SAG/RBX2 E3 ligase complexes with UBCH10 and UBE2S E2s to ubiquitylate β-TrCP1 via K11-linkage for degradation

Peng Kuang[1,2], Mingjia Tan[2], Weihua Zhou[2], Qiang Zhang[2], and Yi Sun[2-4]

[1]Department of Internal Medicine, Beijing University School of Medicine, 38 Xueyuan Road, Beijing, 100191, China

[2]Division of Radiation and Cancer Biology, Department of Radiation Oncology, University of Michigan, 4424B MS-1, 1301 Catherine Street, Ann Arbor, MI 48109, USA.

[3]Institute of Translational Medicine, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People's Republic of China.

[4]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou, P.R. China;

[#]Corresponding author: email: sunyi@umich.edu or yisun@zju.edu.cn

Running title: SAG ubiquitylates βTrCP for degradation

Key words: β-TrCP, CRL5, RBX1, SAG, UBE2S, and UBCH10

Supplemental Figure legends:

Figure S1. Inverse relationship between SAG and βTrCP1, and their binding to cullins.

(A) Inverse correlation between SAG and βTrCP: Cell lysates were prepared from various lung cancer cell lines, followed by IB using indicated Abs.

(B) SAG-βTrCP1-cullins form the complex: The 293T cells were transfected with FLAG-tagged CUL1 or CUL5, followed by IP with anti-FLAG Ab or IgG control, and IB with indicated Abs.

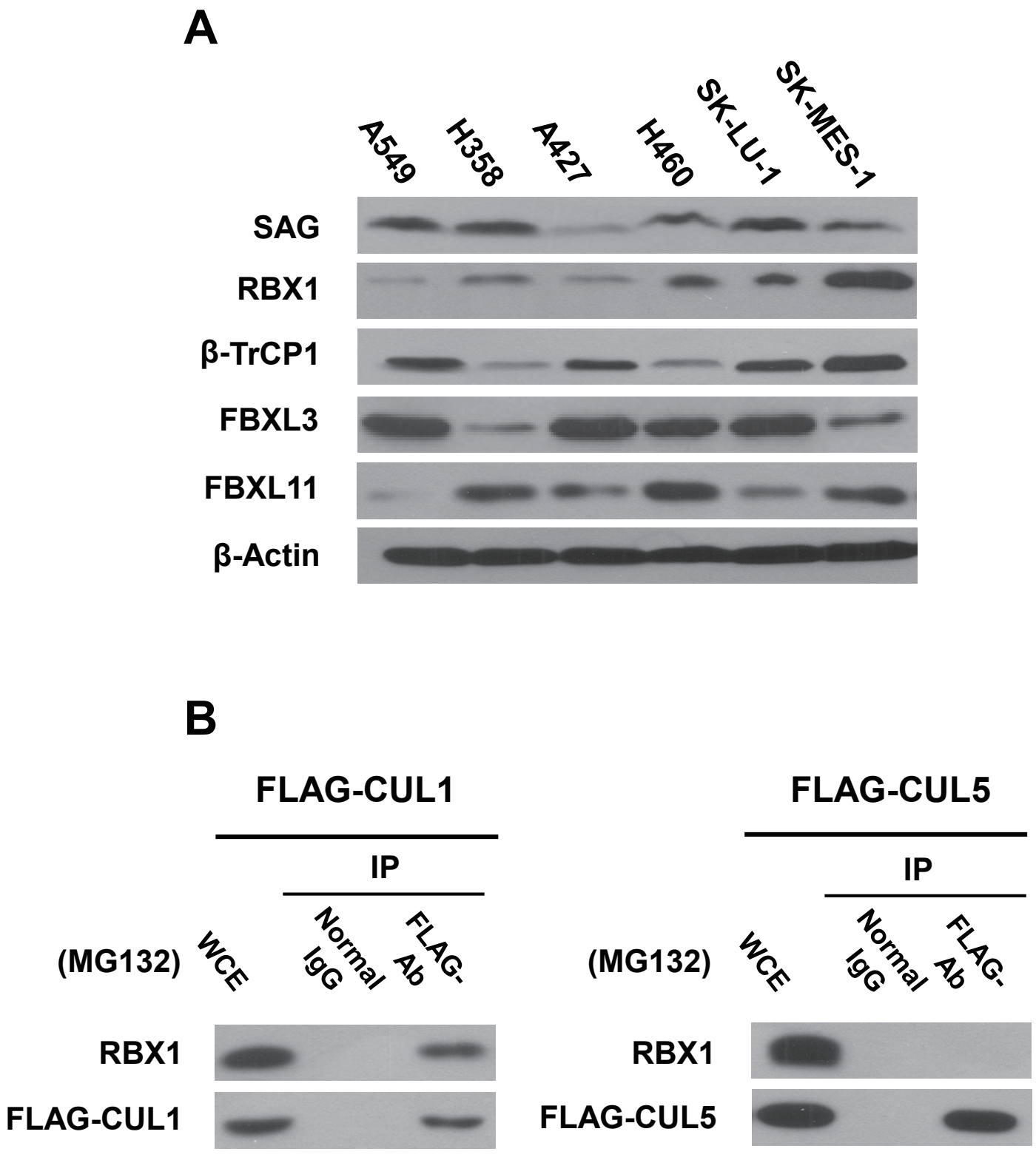
A
A549
H358
A427
H460
SK-LU-1
SK-MES-1
SAG
RBX1
β-TrCP1
FBXL3
FBXL11
β-Actin
B
FLAG-CUL1
IP
(MG132)
WCE
Normal IgG
FLAG-Ab
RBX1
FLAG-CUL1
FLAG-CUL5
IP
(MG132)
WCE
Normal IgG
FLAG-Ab
RBX1
FLAG-CUL5

