Supplemental Information

Extended Methods

Immunoprecipitation

We conducted immunoprecipitation using previously published protocols (2014 Hepatology). In brief, SK-Hep1-shEV and SK-Hep1-shCLIC1 cells were cultured in DMEM with 10% (volume/volume; v/v) FBS and incubated until 80% confluence before being subjected to experiment. The cells were washed with chilled DPBS and lysed with lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitors (Roche, Germany) for 15 minutes. Cell debris was removed by centrifugation at 12 k rpm at 4ºC for 15 minutes. An equal amount of proteins (1 mg) were subjected to immunoprecipitation with corresponding antibodies and Protein G Mag Sepharose Xtra (Blossom Biotenchnologies, Inc.) according to the manufacturer's instructions. The immunoprecipitated complex was rocked at 4℃ for 1 h in an orbital shaker and washed with NETN buffer (20 mM Tris (pH8), 1 mM EDTA, 900 mM NaCl, 0.5% NP-40) five times. The mixture was washed once with NETN buffer containing 100 mM NaCl and removed the liquid portion by aspiration. The beads were added 1x SDS sample buffer and boiled for 5 minutes, and the samples were analyzed by western blot. Non-immune IgG or protein G-sepharose beads alone were used as negative controls. For co-immunoprecipitation assay of CLIC1 and PIP5K, 293T cells were cultured in a 10-cm dish and transfected with pCLIC1-Flag and pPIP5K1A-Myc or empty vector as control. A reciprocal immunoprecipitation assay was performed by transfection with pCLIC1-RFP and pPIP5K1A-Flag. The cell debris was lysed and the immunoprecipitated material was prepared as described above. The Flag-tagged or Myc-tagged protein complexes were immunoprecipitated using anti-Flag M2 beads (Sigma) or anti-Myc antibody (Cell signaling), respectively.

Reseeding assay-nascent adhesion protein

SK-hep1 cells were trypsinized when cell confluence was 90%. After counting, cells were divided to the equal cell numbers and suspended in the medium for 3 hours at room temperature. Cells were reseeded on the ECM-coated cultured dishes for 30 mins. After the reseeding process, for the western blot, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 0.25% Na deoxycholate, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM NAF, 1 mM Na3VO4, 2 mg/mL aprotinin, and 2 mg/ml leupeptin). Cell debris was removed by centrifugation at 13,000 rpm for 15 minutes at 4ºC. Twenty micrograms of protein

were subjected to western blot with antibodies against the target proteins. β-actin or GAPDH as loading control were included for all immunoblots. Proteins were resolved by SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes (PVDF, Schleiche & Schuell, Einbeck, Germany), and then incubated with primary antibodies overnight. After washing with PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-goat, or anti-rabbit IgG antibodies (1: 2,000). Immunoblots was detected by using enhanced chemiluminescence (ECL) kit, (Amersham Pharmacia). The lists of primary antibodies were: anti-ITGA4 (1:1000, #8440, cell signaling Inc.), anti-ITGB4 (1:1000, #14803, cell signaling Inc.), anti-phospho-FAK (1:1000, #8506, cell signaling Inc.), anti-FAK (1:1000, #3285, cell signaling Inc.), anti-phospho -Src (1:1000, #2105, cell signaling Inc.), anti-Src (1:1000, #2109, cell signaling Inc.), anti-phsopho-paxillin (1:1000, #2541, cell signaling Inc.), anti-paxillin (1:1000, #12065, cell signaling Inc.), anti-phosphor-ERK1/2 (1:1000, #9101, cell signaling Inc.), anti-ERK1/2 (1:1000, #4695, cell signaling Inc.), anti-Talin1 (1:1000, 4021, cell signaling Inc.), anti-PIP5K1C (1:1000, #3296, cell signaling Inc.), anti-CLIC1 (1:1000, SAB140-3677, Sigma).

Subcellular fractionation

We follow the procedure of Subcellular protein fractionation kit for culture cells from ThermoScientific Co.(cat No. 78840) Briefly, cells were harvested with trypsin and centrifuge at 500x g for 5 minutes. After ice-cold PBS washing, transfer the optimum amounts of cells to the new tube and add ice-cold cytoplasmic extraction buffer (CEB) containing protease inhibitors to cell pellet. After being incubated at 4c for 10 minutes with gentle mixing, cells were centrifuged at 500 x g for 5 minutes and transfer the supernatant (cytoplasmic extract) to the new tube on ice. Then add ice-cold membrane extraction buffer (MEB) to cell pellets and vortex the tube for 5 seconds. Cells were incubated at 4c for 10 minutes with gentle mixing and centrifuge at 3000 x g for 5 min. Transfer the supernatant (membrane extract) to the new tube.

Antibodies used in this study

Table for antibodies list

Antibody	Dilution	Company	Cat No.
ITGA4	1:1000	cell signaling	#8440
ITGB4	1:1000	cell signaling	#14803
p-FAK	1:1000	cell signaling	#8506

FAK	1:1000	cell signaling	#3285
p-paxillin	1:1000	cell signaling	#2541
paxillin	1:1000	cell signaling	#12065
p-Src	1:1000	cell signaling	#2105
Src	1:1000	cell signaling	#2109
Talin1	1:1000	cell signaling	#4021
p-ERK1/2	1:1000	cell signaling	#9101
ERK1/2	1:1000	cell signaling	#4695
PIP5K1C	1:1000	cell signaling	#3296
PIP5K1A	1:1000	Proteintech	15713-1-AP
CLIC1	1:2000	Sigma	SAB1403677
Na/K ATPase	1:1000	Abcam	ab7671
GAPDH	1:5000	cell signaling	#5174
ITGα6	1:50	Abcam	ab75737
ITGβ1	1:1000	Cell signaling	#4706

