Identifying the ubiquitination targets of E6AP by orthogonal ubiquitin transfer

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E3 ubiquitin (UB) ligases are the ending modules of the E1-E2-E3 cascades that transfer UB to cellular proteins and regulate their biological functions. Identifying the substrates of an E3 holds the key to elucidate its role in cell regulation. Here, we construct an orthogonal UB transfer (OUT) cascade to identify the substrates of E6AP, a HECT E3 also known as Ube3a that is implicated in cancer and neurodevelopmental disorders. We use yeast cell surface display to engineer E6AP to exclusively transfer an affinity-tagged UB variant (xUB) to its substrate proteins. Proteomic identification of xUB-conjugated proteins in HEK293 cells affords 130 potential E6AP targets. Among them, we verify that MAPK1, CDK1, CDK4, PRMT5, β-catenin, and UbxD8 are directly ubiquitinated by E6AP in vitro and in the cell. Our work establishes OUT as an efficient platform to profile E3 substrates and reveal the cellular circuits mediated by the E3 enzymes.

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ubiquitin (UB), a 76-residue protein riding on a E1–E2–E3 enzymatic cascade, is a key messenger in cell signaling. UB attachment to cellular proteins regulates many key processes such as protein degradation, subcellular trafficking, enzymatic turnover, and complex formation. E1 activates UB with the formation of a thioester linkage between a catalytic Cys of E1 and the C-terminal Gly of UB. UB bound to E1 is loaded on an E2 in a thioester exchange reaction to form a UB–E2 conjugate (~7 designates the thioester bond). E2 then carries UB to an E3 that recruits target proteins for UB conjugation. The human genome encodes 2 E1s, at least 40 E2s and more than 600 E3s, and various E3s transfer UB to an overlapping pool of protein ubiquitination targets, which a UB variant (xUB) is confined to the substrate of a specific E3. Since E3s recognize protein ubiquitination targets, they often play key regulatory roles, and their malfunction drives the development of many diseases including cancer, neurodegeneration, and inflammation. For example, E6AP, also known as Ube3a, is a E3 with a signature HECT domain for E2 binding. E6AP is a critical regulator of neuron development; loss of its activity results in Angelman syndrome (AS), and duplications of the chromosomal region 15q11-13 including its encoding gene Ube3a are associated with autism spectrum disorders (ASD). E6AP promotes tumorigenesis upon infection of high-risk human papillomavirus—it forms a complex with the viral oncoprotein E6 to ubiquitinate p53 and induce its degradation. Other non-HECT E3s may bind the E2–UB conjugate through a Ring, Ring-between-Ring (RBR) or U-box motif. Regardless of the sequence homology between the human and yeast Uba1, we decided to engineer human Uba1 as the xE1 for the OUT cascade. Based on the sequence homology between the human and yeast Uba1, we identified residues Q608, S621 and D623 in the adenylation domain and E1004, D1014, and E1016 in the UFD domain of human Uba1 matching the sites of mutations in the yeast Uba1 (Supplementary Fig. 1d, e). We mutated these residues to R or K of the opposite charge to generate human xUba1 (Table 1). As expected, we found human xUba1 is reactive with xUB by forming xUB–xUba1 conjugate, yet it rejects wt UB in the activation reaction (Fig. 1a). Moreover, xUba1 cannot transfer xUB to wt human E2s such as UbcH7 due to the mutations in the UFD domain of Uba1.

