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Supplementary Materials for

Identifying the substrate proteins of U-box E3s E4B and CHIP by orthogonal ubiquitin transfer

Karan Bhuripanyo, Yiyang Wang, Xianpeng Liu, Li Zhou, Ruochuan Liu, Duc Duong, Bo Zhao, Yingtao Bi, Han Zhou, Geng Chen, Nicholas T. Seyfried, Walter J. Chazin, Hiroaki Kiyokawa, Jun Yin

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Supplementary Materials

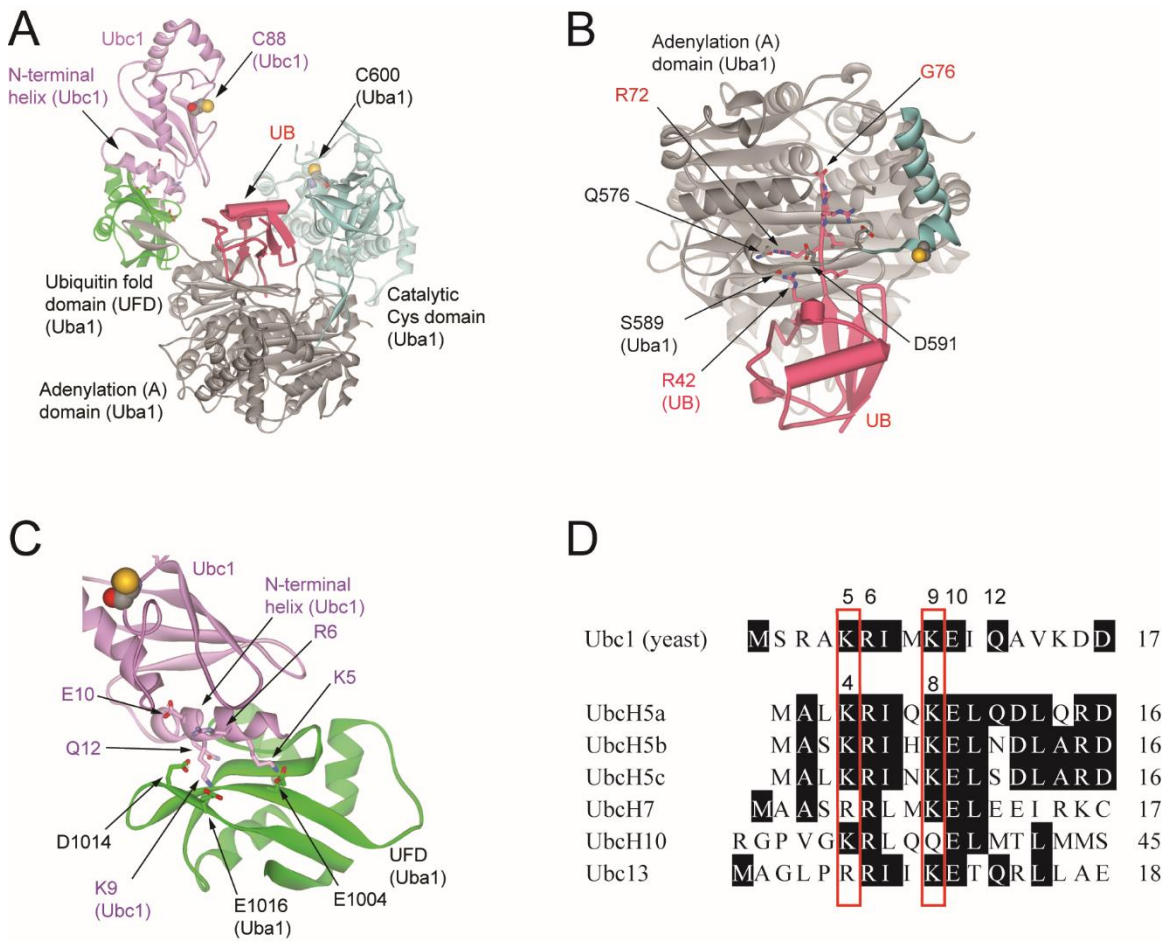

fig. S1. Design of the xUB-xUba1 and xUba1-xUbcH5b pairs for the OUT cascade. (**A**) Crystal structure of the yeast E1 Uba1 in complex with UB (PDB ID 3CMM). The binding of yeast E2 Ubc1 with Uba1 was modeled in the structure (*74*). Cys600 and Cys88 are the catalytic Cys residues for UB conjugation in Uba1 and Ubc1, respectively. To initiate UB transfer, UB is first bound to the adenylation domain of Uba1. The energy of ATP hydrolysis is then used to form a thioester conjugate between the UB C-terminal carboxylate and the catalytic Cys residue of Uba1. UB in the UB~Uba1 conjugate ("~" designates the thioester bond) is then transferred to a catalytic Cys residue of E2 that is bound to the UB fold domain (UFD) of Uba1. (**B**) UB binding with key residues in the adenylation (A) domain of Uba1. R42 and R72 in UB were mutated to Glu to generate xUB, and correspondingly, Q576, Ser589 and D591 in Uba1 were mutated to Arg to generate xUba1(A) for activation of xUB (*12*). xUB could react with xUba1(A) in the presence of ATP to form xUB~xUba1(A) thioester conjugates. In contrast, xUB could not be activated with wt Uba1, and vice versa, wt UB was not reactive with xUba1(A). So the xUB-xUba1(A) and wt UB-wt Uba1 pairs were orthogonal to each other. (**C**) Binding of the N-terminal helix of Ubc1 with the UFD domain of Uba1. To generate orthogonal xE1-xE2 pair, we introduced mutations