Supplemental Figures

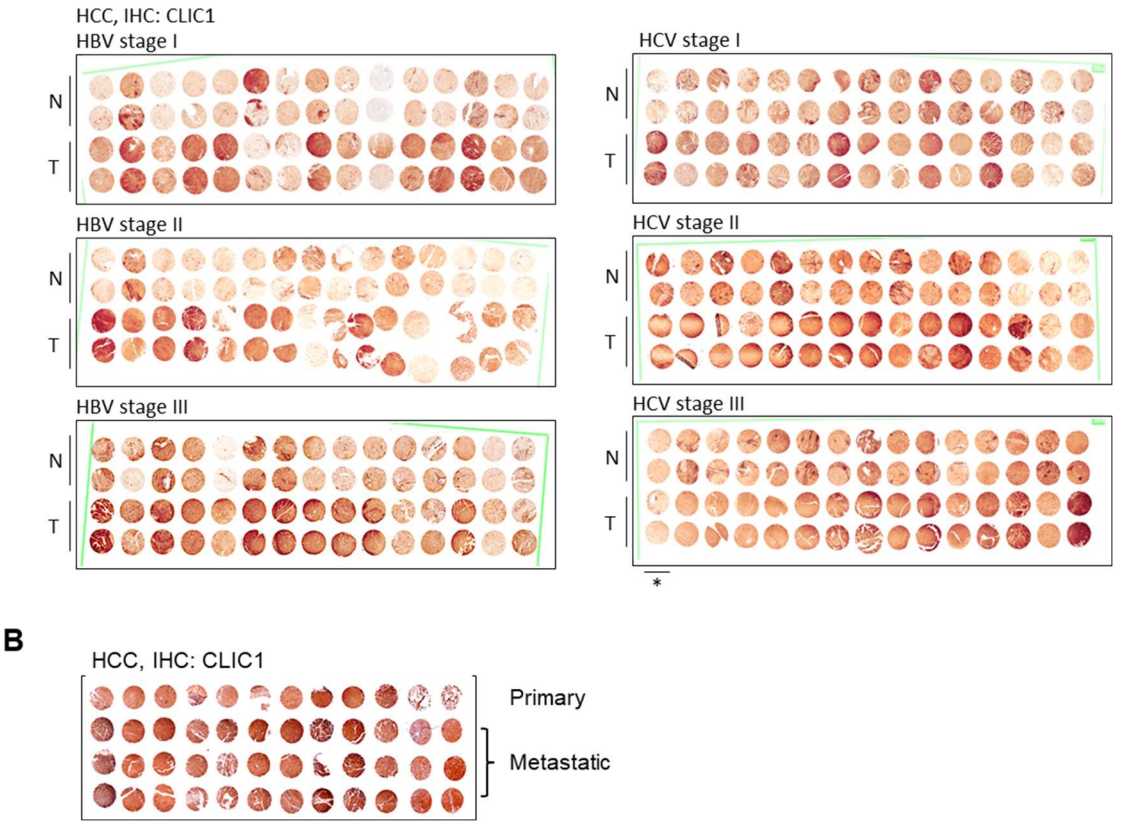


Figure 1. IHC on tissue microarrays. (**A**) Immunohistochemistry for CLIC1 expression on tissue microarrays (TMA) containing 89 paired tumor (T) and para-tumor (N) liver tissues in duplicate from HCC patients of chronic hepatitis B (45 cases) and C (44 cases) in tumor stage I, II, and III (7^{th} ed. AJCC). IHC scores for CLIC1 was determined by percentage of positive hepatocytes × IHC intensity (0-3) with a maximum of 300, which were measured by an automation system (inForm® Advanced Image Analysis Software, version 2.3, PerkinElmer, MA). (**B**) IHC for CLIC1 in 12 paired primary and metastatic tumors (1:3 sections). IHC scores were determined by an automation system (inForm® Advanced Image Analysis Software, version 2.3, PerkinElmer, MA).

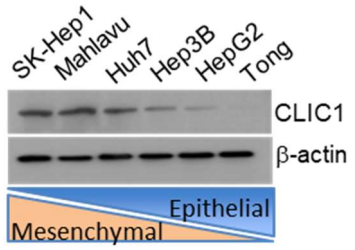
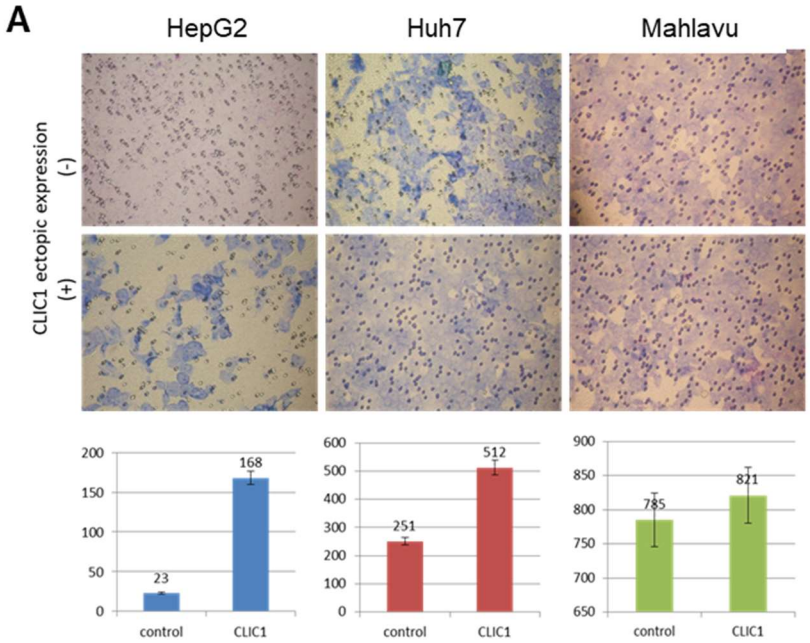
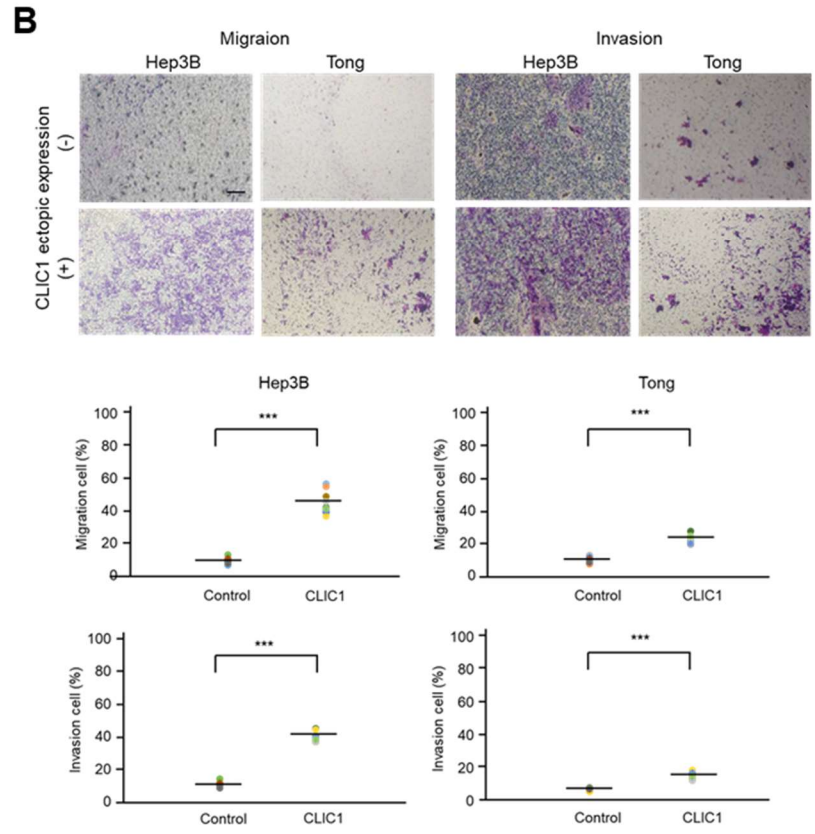


Figure 2. Immunoblots for the relative levels of CLIC1 in a panel of HCC cell lines. Interestingly, the CLIC1 levels were positively correlated to the mesenchymal traits of the cells.


A
HepG2
Huh7
Mahlavu
CLIC1 ectopic expression
(-)
(+)
200
150
100
50
0
168
23
control
CLIC1
600
500
400
300
200
100
0
512
251
control
CLIC1
900
850
800
750
700
650
821
785
control
CLIC1


B
Migraion
Invasion
Hep3B
Tong
Hep3B
Tong
CLIC1 ectopic expression
(-)
(+)
Hep3B
Tong
***
Migration cell (%)
Invasion cell (%)
100
80
60
40
20
0
Control
CLIC1

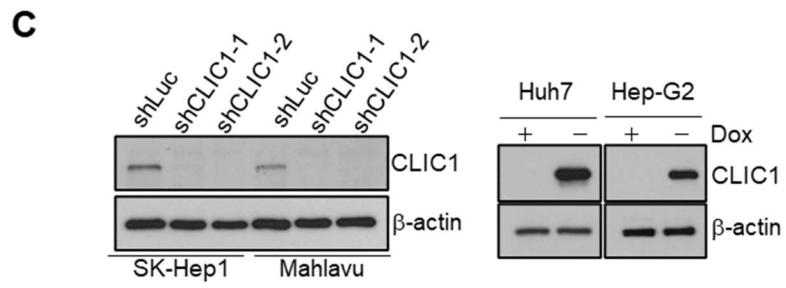

C
shLuc
shCLIC1-1
shCLIC1-2
shLuc
shCLIC1-1
shCLIC1-2
CLIC1
β-actin
SK-Hep1
Mahlavu
Huh7
Hep-G2
+
−
+
−
Dox
CLIC1
β-actin

Figure 3. Ectopic expression of CLIC1 promoted tumor cell migration and invasion. Tumor cells were constitutively transfected with a CLIC1 cDNA under control by a tet-off inducible promoter system. Removal of doxycycline from media would induce CLIC1 expression. (**A**) Migration assays by using Boyden chambers. Notably, the efficiency of promoted motility in different types of cells (7.3, 2.0, and 1.05 folds for HepG2, Huh7, and Mahlavu, respectively) was inverse with the endogenous CLIC1 level among HepG2, Huh7, and Mahlavu cells (by using the same empty vector and CLIC1 expression clones as those used in Figure 4C). (**B**) Migration and invasion assays. Scale bar = 200 μm. ***$P < 0.001$. n = 6. (**C**) Immunoblots for the silencing and ectopic expression efficiency in HCC cells. Ectopic expression of CLIC1 was driven by an inducible promoter (Tet-off). Removing of doxycycline from culture media induced the expression of CLIC1.