Figure S2. SAG negatively regulates β-TrCP1 protein level.

(A) SAG-CUL5 reduces βTrCP1 level: The 293T cells were transfected with indicated plasmids. Whole cell extracts were analyzed by IB with indicated antibodies. FLAG-1: CUL1 or CUL5; FLAG-2, SAG or RBX1.

(**B&C**) Accumulation of βTrCP1 and p27: Two indicated lung cancer lines were treated with various concentrations of MLN4924 for 24 hrs, followed by IB, using indicated Abs.

(**D&E**) SAG-CUL5 shortens the half-life of βTrCP1 and βTrCP1ΔF: After transfection with indicated plasmids for 12 hrs, cells were switched to fresh medium (10% FBS) containing cycloheximide (CHX) and incubated for indicated time periods before being harvested for IB using indicated Abs.

(**F**) Manipulation of SAG-CUL5 has no effect on β-TrCP mRNA level. A427 cells were transfected with indicated plasmid or siRNA oligoes. Forty-eight hrs post-transfection, total RNA was isolated and subjected to RT-PCR analysis for β-TrCP mRNA levels.

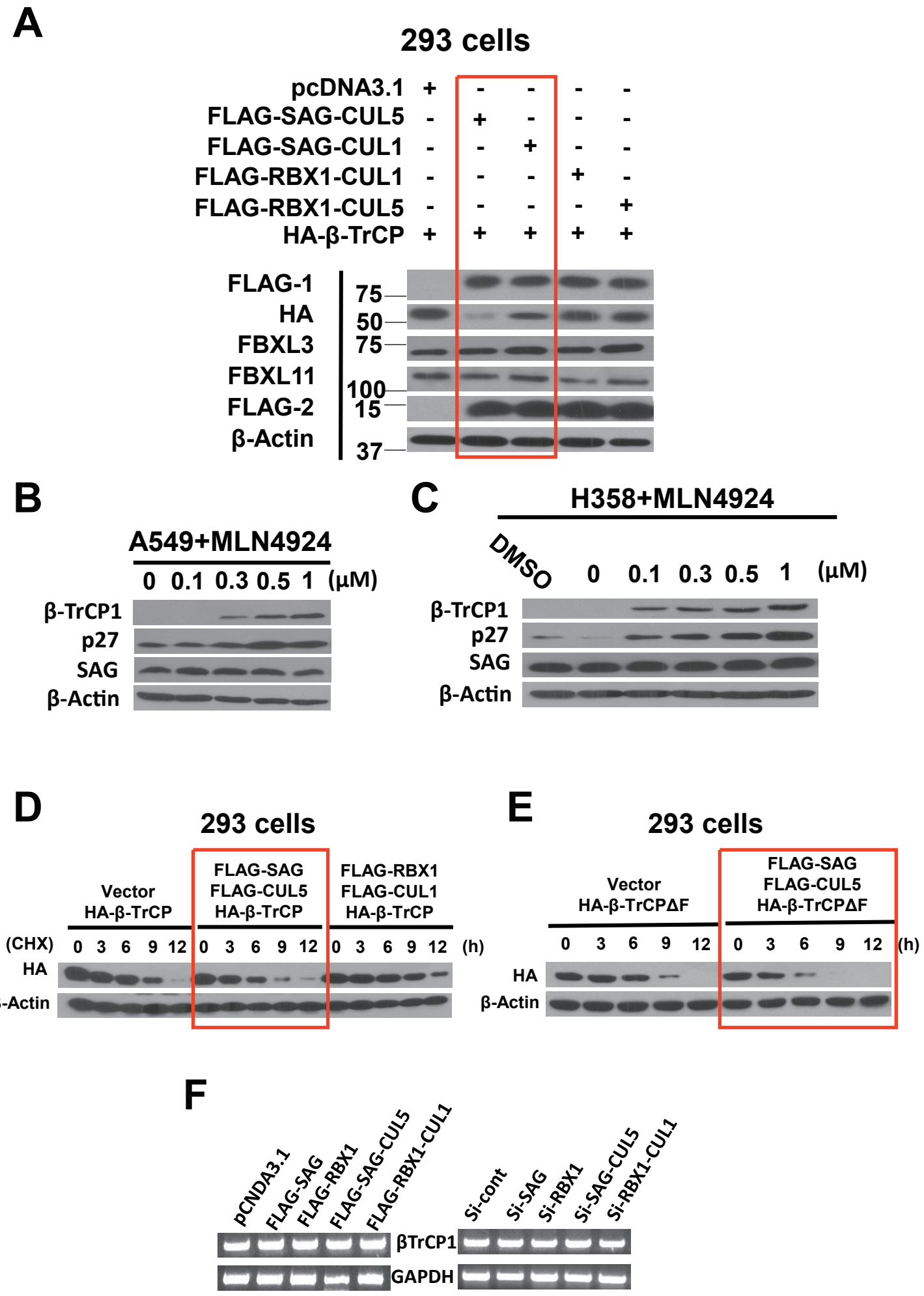
A
293 cells
pcDNA3.1 + - - - -
FLAG-SAG-CUL5 - + - - -
FLAG-SAG-CUL1 - - + - -
FLAG-RBX1-CUL1 - - - + -
FLAG-RBX1-CUL5 - - - - +
HA-β-TrCP + + + + +
FLAG-1
HA
FBXL3
FBXL11
FLAG-2
β-Actin
75
50
75
100
15
37
B
A549+MLN4924
0 0.1 0.3 0.5 1 (μM)
β-TrCP1
p27
SAG
β-Actin
C
H358+MLN4924
DMSO 0 0.1 0.3 0.5 1 (μM)
β-TrCP1
p27
SAG
β-Actin
D
293 cells
Vector
HA-β-TrCP
FLAG-SAG
FLAG-CUL5
HA-β-TrCP
FLAG-RBX1
FLAG-CUL1
HA-β-TrCP
(CHX) 0 3 6 9 12 0 3 6 9 12 0 3 6 9 12 (h)
HA
β-Actin
E
293 cells
Vector
HA-β-TrCPΔF
FLAG-SAG
FLAG-CUL5
HA-β-TrCPΔF
0 3 6 9 12 0 3 6 9 12 (h)
HA
β-Actin
F
pCNDA3.1
FLAG-SAG
FLAG-RBX1
FLAG-SAG-CUL5
FLAG-RBX1-CUL1
Si-cont
Si-SAG
Si-RBX1
Si-SAG-CUL5
Si-RBX1-CUL1
βTrCP1
GAPDH