E1004K, D1014K, E1016K into the UFD domain of yeast Uba1 to construct mutant xUba1 (UFD) (*12*). It could still activate wt UB to form wtUB-xUba1(UFD) thioester conjugates but it was incapable of transferring xUB to wt Ubc1 since the UFD domain mutations blocked the binding of the N-terminal helix of the E2 enzymes. To restore E2 interaction with xUba1(UFD), we used phage selection to screen a Ubc1 library with randomized N-terminal helix residues. We identified a Ubc1 mutant (xUbc1) with mutations K5D, R6E, K9E, E10Q, Q12L that could restore wt UB transfer from xUba1(UFD) to xUbc1 (*12*). By combining the mutations in xUba1(A) and xUba1(UFD), the newly generated xUba1 mutant could transfer xUB to xUbc1 for the formation of xUB~xUbc1 conjugate. xUB could not be transferred by xUba1 to wt E2 enzymes. So the xUba1-xUbc1 pair was orthogonal to the wt E1-E2 pairs in xUB transfer. (**D**) Alignment of the peptide sequences of the N-terminal helices of the E2 enzymes. K4 and K8 of UbcH5b were mutated to Glu to generate xUbcH5b for pairing with human xUba1.

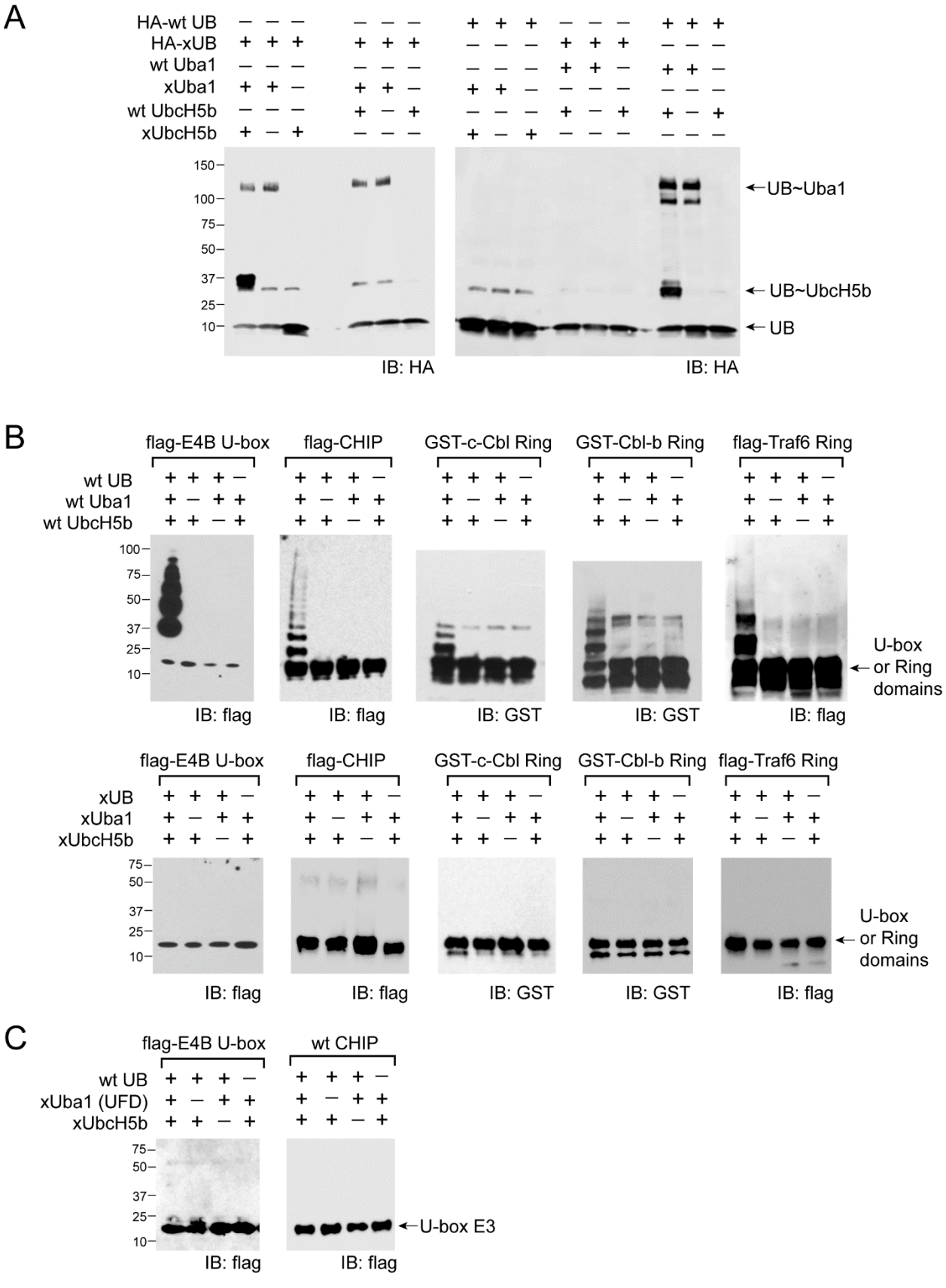
A
HA-wt UB
HA-xUB
wt Uba1
xUba1
wt UbcH5b
xUbcH5b
150
100
75
50
37
25
10
UB~Uba1
UB~UbcH5b
UB
IB: HA
IB: HA
B
flag-E4B U-box
flag-CHIP
GST-c-Cbl Ring
GST-Cbl-b Ring
flag-Traf6 Ring
wt UB
wt Uba1
wt UbcH5b
100
75
50
37
25
10
U-box
or Ring
domains
IB: flag
IB: flag
IB: GST
IB: GST
IB: flag
flag-E4B U-box
flag-CHIP
GST-c-Cbl Ring
GST-Cbl-b Ring
flag-Traf6 Ring
xUB
xUba1
xUbcH5b
75
50
37
25
10
U-box
or Ring
domains
IB: flag
IB: flag
IB: GST
IB: GST
IB: flag
C
flag-E4B U-box
wt CHIP
wt UB
xUba1 (UFD)
xUbcH5b
75
50
37
25
10
U-box E3
IB: flag
IB: flag