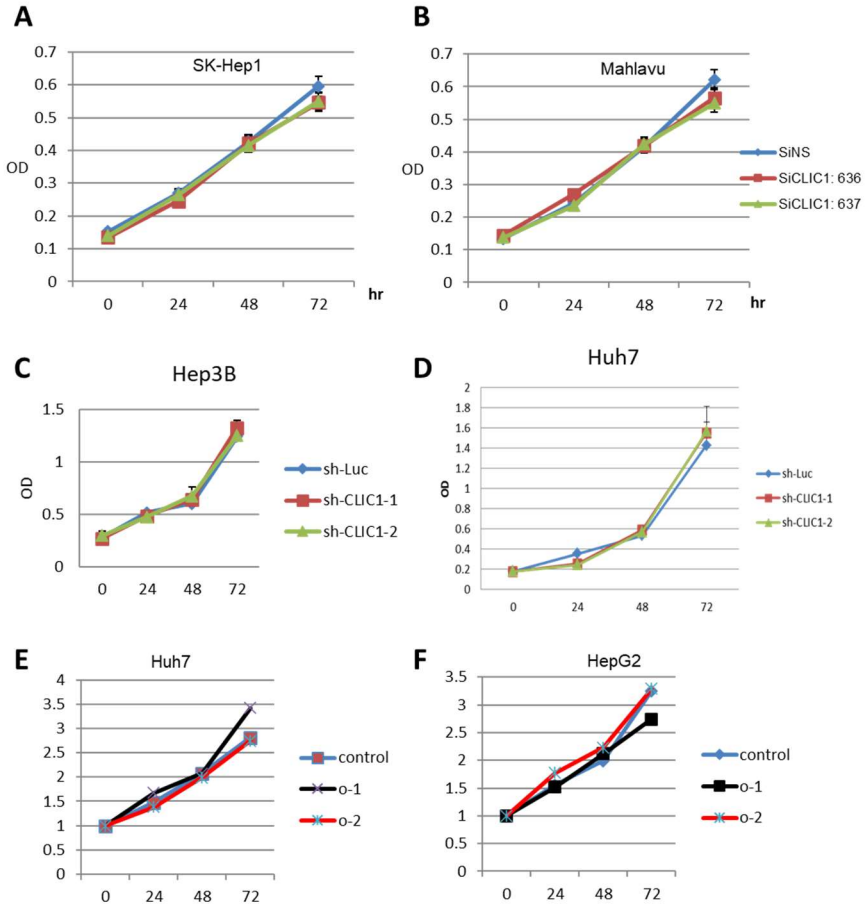


Figure 4. No apparent effects of CLIC1 on cell proliferation. (**A**-**D**) The expression of CLIC1 was silenced by transducing the cells with two clones of siRNA (siCLICs: 636 and 637, **A** and **B**) or two clones of shRNA (shCLIC1-1 and -2, **C** and **D**) targeting CLIC1. Cells transduced with siRNA containing scrambled sequences (siNS, **A** and **B**) or shRNA targeting luciferase gene (shLuc, **C** and **D**) were used as controls. The relative amounts of viable cells were measured by tetrazolium reduction assays (XTT Cell Proliferation Kit, Merck). (**E**, **F**) We also did the ectopic expression of CLIC1 in two HCC cell lines by transducing with CLIC1 cDNA under the control of the tetracycline-inducible promoter (control: cells transfected with an empty vector; 1, CLIC1 not induced as treatment with doxycycline; 2, induction of CLIC1 expression by removal of doxycycline from the media). See also Supplemental Figure 3C for the efficiency of silencing and ectopic expression of CLIC1 in transduced cells.

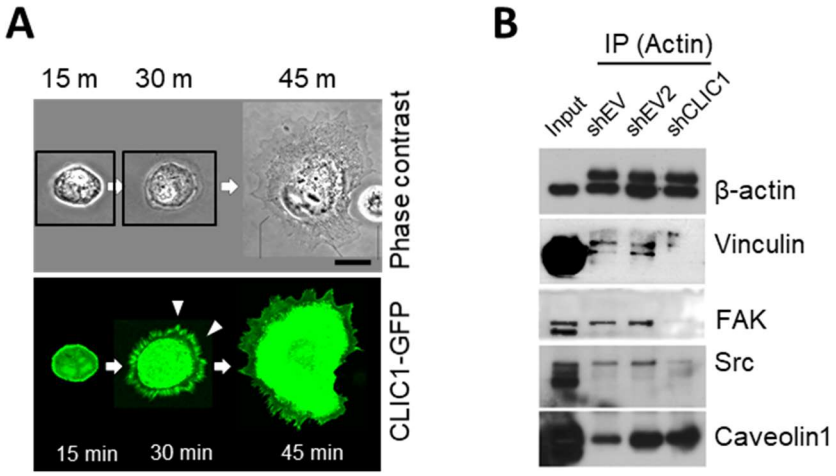


Figure 5. (**A**) Time-lapse tracking of the formation of nascent cell-matrix adhesions and subcellular distribution of CLIC1-GFP in Huh7 cells after reseeding on laminin-coated plates. Arrowheads: nascent cell-matrix adhesions 30 min after reseeding. (**B**) IP by using anti-beta actin antibodies in 293T cells with and without CLIC1 depletion.

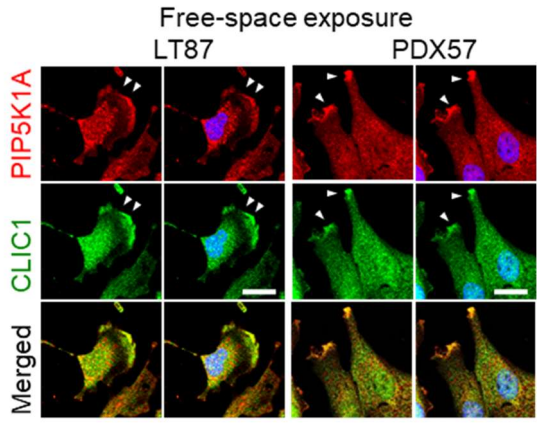


Figure 6 CLIC1 transports PIP5K1A and PIP5K1C to the front end of lamellipodia and nascent adhesions. Immunofluorescence demonstrates co-targeting of CLIC1 (Alexa 594, green) and PIP5K1A (Alexa 488, red) to the tips of lamellipodia of two primary HCC cells derived from two patients of HCC in response to free-space exposure. Scale bars: 4 μm.

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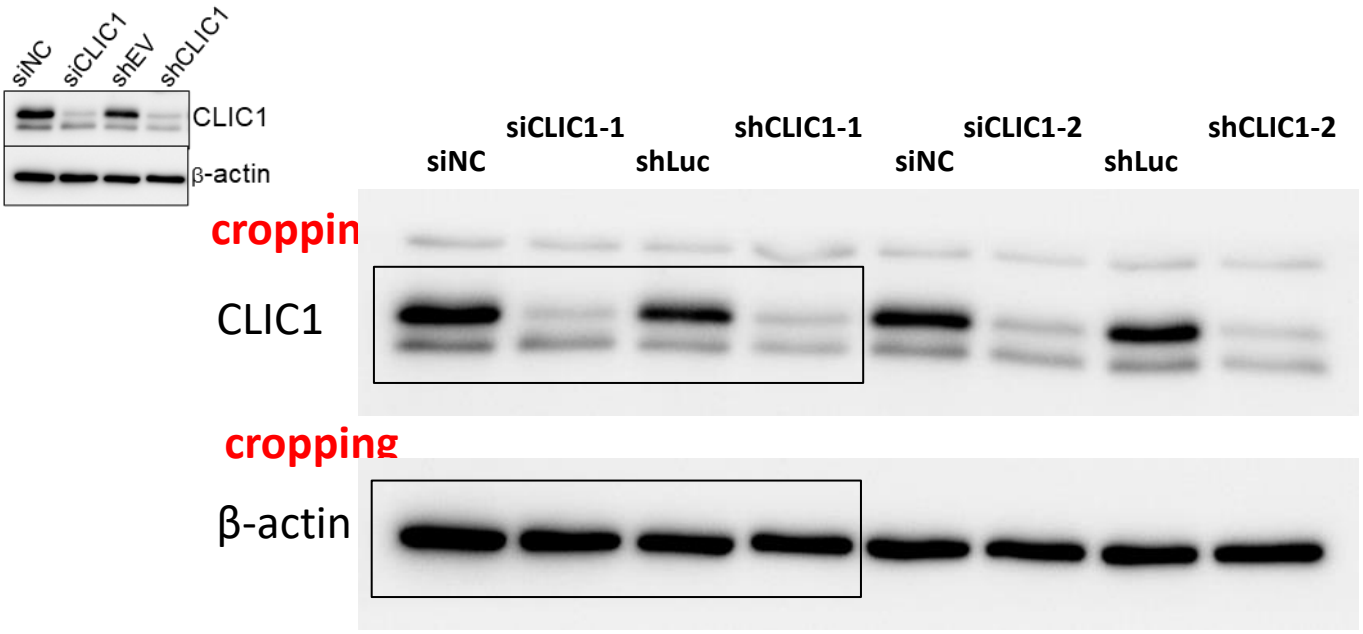