To restore xUB transfer to E2s, we generated orthogonal xE1–xE2 pairs based on the sequence homology between the yeast and human E2s. The N-terminal helix of E2 plays a key role in binding the UFD domain of the E1 as shown in the crystal structures of yeast S. pombe Uba1 in complex with E2 Ubc4, and the modeled structure of S. cerevisiae Uba1 bound with Ubc1 (Supplementary Fig. 1a, c). The sequences of the N-terminal helices of E2s from yeast and human align well with highly conserved K or R residues at positions 5, 6, and 9 (UbcH7 numbering) (Supplementary Fig. 1f). Based on the sequence alignment, we mutated R5 and K9 in UbcH7 to Glu following the mutations in yeast Ubc1 and found the newly constructed xUbcH7 can pair with xUba1 to accept xUB transfer (Fig. 1a and Table 1). We have thus constructed an xUba1–xUbcH7 pair for xUB transfer through the OUT cascade. Since UbcH7 partners with HECT E3 in the cell, the exclusive delivery of xUB by xUba1 restores the activity of xUB with E1 to form xUB–E1 thioester conjugates (Supplementary Fig. 1a, b). We also introduced mutations E1004K, D1014K and E1016K into the UFD domain of the yeast Uba1 to block its interaction with the wt E2s (Supplementary Fig. 1c). We then used phage display to identify mutations in the N-terminal helix of the Ubc1, a yeast E2, to restore E1–E2 interaction and enable UB transfer to the E2 enzyme. By combining the mutations in the adenylation and the UFD domains of yeast Uba1, we generated the E1 mutant xUba1 that can specifically transfer xUB to xUbc1, the E2 mutant from phage selection (Table 1). In contrast, xUB cannot be activated by wt Uba1 for its transfer to wt E2s. xUba1 cannot activate wt UB, neither can it transfer xUB to wt E2. Thus, the xUB–xE1 and xE1–xE2 pairs are orthogonal to their native partners, and they can assemble a two-step cascade to transfer xUB to a designated E2.

Our success in engineering the xUB–xE1 and xE1–xE2 pairs with the yeast system is instrumental for constructing the OUT cascade in the human cell. We found xUB is not catalytically active with either of the human E1s, Uba1 (also known as Ube1), or Ube4. Since Uba1 plays a major role in supporting protein ubiquitination in the human cell, we used yeast cell surface display to engineer an orthogonal xE2, and various E3s transfer UB to an overlapping pool of substrates. The complex cross-reactivities among E2, E3, and substrates make it a significant challenge to profile the substrates of a specific E3 to map it on the cell signaling network.

We envision an “orthogonal UB transfer (OUT)” pathway in which a UB variant (xUB) is confined to a single track of engineered xE1, xE2, and xE3 would guide the transfer of xUB exclusively to the substrate of a specific E3 (x”designates engineered UB or enzyme variants orthogonal to their native partners). By expressing xUB and the OUT cascade of xE1–xE2–xE3 in the cell and purifying cellular proteins conjugated to xUB, we would be able to identify the direct substrates of an E3. The development of the OUT cascade removes the cross-reacting paths among various E2s and E3s. It enables the assignment of E3 substrates by directly following xUB transfer through the E3 instead of reading some indirect indicators of protein ubiquitination such as affinity binding with E3, or change of protein stability or ubiquitination levels upon E3 expression.

To implement OUT, we need to engineer orthogonal pairs of xUB–xE1, xE1–xE2, and xE2–xE3 that are free of cross-reactivities with native E1, E2, and E3 to secure the exclusive transfer of xUB to the substrates of an E3 in the cell. We previously reported engineering orthogonal xUB–xE1 and xE1–xE2 pairs by phage display. We also generated the xUB–xE1 pairs with the two human E1, Uba1, and Uba6, respectively, to differentiate their targets of UB transfer in the cell. Here we report that we have accomplished the last leg of OUT engineering: we used yeast cell surface display to engineer an orthogonal xE2–xE3 pair with the HECT E3 E6AP; we expressed the OUT cascade in HEK293 cells to profile E6AP substrates; and we identified a number of key signaling proteins as E6AP substrates and established regulatory circuits mediated by UB transfer through E6AP.

### Results

**Constructing the xUB–xUba1 and the xUba1–xUbcH7 pair.** We previously generated an xUB–xE1 pair with the E1 enzyme Uba1 from S. cerevisiae. Using phage selection, we found that the two mutations in xUB (R42E and R72E) would block xUB recognition by wt Uba1, yet by incorporating mutations Q576R, S589R and D591R into the adenylation domain of yeast Uba1, we could restore the activity of xUB with E1 to form xUB–E1 thioester conjugates (Supplementary Fig. 1a, b). We also introduced mutations E1004K, D1014K and E1016K into the UFD domain of the yeast Uba1 to block its interaction with the wt E2s (Supplementary Fig. 1c).