fig. S2. xUB transfer through the human xUba1-xUbcH5b pair. (**A**) xUB could be activated by xUba1 to form xUB~xUba1 thioester and be further transferred to xUbcH5b to form the xUB~xUbcH5b conjugate. Crossing xUba1 with wt UbcH5b would only generate xUB~xUba1 but the transfer of xUB to wt UbcH5b was blocked. Neither could xUB be activated by wt Uba1, nor wt UB be activated by xUba1. (**B**) xUB transfer through xUba1-xUbcH5b to the wt U-box or Ring domains of the E3s E4B, CHIP, c-Cbl, Cbl-b, and Traf6 was blocked. In contrast, wt UB transfer through wt Uba1-UbcH5b pair to the E3s was quite efficient. (**C**) wt UB transfer through xUba1(UFD)-xUbcH5b pair to the U-box domains of E4B and CHIP was also blocked suggesting a defective E2-E3 interface between xUbcH5b and wt U-box domains.

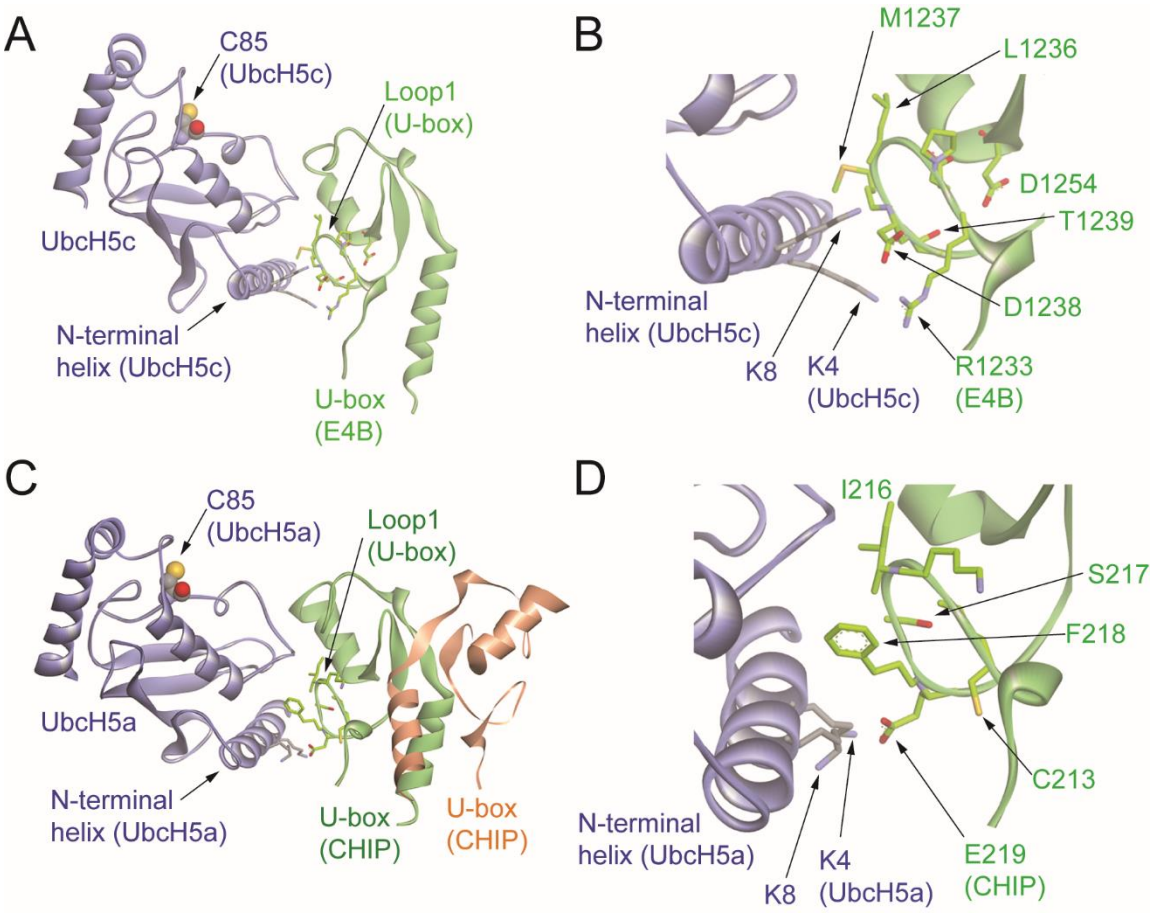

fig. S3. Structure analysis of the U-box domains of E4B and CHIP interacting with the UbcH5c and UbcH5a, respectively. (**A**) Crystal structure of UbcH5c in complex with the U-box domain of E4B (PDB ID 3L1Z) (*16*). Cys85 is the catalytic Cys for UB conjugation in UbcH5c. N-terminal helix of UbcH5c interacts with loop1 of the U-box domain of E4B. (**B**) Interaction between K4 and K8 of UbcH5c with the loop1 residues in the U-box domain of E4B. K4 and K8 in UbcH5b were mutated to Glu to generate xUbcH5b and they mainly engage loop1 residues