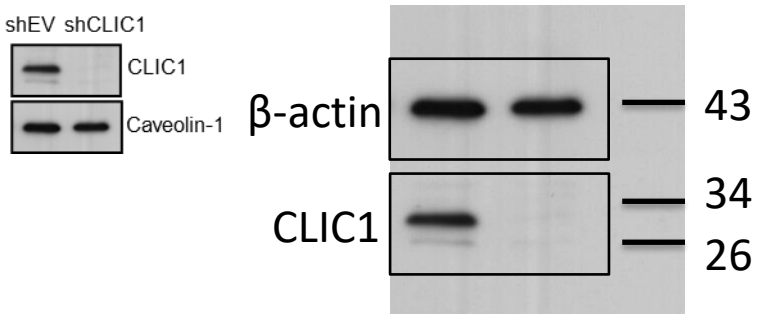

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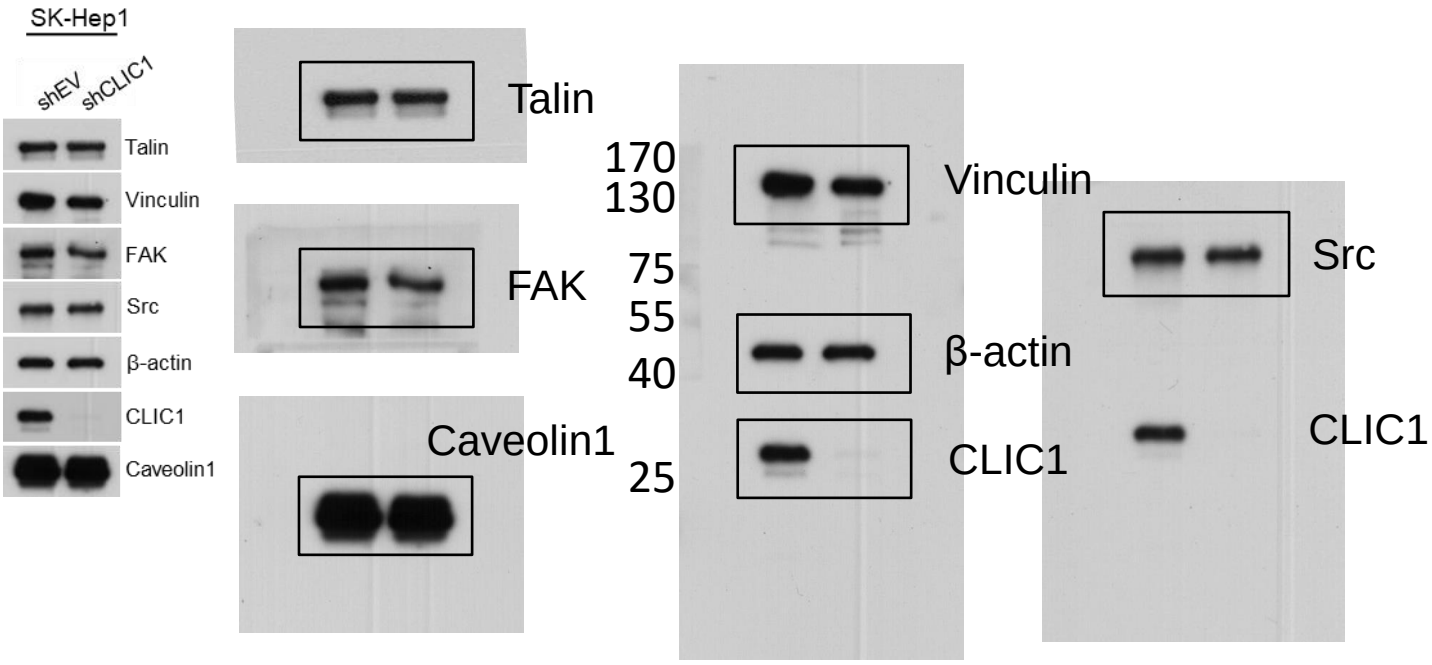

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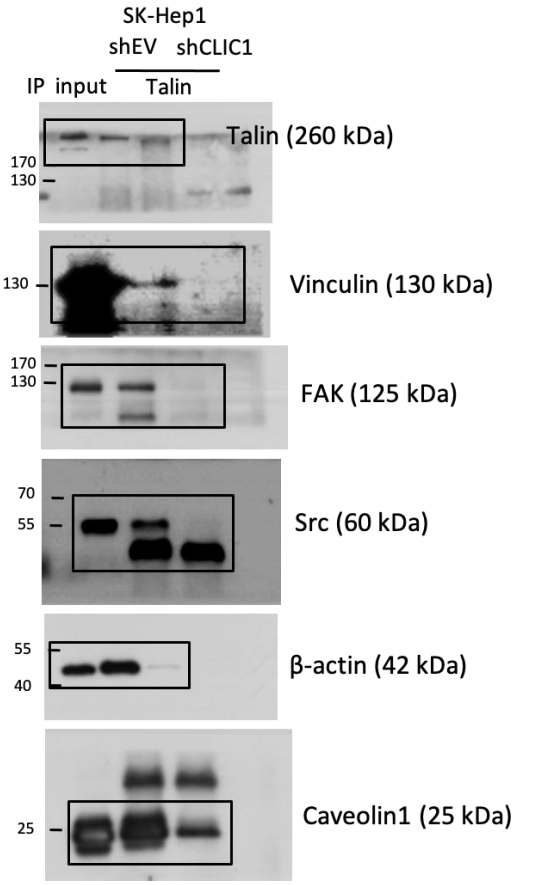

