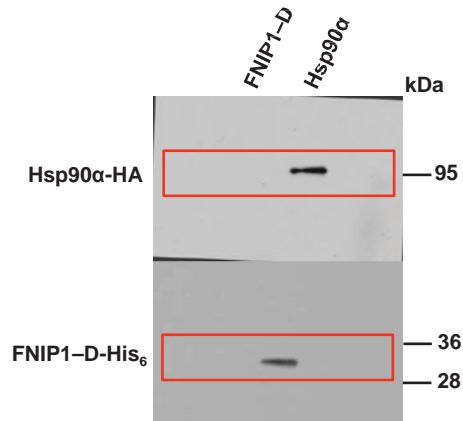
Supp. Figure 1a



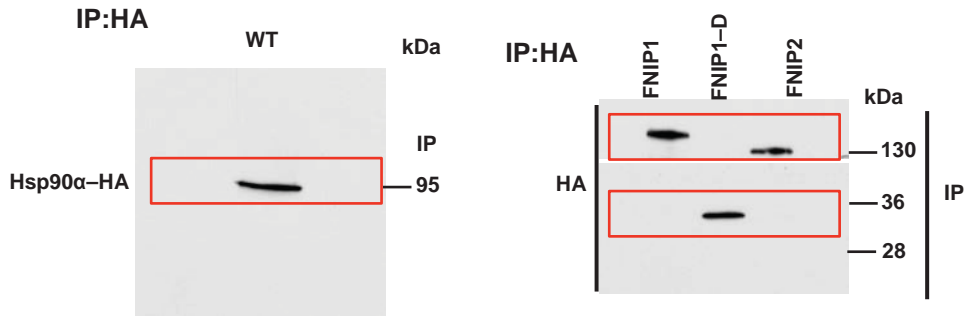
Supp. Figure 2a



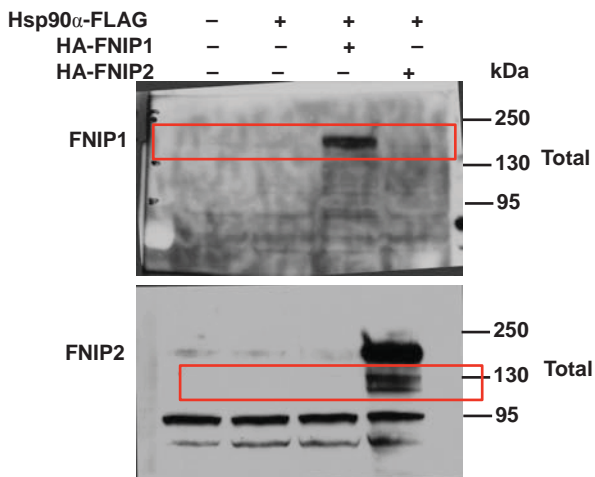
Supp. Figure 2c



Supp. Figure 4a



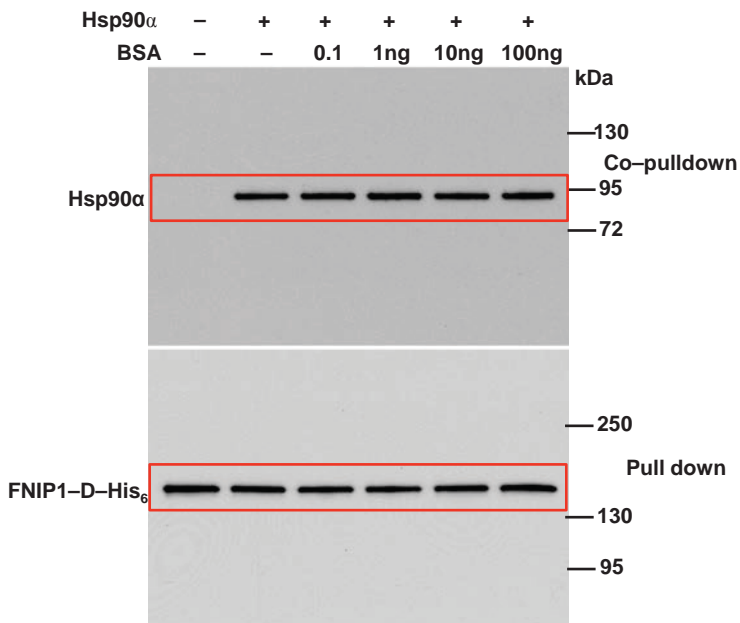
Supp. Figure 5a



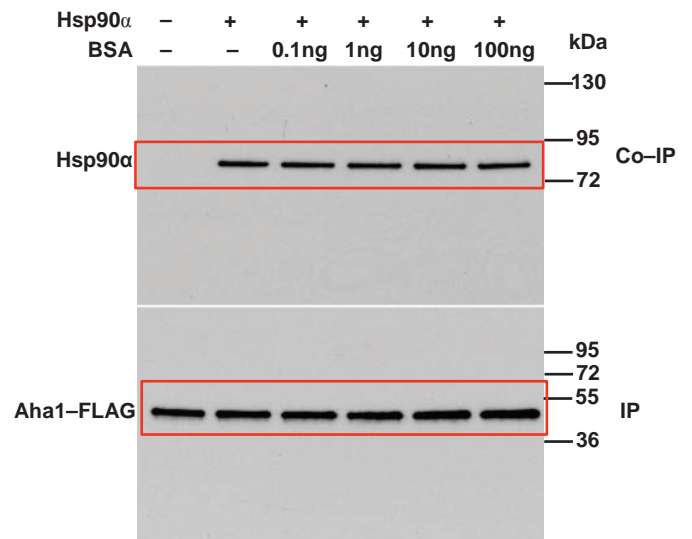
Supp. Figure 5f



Supp. Figure 5h



Supp. Figure 5i



Supplementary Table 1. Cellular proteins associated with FLCN.

Protein	MW	Protein Score	C.I.%	Total Ion Score	C.I.%	Peptides	Accession Number
Spliceosomal protein SAP 155	145723	158	100	124	100	HFVQHR VLPPPAGYVPIR MTPPIKDLLPR RATVNTFGYIAK LDDLVRPYVHK DTPGHGSGWAETPR IVDDLKDEAEQYRK AGELKVVNGAAASQPPSK IWDPTPSHTPAGAATPGR EWMRICFELLELLK TPDPKMNVRTYMDVMR DYIYAVTPLLEDALMDR LLVDVDESTLSPEEQKER NRPLSDEELDAMFPEGYK EDEYKHKHRTMISPER YNSIYIGSQDALIAHYPR AIGYLIPLMDAEYANYTR IYNDKNTYIRYELDYIL KLTATPTPLGGMTGFHMQTEDR KPGYHAPWALLNDIPQSTEQYDPPFAEHRPPK EIQGKKAALDEAQQVGLDSTGYDQEIYGGSDSR	gi 4033735
Splicing factor 3b, subunit 3, 130kDa	135432	N/A	N/A	24	88	FLAVGLVDNTR	gi 46362557
Heat shock protein HSP 90-alpha	98180	89	100	83	100	VILHLKEDQTEYLEER HSQFIGYPITLFEKER	gi 153792590
Heat shock 70 kDa protein 1A/1B	70009	222	100	173	100	DEFEHK LLQDFNFR DAGVIAGLNVLR LVNHFVEEFKR LLQDFNFRDLNK ATAGDTHLGGEDFDNR IINEPTAAAIAYGLDR NQVALNPONTVFDADR LDKAIHDLVLVGGSTR RLIGRKFQDPVQSDMK GRLSKEIERMVQEAEK STGKANKITITNDKGRLSK QTQIFTTYSDNQPGVLIQVYEGER TLSSSTQASLEIDSLFEGIDFYTSITR MKEIAEAYLGPVTVNAVITVPAYFNDSQR VEIANDQGNRTTPSYVAFTDTERLIGDAAK	gi 167466173