Figure S3. SAG-CUL5, but not RBX1-CUL1, promotes the ubiquitylation of βTrCP1 via K11 linkage.

(A) SAG-CUL5 promotes polyubiquitylation of β-TrCP1 and β-TrCP1ΔF *in vivo*: H1299 cells were transfected with indicated plasmids, lysed under denatured condition by 6M guanidinium solution, followed by Ni-bead pull-down. Washed beads were boiled and subjected to IB, along with whole cell lysates, using indicated Abs.

(B-E) SAG/CUL5 promotes poly-ubiquitylation of β-TrCP1 via K11 linkage: H1299 (B&D) or 293T (C&E) cells were cotransfected with indicated plasmids alone or in combination, along with ubiquitin and various ubiquitin mutants. Whole cell extracts and Ni-NTA affinity purified fractions were analyzed by IB using indicated Abs.

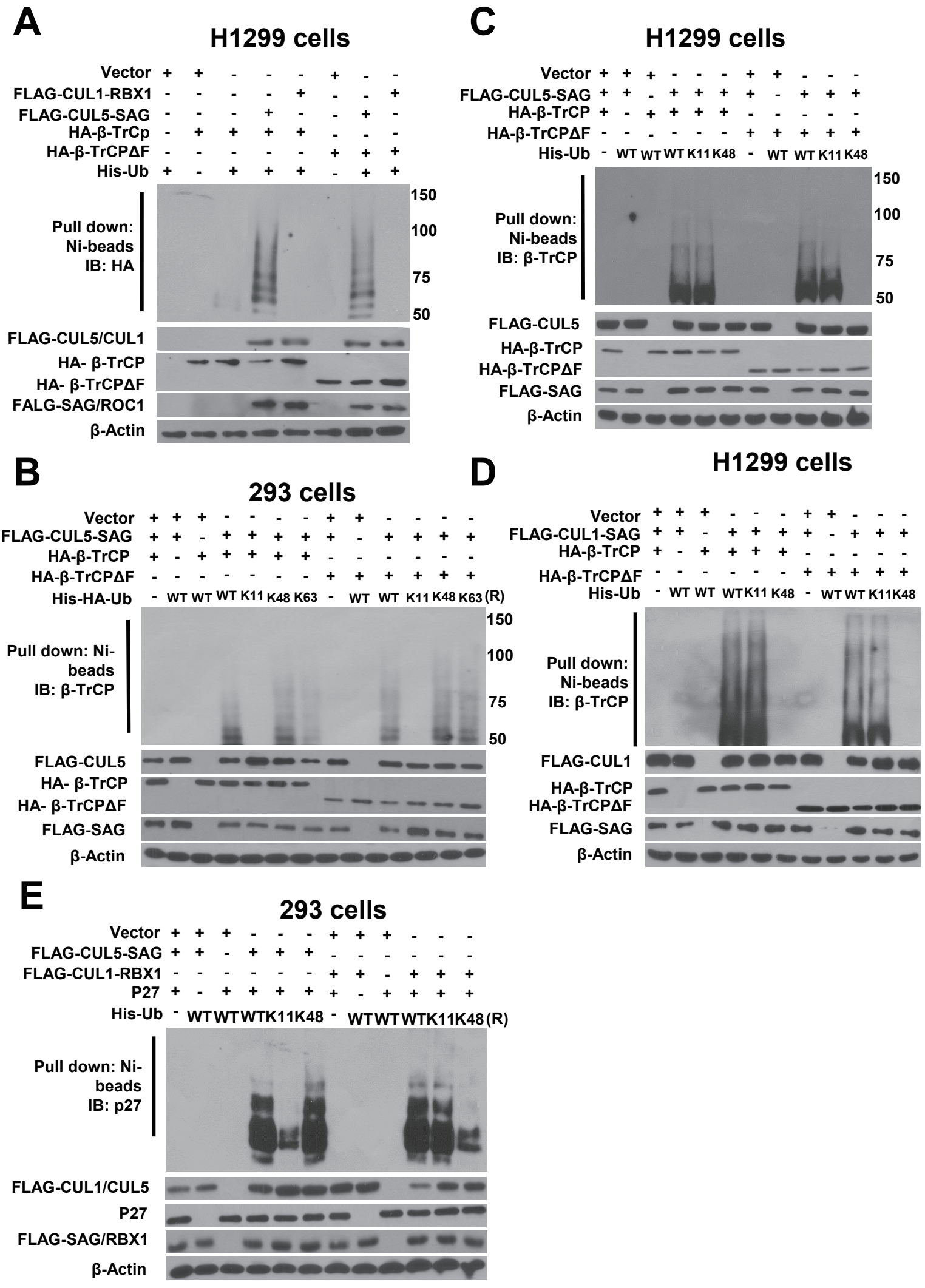
A
H1299 cells
Vector
FLAG-CUL1-RBX1
FLAG-CUL5-SAG
HA-β-TrCp
HA-β-TrCPΔF
His-Ub
Pull down: Ni-beads IB: HA
150
100
75
50
FLAG-CUL5/CUL1
HA- β-TrCP
HA- β-TrCPΔF
FALG-SAG/ROC1
β-Actin
C
H1299 cells
Vector
FLAG-CUL5-SAG
HA-β-TrCP
HA-β-TrCPΔF
His-Ub
WT WT WT K11 K48 - WT WT K11 K48
Pull down: Ni-beads IB: β-TrCP
150
100
75
50
FLAG-CUL5
HA-β-TrCP
HA-β-TrCPΔF
FLAG-SAG