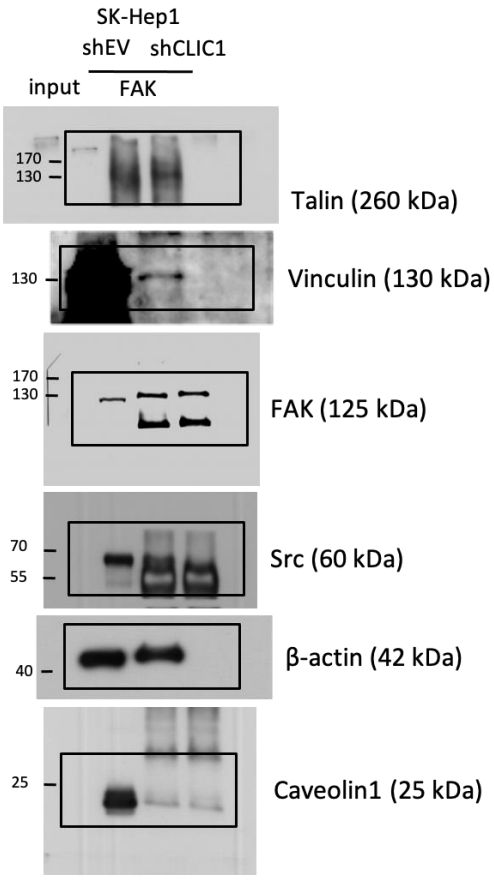

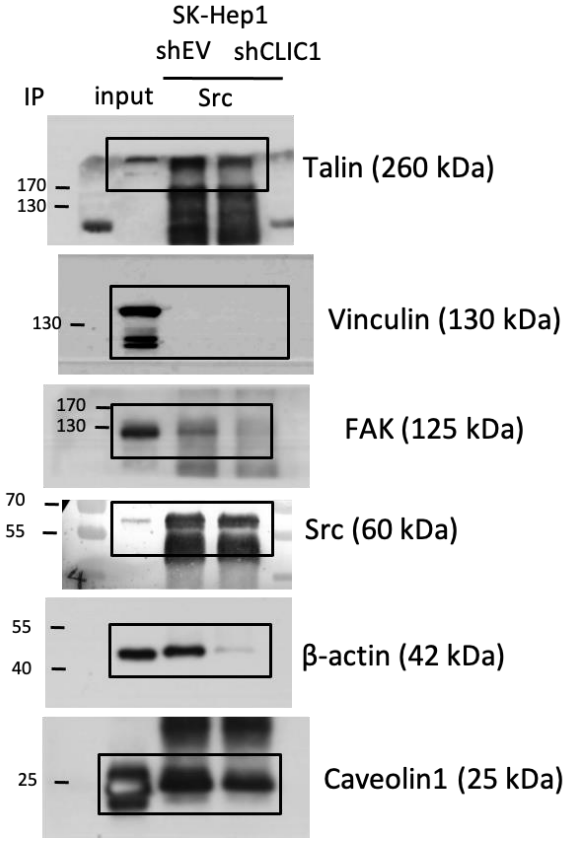

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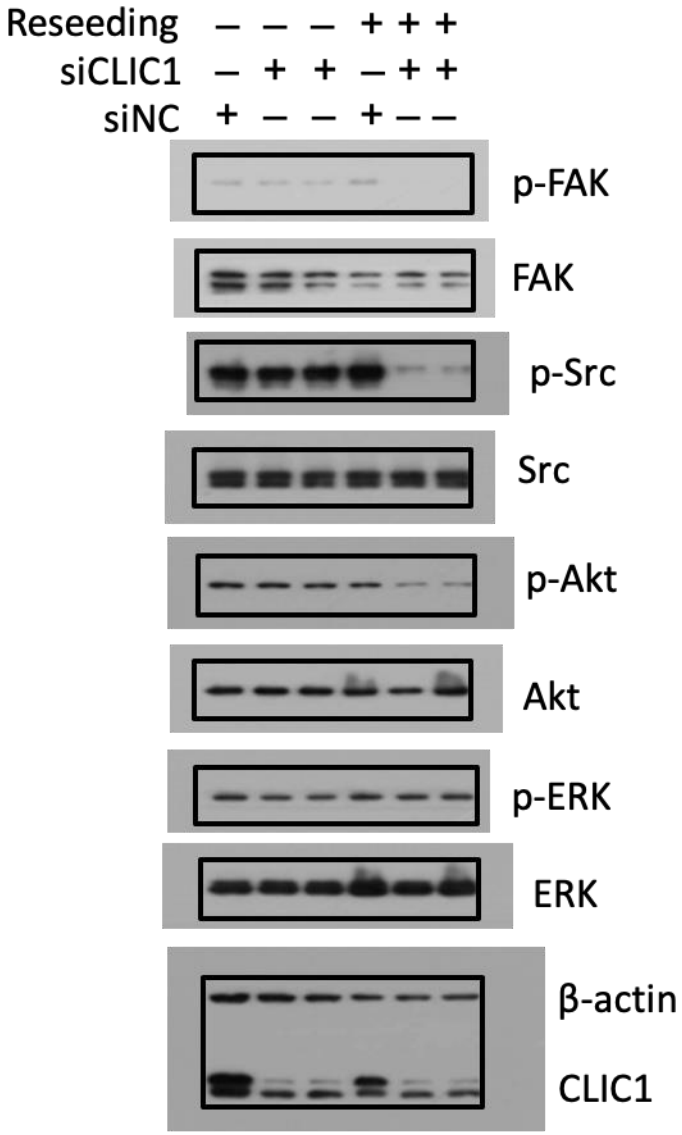

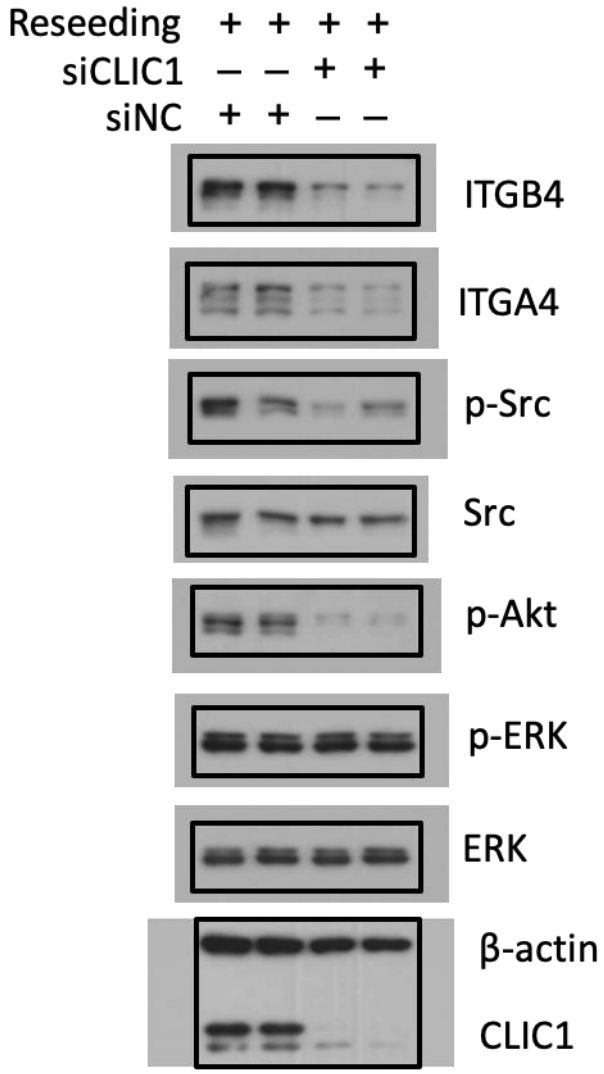

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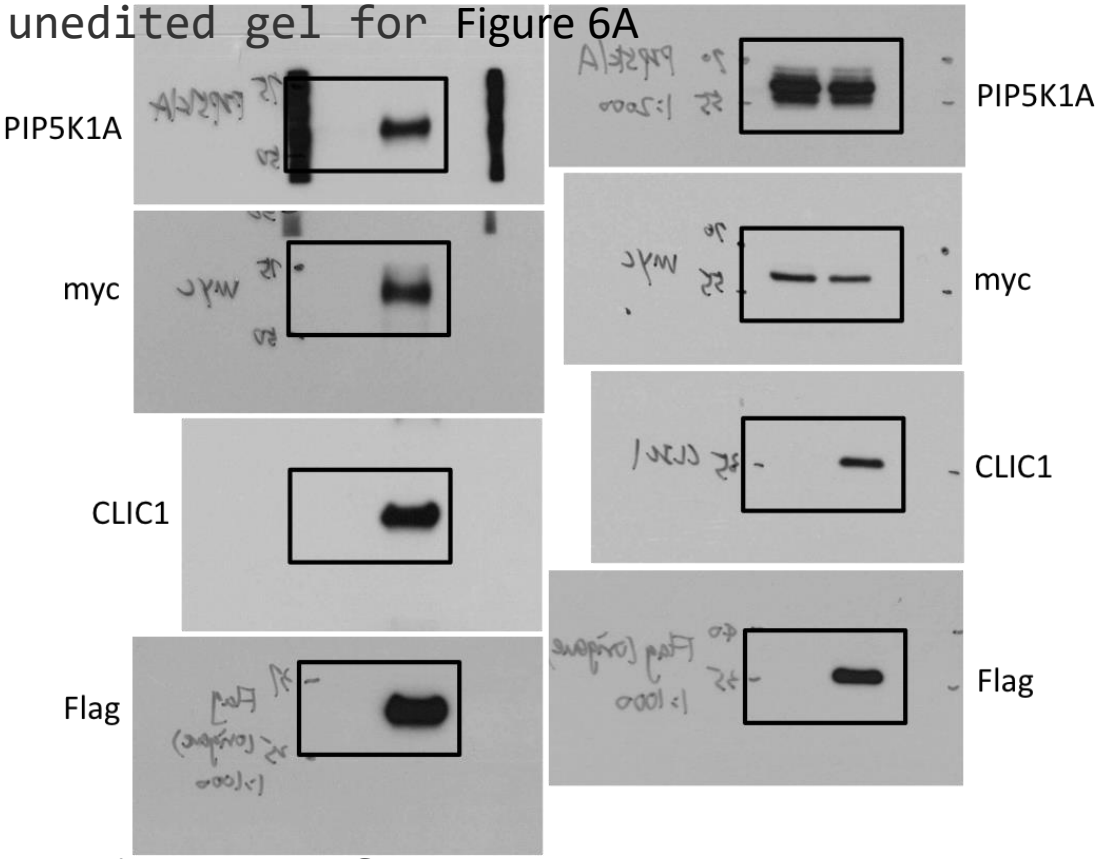