R1233, L1236, M1237, D1238, and T1239 of the U-box. The ε-NH_2 of K8 of UbcH5c is 3.5 Å away from the carboxylate oxygen of D1238 of the U-box; the two groups may interact by a salt bridge or a hydrogen bond. The sidechain of K8 is also close to L1236 and M1237, which are 5-6 Å away. The ε-NH_2 of K4 may be engaged in a salt bridge interaction with D1238 of the U-box loop1, and/or interact with nearby R1233 and T1239 ($\leq$ 6 Å). The hydroxyl group of the T1239 sidechain may bind to the carboxylate of D1254 with hydrogen bonding interactions to stabilized the U-box fold. Based on the structural analysis, U-box residues R1233, L1236, M1237, D1238 and T1239 were randomized to construct the U-box library for phage selection. (**C**) Crystal structure of UbcH5a binding to the homodimer of the U-box domain of CHIP (PDB ID 2OXQ) (*18*). Cys85 is the site of UB conjugation in UbcH5a. The two CHIP U-box domains in the dimer are shown in green and orange,

respectively. (**D**) Interaction between K4 and K8 of UbcH5a with loop 1 of CHIP U-box is similar to UbcH5c interacting with the E4B U-box. E219 in CHIP is sandwiched between K4 and K8 residues of UbcH5a and may play a major role in binding to E2. Residues between Cys213 and Glu219 were replaced with the corresponding loop1 residues in the KB2 and KB12 mutants of E4B U-box to generate CHIP-KB2 and CHIP-KB12 to restore CHIP interaction with xUbcH5b.