β-Actin
B
293 cells
Vector
FLAG-CUL5-SAG
HA-β-TrCP
HA-β-TrCPΔF
His-HA-Ub
- WT WT WT K11 K48 K63 - WT WT K11 K48 K63 (R)
Pull down: Ni-beads IB: β-TrCP
150
100
75
50
FLAG-CUL5
HA- β-TrCP
HA- β-TrCPΔF
FLAG-SAG
β-Actin
D
H1299 cells
Vector
FLAG-CUL1-SAG
HA-β-TrCP
HA-β-TrCPΔF
His-Ub
- WT WT WT K11 K48 - WT WT K11 K48
Pull down: Ni-beads IB: β-TrCP
FLAG-CUL1
HA-β-TrCP
HA-β-TrCPΔF
FLAG-SAG
β-Actin
E
293 cells
Vector
FLAG-CUL5-SAG
FLAG-CUL1-RBX1
P27
His-Ub
- WT WT WT K11 K48 - WT WT WT K11 K48 (R)
Pull down: Ni-beads IB: p27
FLAG-CUL1/CUL5
P27
FLAG-SAG/RBX1
β-Actin

Figure S4. SAG and RBX1 form the complex with different E2s.

(A) H1299 cells were transfected with FLAG-SAG or FLAG-RBX1, followed by IP using FLAG Ab or normal IgG control, and IB with indicated Abs.

(B&C) Whole cell extracts (WCE) from A427 cells were subjected to IP and IB, or directly subjected to IB with indicated Abs.

(**D**) The 293T cells were transfected with indicated plasmids alone or in combination, and subjected to in vitro ubiquitylation as described in M&M. The reaction mixture was then loaded onto PAGE gel for IB using anti-Erbin Ab.

Kuang et al., Figure S4