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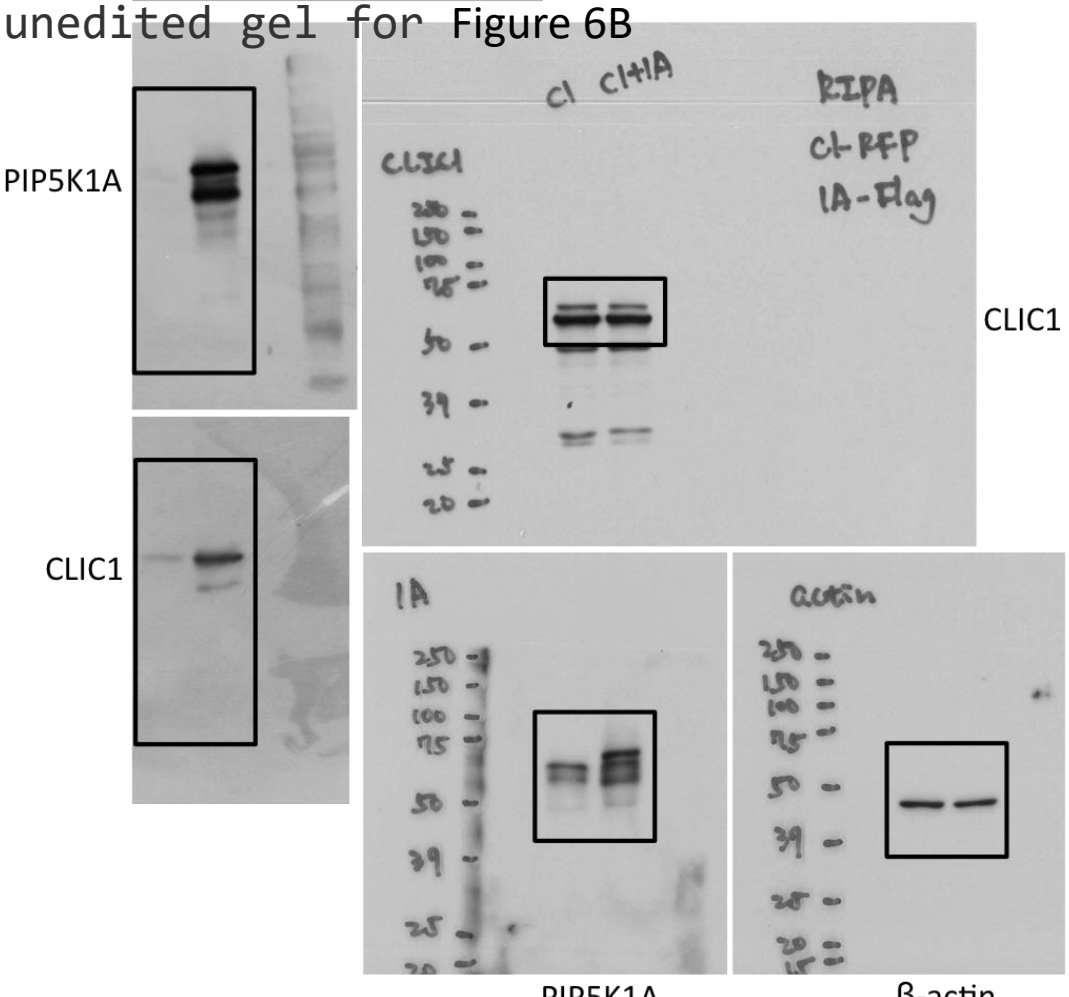

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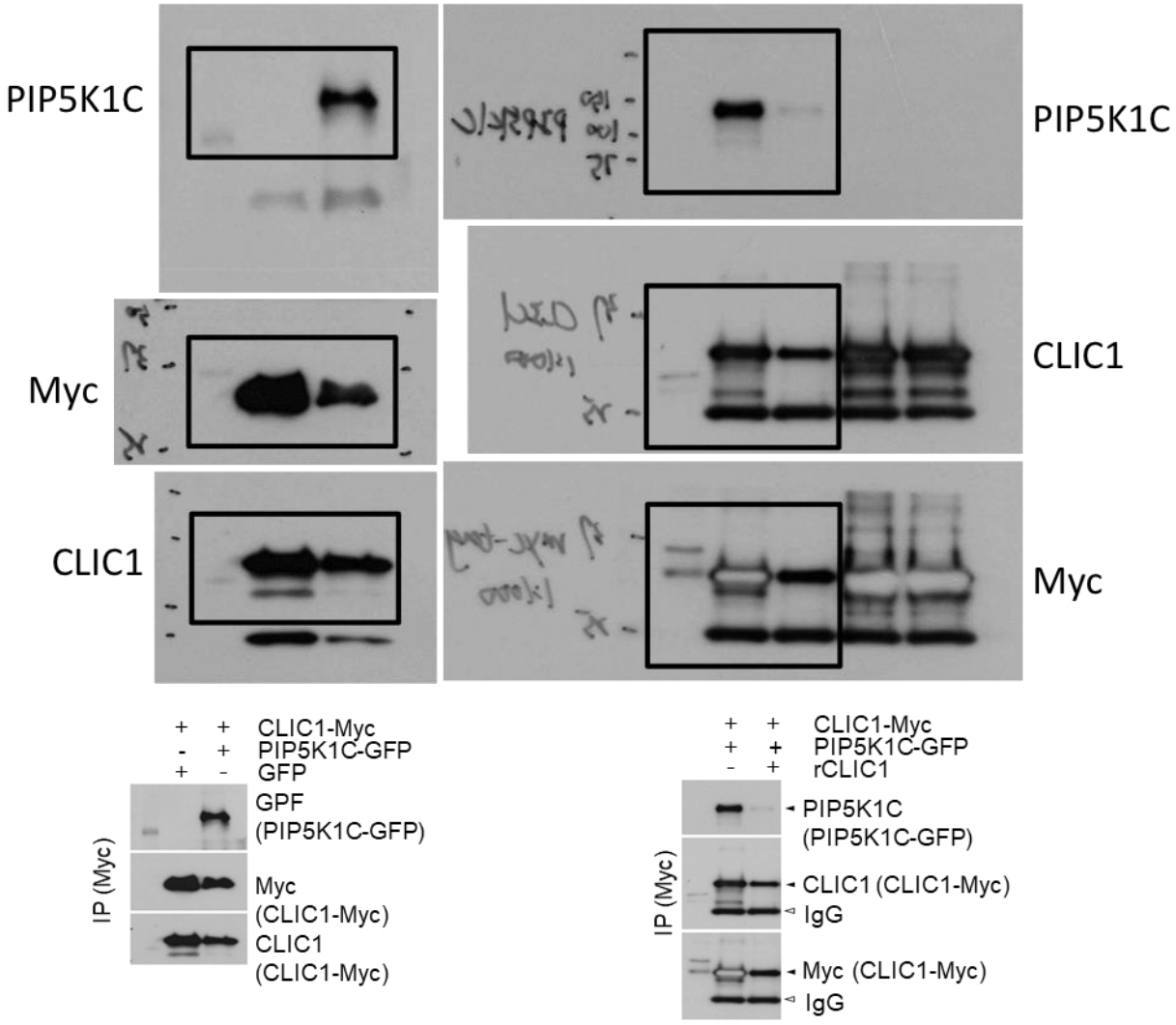