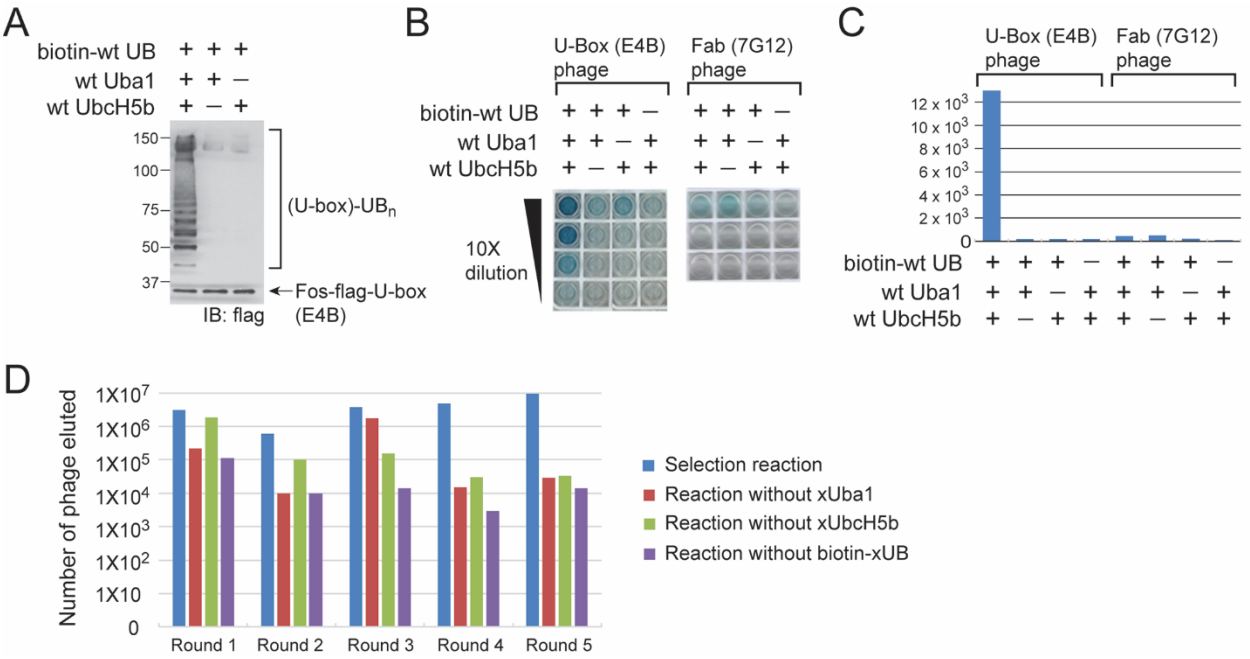

fig. S4. Model selection of E4B U-box domain displayed on M13 phage. (**A**) E4B U-box retained its activity upon displaying on M13 phage surface as a fusion with the Fos peptide. wt U-box domain anchored on phage surface could be ubiquitinated by wt UB through the wt Uba1-UbcH5b pair with high efficiency. (**B**) Phage ELISA to measure the efficiency of phage selection based on UB transfer. M13 phage displaying wt U-box domain of E4B were added to a UB loading reaction including biotin-wt UB, wt Uba1 and UbcH5b. Control reactions were set up to exclude either Uba1 or UbcH5b from the reaction mixture. After the reaction, the phage mixture was added to the 96-well plate coated with streptavidin. Serial 10-fold dilution was carried out across the plate. The plate was washed and the amount of biotin-labeled phage bound to the streptavidin plate was revealed by binding to a mouse anti-phage antibody followed by an anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP). Development of the plate with a chromogenic substrate suggested that the amount of phage bound to the streptavidin plate after biotin-UB loading was more than 100-fold higher than the controls missing wt Uba1, UbcH5b or biotin-wt UB in the reaction. In another control, phage displaying the Fab fragment of the 7G12 antibody were used (*75*) and ELISA showed the phage could not be loaded with biotin-UB after incubation with wt Uba1 and UbcH5b. (**C**) Model selection of phage displaying the U-box domain of E4B after biotin-UB transfer. Similar to (**B**), after the UB loading reaction, phage were bound to the streptavidin plate. The plate was washed and the phage were eluted by dithiothreitol (DTT) that would cleave the disulfide bond between the Jun and Fos peptide for U-box anchoring on phage surface. The numbers of phage eluted from the biotin-UB loading reaction and controls were determined by phage titering. Results showed that

the number of phage eluted from the UB loading reaction with wt Uba1 and UbcH5b was at least 200-fold higher than the number of phage eluted from the control reactions missing either wt Uba1 or UbcH5b. Furthermore, when phage displaying the Fab fragment of the 7G12 antibody were mock reacted with biotin-UB and the wt Uba1-UbcH5b pair, no phage enrichment was found comparing to controls without wt Uba1 or UbcH5b. Such results demonstrated that phage selection based on UB transfer was targeting the U-box domains on phage. (**D**) Phage library of the E4B U-box underwent five round of selection based on biotin-UB transfer through the xUba1(UFD)-xUbcH5b pair. Control reactions without xUba1(UFD), xUbcH5b or biotin-wt UB were set up. As the selection proceeded, there was an increasing difference in the numbers of phage eluted from the selection and control reactions.