| Table 1 Mutants for the assembly of the OUT cascade with E6AP |
|---------------------------------------------------------------|
| **xUB (human)** | R42E, R72E |
| **xE1** | Q576R, S589R, D591R, E1004K, D1014K, E1016K |
| **xUba1 (yeast)** | Q608R, S621R, D623R, E1037K, D1047K, E1049K |
| **xUba1 (human)** | R5, K9 |
| **xE2** | KSD, R6, K9, E10Q, Q12L |
| **xUbc1 (yeast)** | R5, K9 |
| **xUbc7 (human)** | R5, K9 |
| **xE5** | R5, K9 |
| **xE6AP (YW6)** | D651R, D652E, M653W, M654H |


to xUbcH7 paved the way for transferring xUB to a specific HECT E3 to profile its substrate proteins.

**Constructing the xUbcH7-xHECT pair with E6AP.** The N-terminal helix of UbcH7 is a key element not only for interaction with E1s but also for interaction with E3s (Fig. 2a, b). We found the R5E and K9E mutants in the N-terminal helix of xUbcH7 interfered with the transfer of xUB to wt HECT E3s such as E6AP, Nedd4, Smurf1, and Smurf2 (Fig. 1b–d). This is advantageous for the construction of the OUT cascade since it is preferred that xE2 would not pair with wt E3s to randomly transfer xUB to any E3 substrates in the cell. Our goal was to bridge xUB transfer through the last step of the OUT cascade by engineering an orthogonal xUbcH7–xE6AP pair. For this purpose, we used yeast cell surface display to select for HECT mutants of E6AP that would restore binding with xUbcH7 to enable xUB loading on the HECT domain (Fig. 2c). For yeast selection, a HECT library of E6AP was expressed as fusions to the yeast cell surface protein Aga2P with each yeast cell displaying a specific member of the HECT library. The yeast library was then reacted with biotin-labeled xUB, xUba1, and xUbcH7. HECT mutants catalytically active with xUbcH7 were loaded with xUB through the formation of xUB–HECT thioester conjugate. The catalytically active HECT mutants were further auto-ubiquitinated by xUB through Lys modification.

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**Fig. 1** Orthogonality of the xUba1–xUbcH7 pair with the wt UB transferring enzymes. **a** Wt UB can be transferred to wt UbcH7 by wt human Uba1, but it cannot be activated by human xUba1 for transferring to xUbcH7. Vice versa, xUB can be activated by xUba1 for transferring to xUbcH7, but it cannot be activated by wt Uba1 for transferring to wt UbcH7, nor can it be transferred to wt UbcH7 by xUba1. The protein gels were run under non-reducing conditions to preserve the thioester conjugates of UB~E1 and UB~E2. **b–d** HECT domains of wt E6AP, Nedd4, Smurf1, and Smurf2 can be loaded with wt UB through the wt Uba1–UbcH7 pair. Yet they are not reactive with xUB though the xUba1–xUbcH7 pair. The protein gels were run under reducing conditions to probe the auto-ubiquitination of the HECT enzymes.
with phycoerythrin (PE). For selection of yeast cells displaying the HECT domain, a mouse anti-HA antibody was used to bind to the HA tag at the N-terminus of the HECT and it was subsequently labeled with an anti-mouse IgG conjugated with Alexa 647. Fluorescence-activated cell sorting (FACS) was used to enrich the cells that were double labeled with the PE and Alexa 647. In this way, the sorting was to enrich cells that displayed catalytically active HECT domains capable of bridging xUB transfer from xUbcH7. We validated the selection protocol by displaying the wt HECT domain of E6AP on the yeast cell surface, and demonstrating the efficient labeling of the yeast cells by biotin-wt UB transferred through the wt Uba1–UbcH7 pair (Supplementary Fig. 2).