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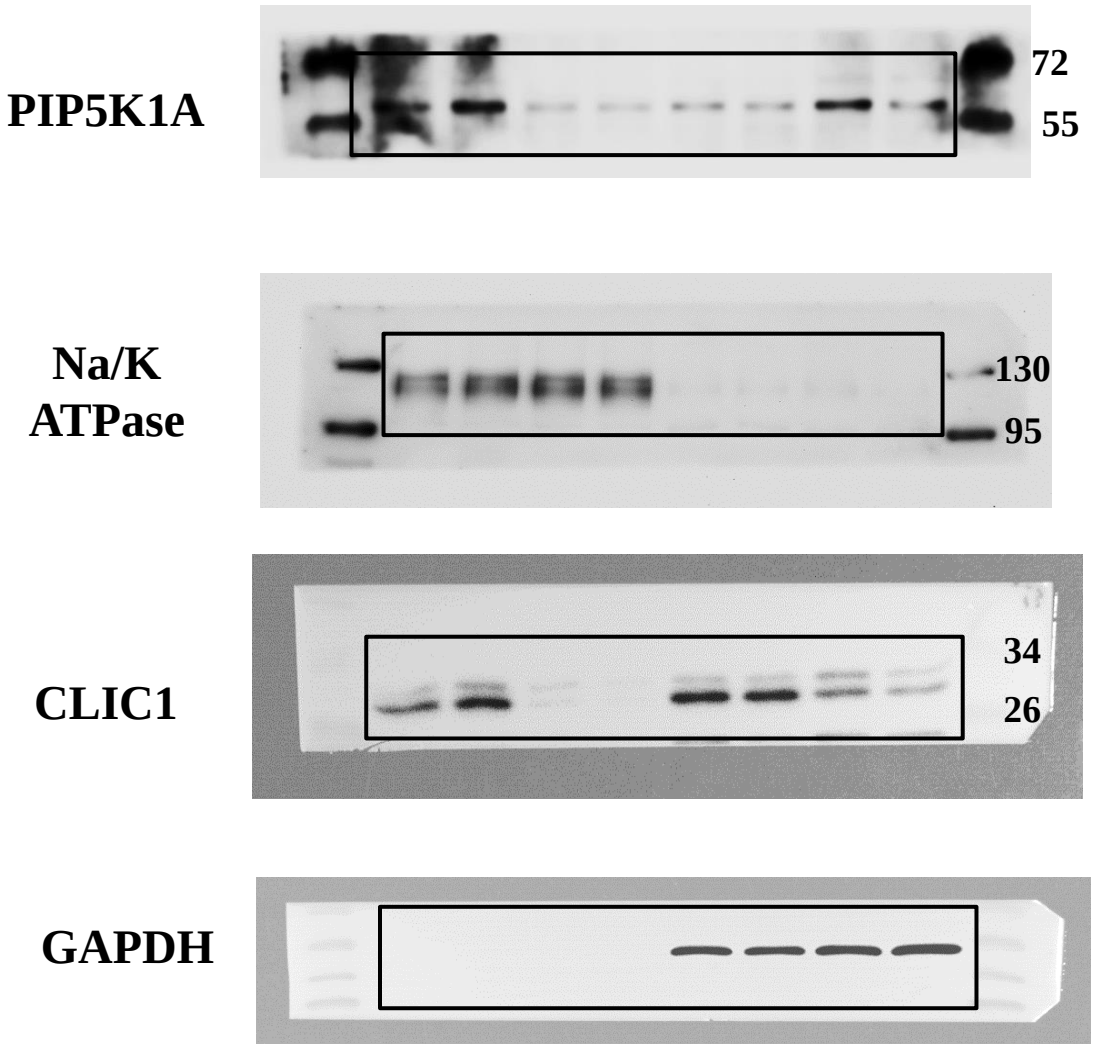

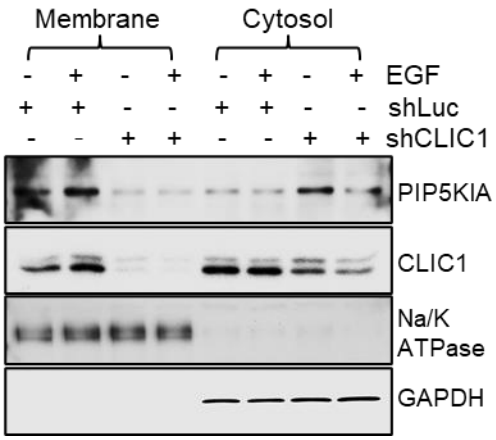

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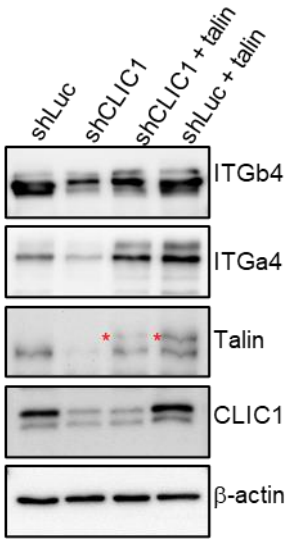

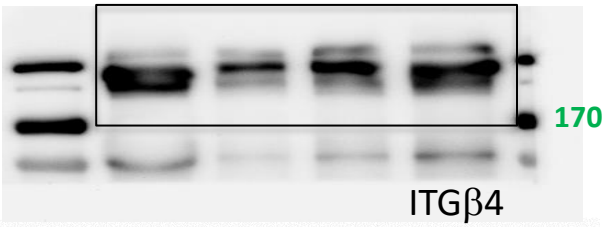

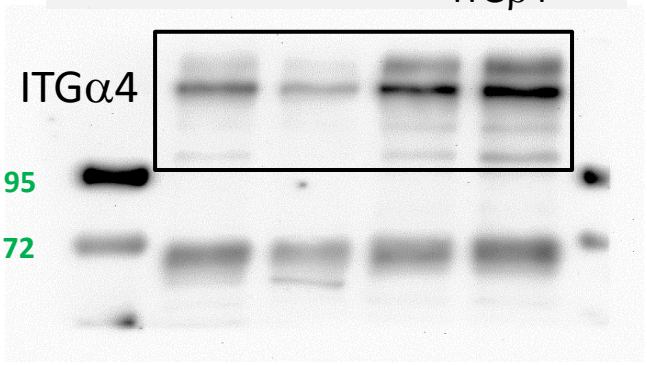

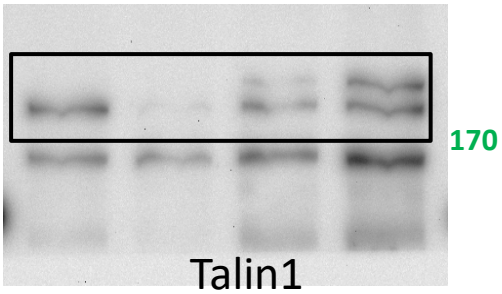

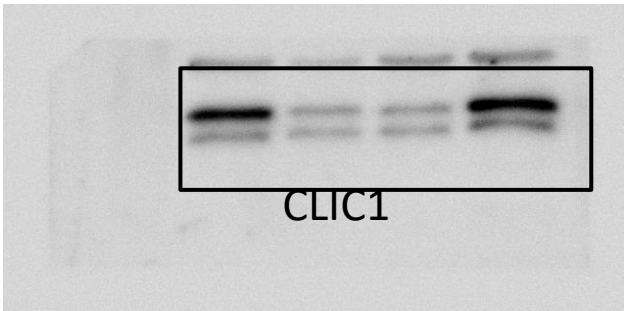

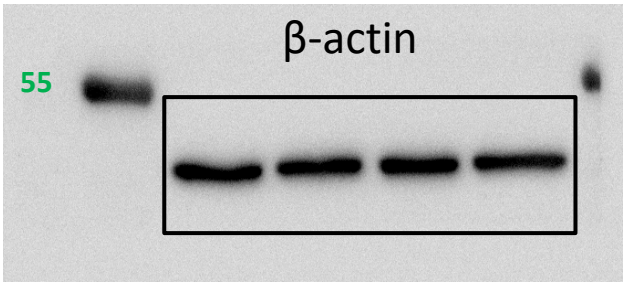

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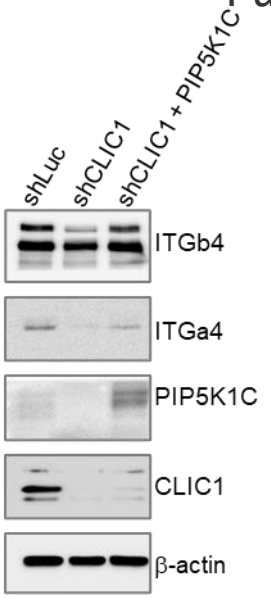