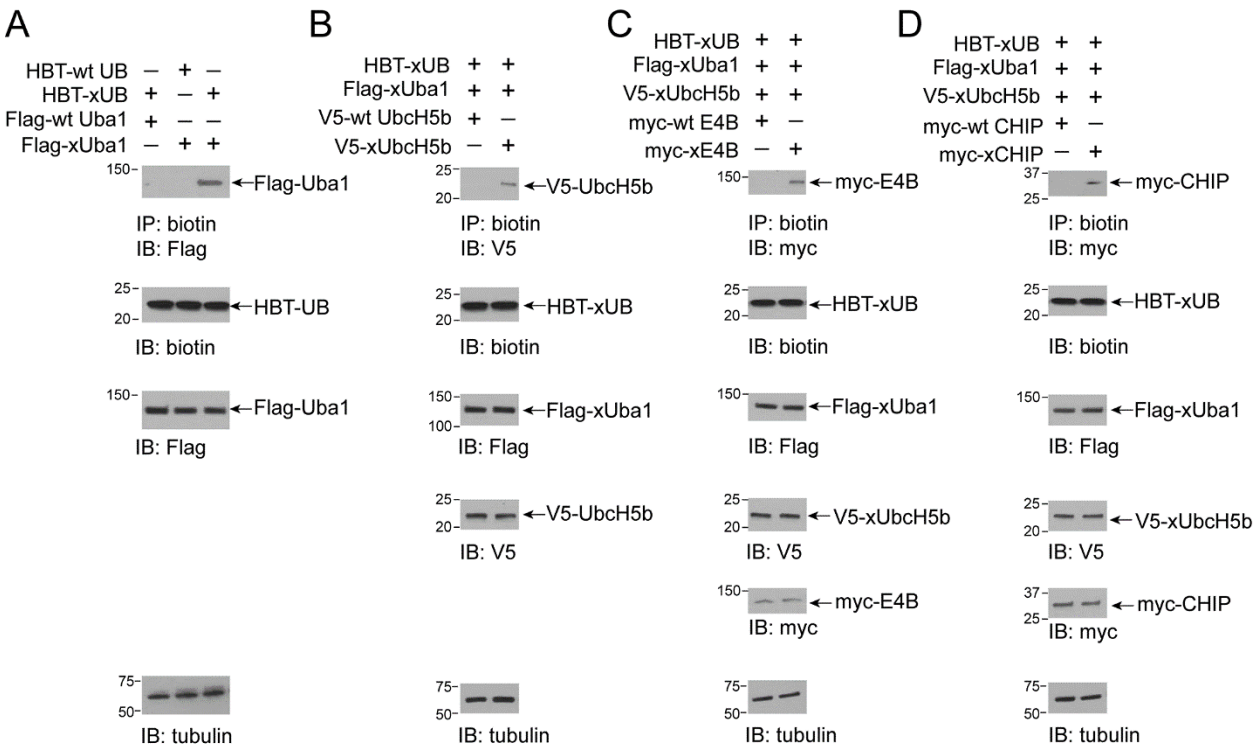

fig. S5. Orthoganality of the OUT cascades of E4B and CHIP with the native UB tranferring enzymes. (**A**) xUB and xUba1 formed xUB~xUba1 conjugate in HEK293 cells. As a result of conjugate formation, flag-xUba1 could be copurified with HBT-xUB by binding to streptavidin beads. In contrast, flag-wt Uba1 could not be copurified with HBT-xUB, neither could flag-xUba1 be copurified with HBT-wt UB in the cells co-expressing the UB-E1 pairs. This suggests that the crossover xUB-wt Uba1 or wt UB-xUba1 pairs were not active in the cell. (**B**) xUB could be transferred through xUba1-xUbcH5b pair to form xUB~xUbcH5b conjugate in the cell. Due to the conjugate formation, V5-xUbcH5b could be copurified with HBT-xUB from cells expressing HBT-xUB, flag-xUba1 and V5-xUbcH5b. In contrast, the expression of HBT-xUB and the crossover pair of xUba1-wt UbcH5b would not generate xUB~wt UbcH5b conjugate, so V5-wt UbcH5b could not be co-purified with HBT-xUB. (**C**) xUB could be transferred through xUba1-xUbcH5b-xE4B cascade to form xUB-xE4B conjugate in the cell. As a result, myc-xE4B could be co-purified with HBT-xUB in cells expressing HBT-xUB and the xUba1-xUbcH5b-xE4B cascade. In contrast, coexpression of HBT-xUB with the crossover cascade of xUba1-xUbcH5b-wt E4B would not generate xUB~wt E4B conjugate, so myc-wt E4B could not be co-purified with HBT-xUB. (**D**) Similarily, xUB could be transferred through xUba1-xUbcH5b-xCHIP cascade to form xUB-CHIP conjugate in the cell. However xUB could not be transferred to wt CHIP by xUba1-xUbcH5b to form xUB-wt CHIP conjugate.