We constructed a HECT library of E6AP based on the crystal structure of the HECT domain with UbcH7 (Fig. 2a, b)22. Residues R5 and K9 in the N-terminal helix of UbcH7 were mutated to Glu in xUbcH7 and the crystal structure shows that these residues mainly interact with a helical turn in the HECT domain of E6AP. R5 of UbcH7 is in close distance (4.6 Å) with the hydroxyl group of T656 of HECT. It also packs on the side chain of M654 that is 3.2 Å away. K9 of UbcH7 may form salt bridge with D651, D652, M653, M654, and T656 in the HECT domain of E6AP were randomized for library selection by yeast cell surface display. HECT mutant YW6 with mutations D651R, D652E, M653W, and M654H was selected and used as xHECT in this study. A mouse anti-HA antibody and an anti-mouse IgG conjugated with Alexa 647 were used to bind to the HA tag fused to the N-terminus of the HECT domain of E6AP and selection of the HECT library based on the transfer of biotin-xUB from xUbcH7 to the HECT domain. Biotin-xUB attached to the HECT domain was labeled with streptavidin-PE, and the HA tag fused to the N-terminus of the HECT domain was labeled with a mouse anti-HA antibody and streptavidin labeling, and cell sorting. Percentages in black designate fraction of yeast cells doubly labeled with PE and Alexa 647. The frames and percentages in blue designate the fraction of yeast cells collected by FACS in each round of selection.

The frames and percentages in blue designate the fraction of yeast cells collected by FACS in each round of selection. e Sequence alignment of the E6AP HECT clones selected by yeast cell surface display. Residues denoted by red stars were randomized in the HECT library.

**Fig. 2** Yeast selection of E6AP library to engineer the xUbcH7-xE6AP pair. a Crystal structure of the E6AP HECT domain in complex with UbcH7 (PDB ID 1C4Z)22. The N-terminal helix of UbcH7 plays a key role in interacting with the HECT domain. The catalytic Cys residues in UbcH7 and the E6AP HECT are shown in CPK models. b Detailed interactions between the N-terminal helix of UbcH7 and the HECT domain of E6AP. To complement the R5E and K9E substitutions in xUbcH7 and the crystal structure shows that the hydroxyl group of T656 of HECT. It also packs on the side chain of M654 that is 3.2 Å away. K9 of UbcH7 may form salt bridge with HECT D651 and D652 that are a short distance apart (4.5 Å). We thus decided to randomize E6AP residues D651, D652, M653, M654, and T656 to generate the HECT library.

We expressed the library on the yeast cell surface and carried out the selection by transferring biotin-xUB to the HECT mutants through the xUba1–xUbcH7 pair. Cells were labeled with streptavidin and antibody conjugates with fluorophores as in the model selection, and FACS was performed to harvest cells that were double labeled with PE and Alexa 647. Cells collected were cultered for next round of biotin-xUB loading, fluorescent labeling, and FACS. After 6 rounds of sorting, 51% of the cells were double labeled with both fluorophores suggesting a population of HECT mutants with efficient xUB transfer activity from xUbcH7 were selected (Fig. 2d). DNA sequencing of the 40 clones from the 6th round of sorting showed a clear pattern of convergence (Fig. 2e). Clones appearing multiple times tend to
Fig. 3 Activity in xUB transfer by the E6AP mutants identified from yeast cell selection. a Activity of the HECT mutants YW1–6 in auto-ubiquitination by xUB through the xUba1–xUbcH7 pair. b Activity of the full-length E6AP mutants fYW1, fYW4, and fYW6 in auto-ubiquitination by the xUba1–xUbcH7 pair (right panel). While wt E6AP can be auto-ubiquitinated by the wt Uba1–UbcH7 pair, it cannot be conjugated to xUB by the xUba1–xUbcH7 pair (right panel). c p53 ubiquitination by wt E6AP and fYW4 and fYW6 in the presence of E6. p53 can be efficiently ubiquitinated by wt UB through the wt Uba1–UbcH7–E6AP cascade in the presence of E6 (left panel). p53 can also be efficiently modified by xUB through the engineered cascade of xUba1–xUbcH7–fYW6 in the presence of E6 (right panels). In contrast, xUB has very low activity in ubiquitinating p53 through the crossover cascade of xUba1–xUbcH7–wt E6AP in the presence of E6. All blots are representative of at least three independent experiments.