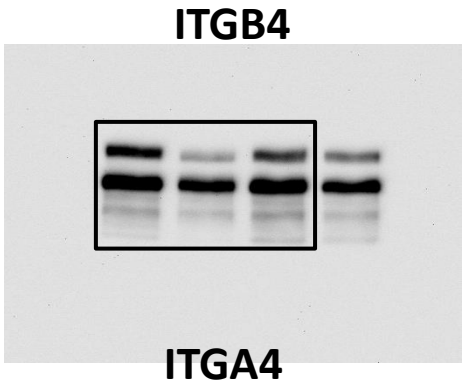

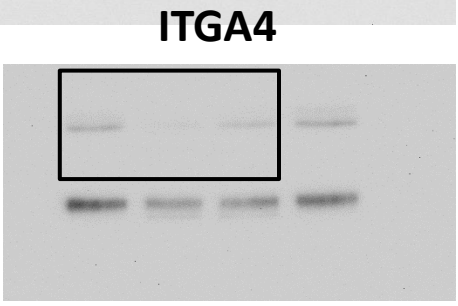

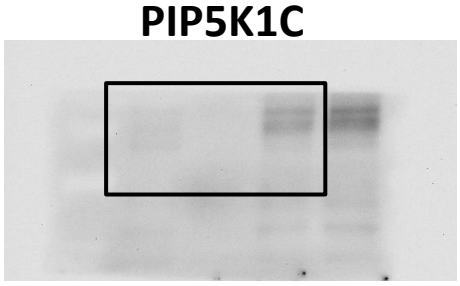

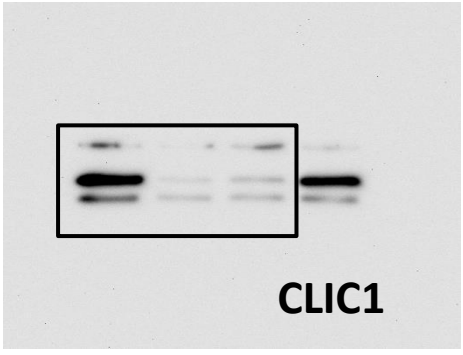

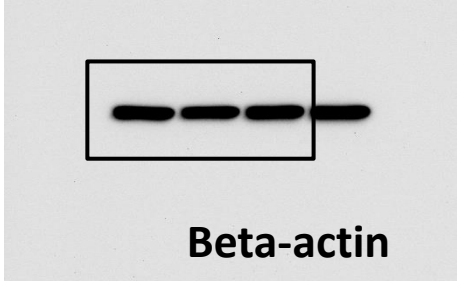

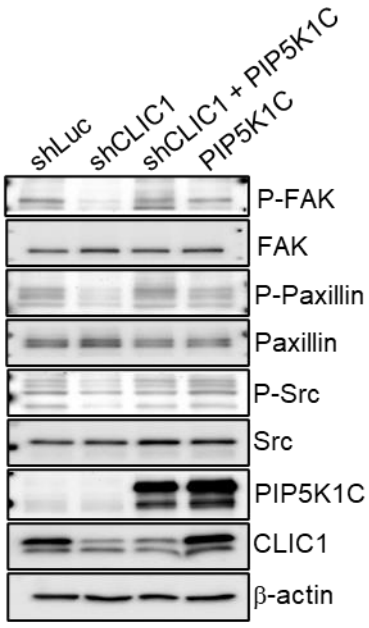

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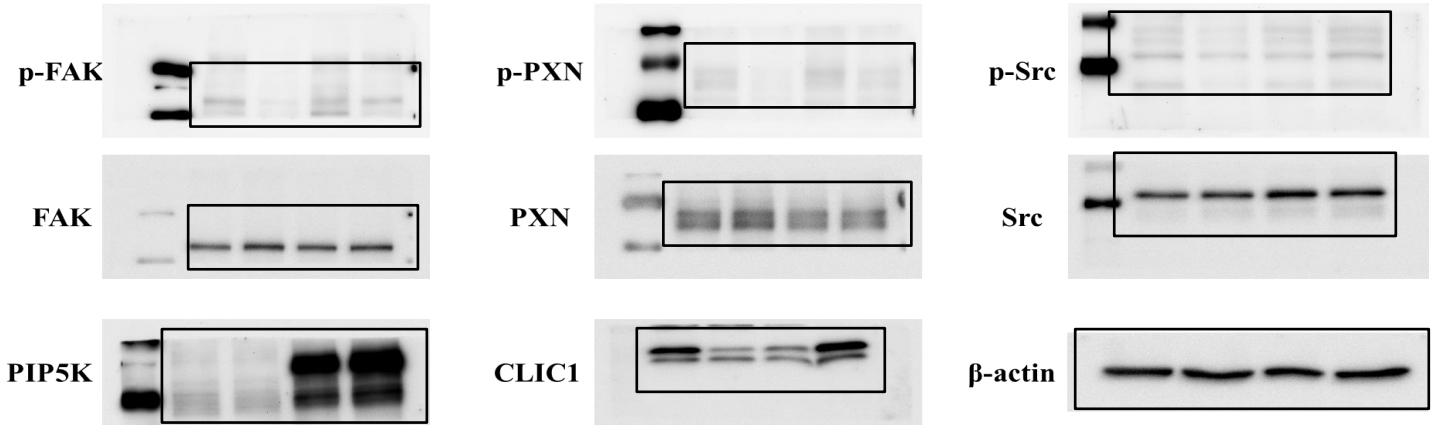

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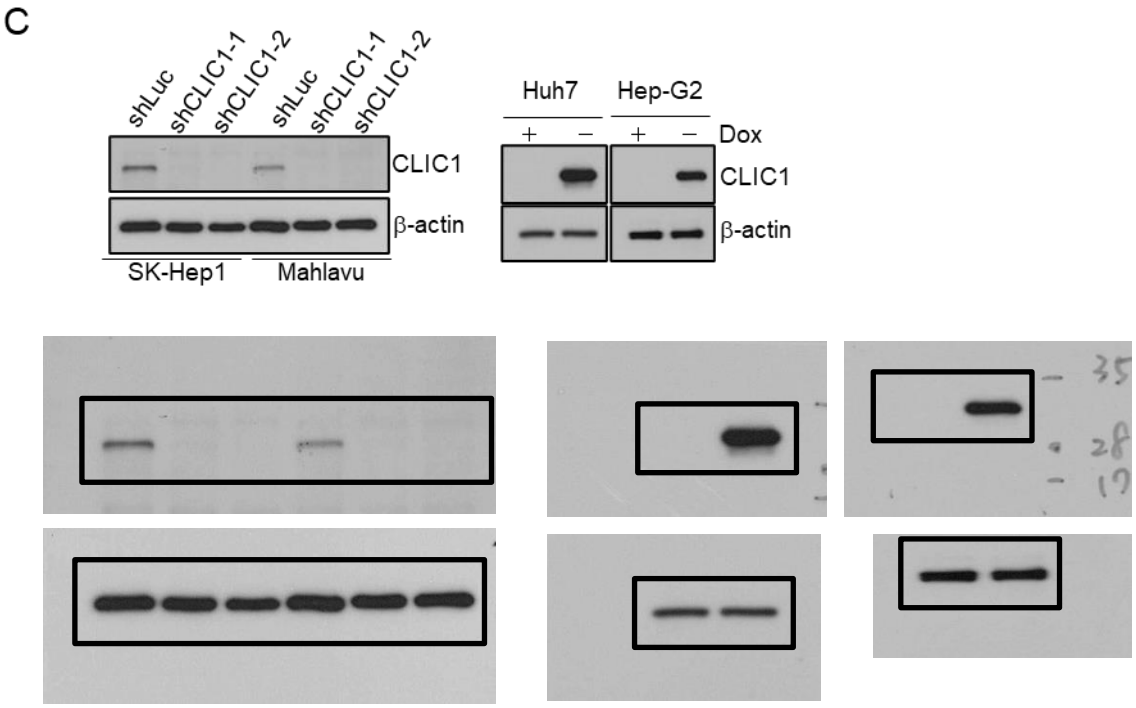

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