Chaperonin containing TCP1, subunit 8 (theta)	59440	171	100	156	100	GYEIACRK FAEAFEAI PR HFSGLEEA VYR LFTVNDAAATLR HEKEDGAISTIVLR MALHVPKAPGFAQMLK VDQIIMAKPAGGPKPPSGKK YNIMLVRLNSKWDLRR VADMALHYANKYNIMLVR TAEELMNFSGEENLMDAQVK ELAQTTRTAYGPNGMNKMVINHLEK RLVPGGGATEIELAKQITSYGETCPGLEQYAIKK	gi 119630329

T-complex protein 1 subunit beta	57424	94	100	27	92	VPDHHPC LIEEVMIGEDK GATQQILDEAER LLTHHKDHFTK QDLMNIAGTTLSSK TPGKEAVAMESYAKALK RQVLLSAAEAAEVILR EALLSSAVDHGSDEVKFR IHPQTIAGWREATKAAR LALVTGGGIASTFDHPPELVK VDSTAKVAEIEHAEKEKMKKEK EGTIGDMAILGITESFQVQR VQDDEVGDGTTSTVLAELLR ALKMLPTIADNAGYDSADLVAQLR RIENAKILIANGMDDTKIKIFGSR	gij197692147
T-complex protein 1 subunit eta	59329	N/A	N/A	60	100	QVKPYVEEGLHPQIIIR	gij5453607
T-complex protein 1 subunit delta	57888	N/A	N/A	47	100	ALIAGGGAPEIELALR	gij38455427
Activator of 90 kDa heat shock protein ATPase homolog	38250	192	100	131	100	WGEGDPR WIVEER TQARPVGVK GIPAPEEERTR LDGEASINNRK NGETELCMEGR LIFFYEWSVK YYFEGIKQTFGYGAR VFTTQELVQAFTHAPATLEADRGGK ADATNVNNWHWTERDASNWSTDKCLK	gij6912280
E3 ubiquitin-protein ligase CHIP	34834	119	100	107	100	LIAAERER VGHFDPVTR NPLVAVYYTNR LNFDDIPSALR RIHQESELHSYLSR ISFELMREPCITPSGITYDR	gij56181387
Ribosomal protein L10	25067	68	96	35	99	GAFGKPKQGTVAR VRLHPFHVIR ICANKYMKSCGK INKMLSCAGADRLQGTGMR SRFCRGVPDAKIRIFDLGR LQTGMRGAFGKPKQGTVARVHIGQVIMSIRTK	gij119593144