Verifying xUB transfer through E6AP mutants. We separately cultured yeast clones YW1-6 and reacted the yeast cells with the xUba1–xUbcH7 pair for biotin–xUB loading (Supplementary Fig. 3). We found yeast clones YW4 and YW6 had the strongest loading of biotin–xUB with 25% and 34% of the cells doubly labeled. To check the activities of individual HECT domains, we expressed mutants YW1-6 in E. coli and foundYW3, YW4, and YW6 could be efficiently auto-ubiquitinated with xUB through the xUba1–xUbcH7 pair while YW1, YW2, and YW5 were not active for xUB transfer (Fig. 3a). We suspect the difference in activities of the HECT domains anchored on yeast cell surface and free in solution may be due to the change in their folding status in different environments. We replaced the wt HECT domain in E6AP with the mutant HECT of YW1, YW4, and YW6 to generate the full-length E6AP mutants fYW1, fYW4, and fYW6. We found fYW4 and fYW6 can be auto-ubiquitinated by xUB through the xUba1–xUbcH7 cascade (Fig. 3b). However, wt E6AP and fYW1 were not active in auto-ubiquitination by xUB in combination with the xUba1–xUbcH7 pair. We then tested the transfer of xUB from the E6AP mutants to p53, a key ubiquitination target recruited by E611, 16. We found fYW6 could efficiently ubiquitinate p53 with xUB through the xUba1–xUbcH7–fYW6 cascade and the ubiquitination was dependent on the E6 protein (Fig. 3c). The activity of xUB transfer to p53 through fYW6 was approaching the activity of wt UB transfer through wt E6AP. In contrast, when the xUba1–xUbcH7 pair was reacted with wt E6AP, we only observed very low activity in transferring xUB to p53 suggesting the minimal cross-reactivity of xUB with native UB transfer pathways (Fig. 3c). Comparing to fYW6, fYW4 was less active in transferring xUB to p53. We thus decided to use fYW6 as xE6AP in the OUT cascade to identify E6AP substrates in the cell (Table 1).

Verifying the orthogonality of OUT cascade in cells. We next tested if xUB could be exclusively transferred through the OUT cascade of xUba1–xUbcH7–xE6AP in the cells without crossing over to the wt UB transfer cascades (Fig. 4a). We constructed a lentiviral vector to express wt UB or xUB with tandem 6x His and biotin tags at the N terminus of UB (HBT-wt UB and HBT-xUB)23. We also screened HEK293 cells that stably expressed wt Uba1 or xUba1 with an N-terminal Flag tag. Transient expression of HBT-wt UB and HBT-xUB in these cells followed by affinity pull-down
with streptavidin beads showed that xUba1 was co-precipitated with HBT-xUB, but wt Uba1 could not be co-precipitated with HBT-xUB, neither xUba1 could be co-precipitated with HBT-wt UB (Fig. 4b). This suggests that xUB was exclusively reactive with xUba1 in the cell, and there is no cross activities between xUB and wt Uba1, or between wt UB and xUba1. To probe the orthogonality at the E1–E2 interface, we co-expressed HBT-xUB with either wt UbcH7 or xUbcH7 in cells stably expressing xUba1. We found V5-tagged xUbcH7 could be purified with the streptavidin beads suggesting the formation of HBT-xUB–xUbcH7 conjugate, yet V5-tagged wt UbcH7 could not be co-puriﬁed with HBT-xUB (Fig. 4c). This proves that xUba1–xUbcH7 was a functional relay for xUB in the cell, but xUba1–wt UbcH7 pair could not mediate xUB transfer to a wt E2. To verify the orthogonality at the E2-E3 interface, we co-expressed HBT-xUB with either wt E6AP or xE6AP in HEK293 cells stably expressing the xUba1–xUbcH7 pair, and puriﬁed the xUB-conjugated proteins by streptavidin beads. Myc-tagged xE6AP was co-puriﬁed with xUB suggesting the formation of xUB–xE6AP conjugate, yet no wt E6AP was conjugated with xUB (Fig. 4d). These results prove that xUba1–xUbcH7–xE6AP is an orthogonal cascade for the transfer of xUB, and the crossover of xUB to wt cascades was eliminated.