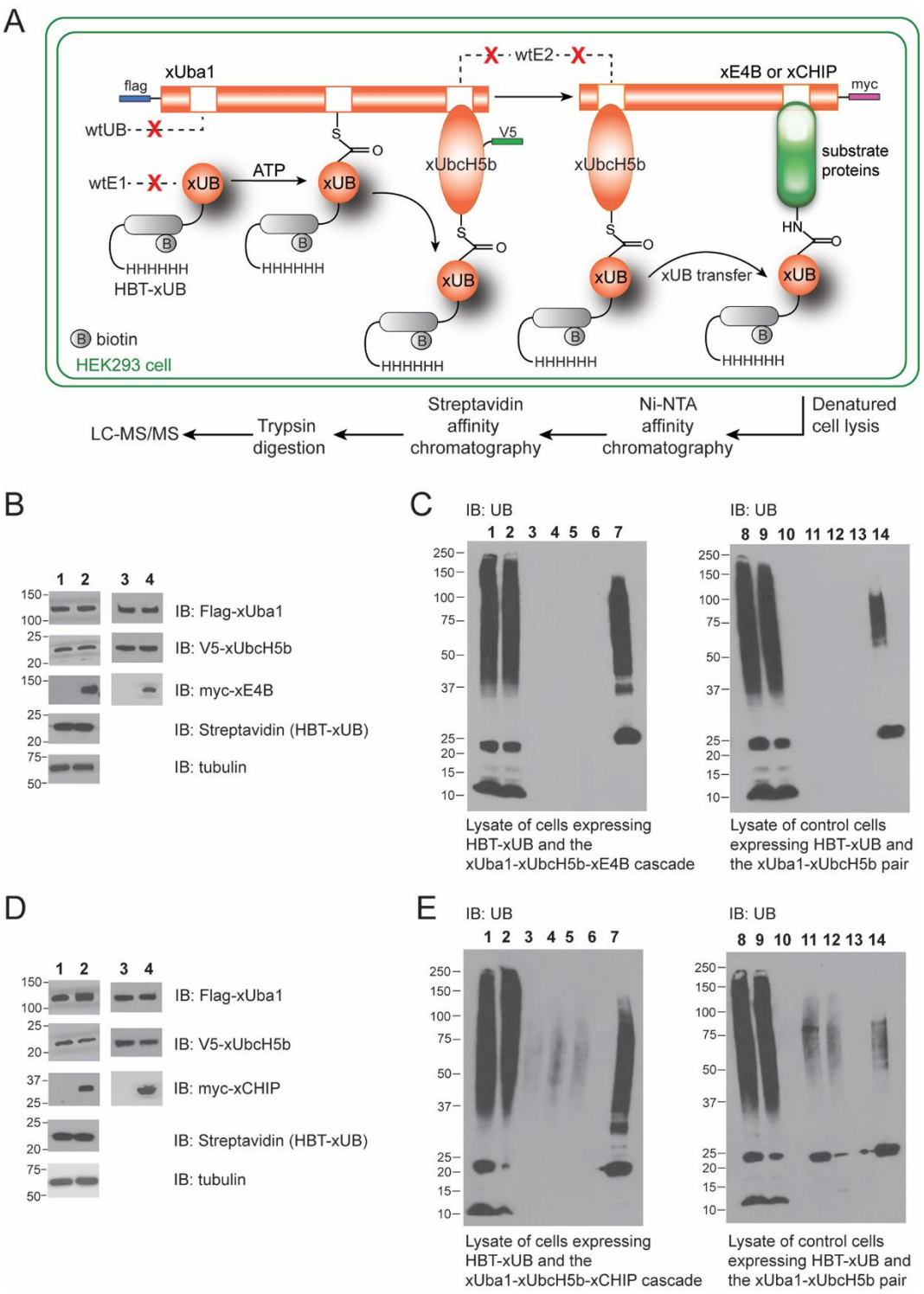

fig. S6. Affinity purification of xUB modified proteins to identify the substrates of E4B and CHIP. (**A**) Scheme to show cells expressing the OUT cascade of E4B and CHIP, tandem affinity purification of xUB-conjugated proteins from the cell lysate, and identification of purified proteins by LC-MS/MS. (**B**) Expressing the OUT cascade of E4B and HBT-xUB in cells. Expression of the components of the xUba1-

xUbcH5b-xE4B cascade was confirmed in HEK293 cells (lane 2). In control cells, the xUba1-xUbcH5b pair was expressed without xE4B (lane 1). To confirm xUB transfer through the OUT cascade, lysates of the cells expressing the OUT cascade of E4B and HBT-xUB were purified by tandem affinity columns of Ni-NTA and streptavidin, and the co-purification of xUba1, xUbcH5b, and xE4B in the xUB-conjugated fraction was confirmed by Western blot (lane 4). Similar purification was done on lysate from cells expressing xUba1-xUbcH5b pair without xE4B. xUba1 and xUbcH5b were co-purified as xUB conjugated proteins (lane 3). (**C**) Purification of xUB conjugated proteins from lysates of cells expressing the full OUT cascade of E4B (lanes 1-7). The Western blots of the gels were probed with an anti-UB antibody. Lane 1, cell lysate; lane 2, flow-through from the Ni-NTA column; lane 3, wash solution of the Ni-NTA column; lane 4, elution from the Ni-NTA column; lane 5, flow-through from the streptavidin column; lane 6, wash solution of the streptavidin column; lane 7, protein bound to the streptavidin beads after washing. Lanes 8-14 were the same as lanes 1-7 respectively, except that xUB-conjugated proteins were purified from control cells with the expression of xUba1-xUbcH5b but without xE4B expression. xUB-conjugated proteins eluted from the Ni-NTA column (lanes 4 and 11) were much diluted comparing to the xUB-conjugated proteins bound to the streptavidin beads (lanes 7 and 14). (**D**) Same as (**B**) to confirm the expressing of OUT cascades of CHIP in cells expressing xUba1-xUbcH5b-xCHIP (lane 2) and in the control cells missing xCHIP expression (lane 1). xUba1, xUbcH5b and xCHIP were purified as xUB-conjugated proteins from lysates of the cell expressing the OUT cascade of CHIP (lane 4). xUba1 and xUbcH5b were purified as xUB-conjugated proteins from control cells without xCHIP expression (lane 3). (**E**) Same as (**C**) except that the purification was performed on cells expressing the OUT cascade of CHIP and on control cells without xCHIP expression.