Supplementary Table 2. siRNA sequences.

Name	Sequences
FNIP1-1	CCCAGGAAUUGUGCGGAAA
FNIP1-2	AAGCAUAACUUGUCUCCAA
FNIP1-3	GCAAAGACUCAUCCAUAUA
FNIP1-4	GCACAAAUACUCCUUUAAU
FNIP2-1	GGACAGUGGCAUUGCUCGA
FNIP2-2	AUAAAGAGGCACCGCAAGA
FNIP2-3	GAAAGAAUUAGGUGUCGUA
FNIP2-4	CCUCGUGGCUGGUGCGAAU
NON-TARGETING POOL-1	UGGUUUACAUGUCGACUAA
NON-TARGETING POOL-2	UGGUUUACAUGUUGUGUGA
NON-TARGETING POOL-3	UGGUUUACAUGUUUUCUGA
NON-TARGETING POOL-4	UGGUUUACAUGUUUCCUA

Supplementary Table 3. Primer sequences. Restriction sites are highlighted in green. Epitope sequences are highlighted in blue.

Primer	Sequence
FLAG-Hsp90-N-BamHI-F	TATGCGGGATCCATGGATTACAAGGATGACGACGATAAGGGACCTGAGGAAACCCAGACCCAAGAC
FLAG-Hsp90-N-XhoI-R	CTCGGTCTCGAGTTACACAAAAAGAGTAATGGATATCCAATAAACTGAGA
FLAG-Hsp90-M-BamHI-F	TATGCGGGATCCATGGATTACAAGGATGACGACGATAAGGGAGATCAAGAAGAGCTCACAAAACA
FLAG-Hsp90-M-XhoI-R	CTCGGTCTCGAGTTATCTTAGGGCTTGAGCTTTCATGATTCTCTCCATGTTT
FLAG-Hsp90-C-BamHI-F	TATGCGGGATCCATGGATTACAAGGATGACGACGATAAGGGAGACAACCTCAACAATGGTTACATG
FLAG-Hsp90-C-XhoI-R	CTCGGTCTCGAGTTAGTCTACTTCTTCCATGCGTGATGTGTCGTCATCTCC
FLCN-Flag-Spe1	CTCGTACACTAGTATGGACTACAAGGACGACGATGACAAGAATGCCATCGTGGCTCTCTGCC
FLCN-R-Xho1	CTAAGGACTCGAGTCAGTTCGAGACTCCGAGGCTGTG
Fnip1-NheI-F	TATGCGCTAGCGCCCCTACGCTGTTCCAGAAGCTCT
Fnip1-XhoI-R	CTCGGTCTCGAGTTAAAGGAGTATTTGTGCAACATATGGAG
Fnip2-NheI-F	TATGCGCTAGCGCCCCGACCCTGCTCCAGAAGCTCTTC
Fnip2-XhoI-R	CTCGGTCTCGAGTTATAAGAGTATTTGAGCCACATAAGGA
Fnip1-NotI-F	TATGCGCGGCCGCGCCCCTACGCTGTTCCAGAAGCTCT
Fnip2-NotI-F	TATGCGCGGCCGCGCCCCGACCCTGCTCCAGAAGCTCTTC
Fnip1-KpnI-FLAG-F	TATGCGGTACCATGGATTACAAGGATGACGATGACAAGGGATCCGCCCTACGCTGTTCCAGAAGCTCT
Fnip2-KpnI-FLAG-F	TATGCGGTACCATGGATTACAAGGATGACGATGACAAGGGATCCGCCCTACGCTGTTCCAGAAGCTCT
Fnip1-SpeI-cMyc-F	TATGCACTAGTATGGAACAAAAGTTAATCTCCGAAGAAGATTTAGCCCCTACGCTGTTCAGAAGCTCTT
Fnip2-SpeI-cMyc-F	TATGCACTAGTATGGAACAAAAGTTAATCTCCGAAGAAGATTTAGCCCCTACGCTGCTCCAGAAGCTCTTCAA