Proﬁling the substrates of E6AP in HEK293 cells by OUT. To express the OUT cascade of E6AP in the cell, we screened cell lines stably expressing xUba1, xUbcH7 and xE6AP by lentiviral infection. Western blots of the cell lysate probed with antibodies against each OUT component suggested their adequate
expression (Supplementary Fig. 4b). We also generated a stable cell line expressing the xUba1–xUbcH7 cascade without xE6AP as a control for the proteomic screen. To initiate xUB transfer through the OUT cascade, we transduced the two stable cell lines with lentivirus carrying the vector to express HBT-xUB. We then purified cellular proteins conjugated with HBT-xUB sequentially by Ni-NTA and streptavidin affinity columns under strong denaturing conditions (Supplementary Fig. 4a). We found xUb1, xUbc1, and xE6AP are among the proteins retained by tandem purification suggesting the loading of HBT-xUB to the engineered E1, E2, and E3 enzymes of the OUT cascade (Supplementary Fig. 4c). We then digested the proteins on the streptavidin beads by trypsin and analyzed the peptide fragments by LC-MS/MS to identify xUB-conjugated proteins. In parallel, we performed tandem purification and proteomic analysis on control cells expressing the xUba1-xUbcH7 pair without xE6AP (Supplementary Fig. 4d, e). By comparing the two proteomic profiles, we identified proteins that had ratios of peptide-spectrum match (PSM) 2-fold or higher between cells expressing the full E6AP OUT cascade and the control cells. We carried out affinity purification and proteomic screen three times. We found 130 proteins repeatedly appearing in all three screens with PSM ratio ≥2 (Supplementary Data 1). These proteins are likely the direct ubiquitination targets of E6AP.

Among the E6AP targets identified, we found previously identified substrates such as the UV excision repair protein HHR23A (RAD23A) and HHR23B (RAD23B), proteasomal ubiquitin receptor ADRM1, 26S proteasome non-ATPase regulatory subunit 4 (PSMD4 or Rpn10), 26S proteasome AAA-ATPase subunit Rpt5 (PSMC3), and E3 ligase RING2 (RNF2 or RING1B)24–27. Ingenuity Pathway Analysis (IPA) of the proteins from the OUT screen showed that E6AP targets have a significant association with a variety of canonical pathways (Supplementary Data 2). It is intriguing that several associated pathways mediate cell cycle control and chromosome replication, matching the role of E6AP in viral onco genesis. IPA also identified 8 protein networks that are significantly associated with E6AP substrates (Supplementary Data 3). The identified networks are related to cell death and survival, DNA replication, recombination, and repair, cellular growth and proliferation, and nervous system development and function.

We used the CRAPome database to evaluate whether proteins non-specifically bound to the affinity resins were among the targets identified by OUT. CRAPome selects non-specific binders in proteomic experiments based on the frequency of their appearance in pull-down experiments with various bait proteins under non-denaturing conditions28. In contrast, we used strong denaturing condition to purify xUB-conjugated proteins in the OUT screen. Nevertheless, we found 2 of the 130 E6AP targets identified by OUT have a frequency higher than 34% in the CRAPome database (Supplementary Data 4). We verified that one of them, PRMT5, is an E6AP target (see below). We also repeatedly identified 35 proteins in control cells without expression of xE6AP, and they were not present among xUB-conjugated proteins purified from cells expressing the full OUT cascade of E6AP (Supplementary Data 5).

In vitro and in vivo validation of the E6AP substrates. We found some key signaling enzymes such as kinases MAPK1, CDK1, CDK4, protein Arg methyltransferase 5 (PRMT5), transcription factor β-catenin, and FAS-associated factor UbxD8 are likely substrates of E6AP (Supplementary Data 1). We thus assayed if E6AP targets them for ubiquitination and regulates their stabilities in the cell. We first used the wt UB transfer cascade Uba1-UbcH7-E6AP to test if the potential substrate proteins could be modified by wt UB in vitro. We expressed and purified the potential substrates from E. coli, and found that they were ubiquitinated by E6AP to different extents: CDK1, CDK4 and β-catenin were strongly ubiquitinated by E6AP with the formation of high molecular weight bands, while MAPK1, PRMT5, and UbxD8 mainly generated species with one or two conjugated UBs (Fig. 5). As a positive control, E6AP-catalyzed ubiquitination of HHR23A, a previously identified E6AP substrate, was confirmed (Fig. 5g)26. Protein expressed in E. coli cells may not bear the proper posttranslational modifications for E6AP recognition, so the in vitro assays may not reflect the real activity of E6AP with the substrate proteins. We thus tested whether the potential substrates are targeted for ubiquitination by E6AP in the cell.