table S5. Primers used in this study.

Primers	Sequences, 5’ to 3’
Bo13	GTACTTGACACAACCG
Bo73	CGGAGCTCGAATTCTCATAGATGAATGGTAATGAAAGGAACTTTAACCTTCTCT CCTTCCTTGTCATCTGCGCAAATTTTGAGAATCATTGTAGATAC
Bo184	CGA GTG GTG ATC CCC TTC CTG ACA GAG TCG TAC AGT TCC CGC CAG CGC CCA CCT GAG AAG TCC ATC CCC
Bo185	CGACTCTGTCAGGAAGGGGATCACCACTCGCACATTGCCTTTGGTGCCCAG
Bo186	GCTCAGCATGGCCGGCCACC
Bo187	GCCAGACTTGGGGGTGAATTC
Kar64	CAATTGGGACTCGAGCATCGCATACTTCTG
Kar65	AGGAGATATCATATGGCTCTGGAAAGAATCCACGAAGAATTGAATGATCTGGC ACGG
Kar77	GGAAGTTCTGCGCGCCGAGCTCGGATCCATAGAGAAG
Kar78	TTTGATCTCGGTACCGTGGTCACTGCTCTG
Kar203	TCTCACGGGATCCGTCATCAGCGCAGCGGCTGCAGGGTCAGCGAACTCGTCCG GGGCATC
Kar204	CCTGCAGCCGCTGCGCTGATGACGGATCCCGTGAGACTGCCC
Kar232	GACATCCCCGACTACCTGAAGGACCCTATTATGCATACGCTGATGCGGGAGCC GTGCATC
Kar233	GACATCCCCGACTACCTGAAGGACCCTATTATGAGGACGCTGATGCGGGAGCC GTGCATC
Kar234	CAGGTAGTCGGGGATGTCTCGCTTCTTCCTCTTCTCATC
Kar238	GAACTCGTCCGGGGC
Kar239	GCCCCGGACGAGTTCAAGGACCCTATTATGCATACGCTGATGACCGATCCCG
Kar240	GCCCCGGACGAGTTCAAGGACCCTATTATGAGGACGCTGATGACCGATCCCG
Kar241	TTCGATGATCTCGAGTCAGTGGTCACTGCTCTGTTTCTCTC
Kar242	GTCTCTGAAGCGGATCCATGAAGTGCAAGAAGAGATG
Kar301	CAGCCCAGACTCGAGTATGGAACAAAAACTCATCTCAGAAGAGGATCTGGCTA GCGAGGAGCTGAGCGCTGAC
Kar302	GATGATACCGGTTCAGTGGTCACTGCTCTGTTTCTCTCTCATCC
Kar303	TGGTGGTGCTCGAGGTCTAGTCAGTAGTCCTCCACCCAGCCATTCTCAGAGATG AATGCG
Kar304	CAGCCCAGAGAATTCTATGGAACAAAAACTCATCTCAGAAGAGGATCTGGCTA GCAAGGGCAAGGAGGAGAAGGAGGGCGGCGCACGG
Kar305	AGAAGGAGATATACATATGAAGGGCAAGG
Kar306	TGGTGGTGCTCGAGGTCGGTACCGTAGTCCTCCACCCAGCC
Karlib1	CCCTCTGGCACCGTCATGGAC
Karlib2	GTCCATGACGGTGCCAGAGGGTAACCTCACGGGATCCGTCATCAGMNNMNNM NNMNNAGGGTCMNNGAACTCGTCCGGGGCATC
WY15	ACTGCTGAATTCACCCCCAAGTCTGGCGTCAAG-3’
WY16	GGCTAGTGGCGCGCCTCATCAGCGGATGGTGTATCG-3’
WY101	TTCACCATGAGCGCTATGGCTCTGGAAAGAATCCACGAAGAATTG
WY102	CCCACCCTTGCTAGCCATGGCATACTTCTGAGT
Jun13	ACTTTTGCTTCCGGCTCGTATGT