We inhibited E6AP expression in HEK293 cells with lentivirus delivering the anti-E6AP shRNA. We also overexpressed E6AP in blank HEK293 cells and cells harboring the anti-E6AP shRNA. Cells were treated with proteasome inhibitor MG132 before harvesting to inhibit protein degradation. Ubiquitination levels of various substrates in different cell populations were revealed by immunoprecipitation with substrate-specific antibodies and immunoblotting with an anti-UB antibody. Comparing to the parental HEK293 cells, cells expressing anti-E6AP shRNA had significantly lower levels of poly-ubiquitinated forms of MAPK1, CDK1, CDK4, PRMT5, β-catenin, and UbxD8 (Fig. 6). The poly-ubiquitination of each target protein in the HEK293 cells harboring the anti-E6AP shRNA can be restored by over-expressing E6AP in the cell. Furthermore, HEK293 cells with over-expression of E6AP gave rise to more intense poly-ubiquitination of MAPK1, CDK1, CDK4, β-catenin, and UbxD8 comparing to the parental HEK293 cells. The known E6AP substrate HHR23A showed similar dependence on E6AP for its ubiquitination in the HEK293 cell. These results prove that the potential E6AP substrates identified by the OUT screen are indeed E6AP targets in the cell.

To probe whether E6AP-mediated ubiquitination would signal protein degradation, we transiently transfected HEK293 cells with varying amounts of wt E6AP plasmid. Western blot of the cell lysates with an anti-E6AP antibody showed an increased E6AP expression had little effect on the level of MAPK1 in the cell. Furthermore, HEK293 cells with over-expression of E6AP gave rise to more intense poly-ubiquitination of MAPK1, CDK1, CDK4, β-catenin, and UbxD8 comparing to the parental HEK293 cells. The known E6AP substrate HHR23A showed similar dependence on E6AP for its ubiquitination in the HEK293 cell. These results prove that the potential E6AP substrates identified by the OUT screen are indeed E6AP targets in the cell.

Discussion
The large number of E3s (>600) encoded in the human genome reflects the key roles they play in cell regulation. On the other hand, their diversity makes it a significant challenge to identify the direct substrates of individual E3s. Current methods screening
E3 substrates fall into three categories—affinity binding to E3, monitoring changes in protein stability or ubiquitination levels in response to E3 perturbation, or trapping E3 substrates by covalent or noncovalent interactions (Supplementary Fig. 5). Affinity-based approaches such as co-immunoprecipitation, yeast two-hybrid system, and protein microarray have been used to screen E3 substrates based on the binding between E3 and substrates (Supplementary Fig. 5a)31–33. They are less specific since the Kd’s of the E3-substrate complexes are around hundreds of μM, and the complexes are transient34. Also, proteins other than substrates can bind to E3s to function as adaptors or regulators. Still, these methods yielded important substrate profiles of HECT E6AP, Ring E3 anaphase-promoting complex (APC), and the Skp1-cullin-F-box (SCF) complex31, 35. A more direct approach to assign E3 substrates is to correlate changes in E3 activity with the changes in stability or ubiquitination level of cellular proteins (Supplementary Fig. 5b). One method known as “global protein stability profiling (GPS)” tracks the stability of thousands of proteins with a fused fluorescence protein tag, and it has been used to screen substrates of SCF E3s35–37. The development of anti-diGly antibody allows affinity enrichment of substrate peptides containing the ubiquitination sites, and comparison of protein ubiquitination levels upon perturbation of E3 activity38. Using the quantitative diGly proteomics (QdiGly), substrate profiles of cullin-Ring and Parkin E3s were generated39, 40. E3s are also converted to substrate traps so the substrate proteins would still be bound to E3 after UB transfer. This would enable the co-purification of the substrate proteins with E3s. To create “UB-activated interaction traps (UBAIT)”, UB was fused to HECT and Ring E3s and it can attack the substrates bound to the E3-UB fusion to generate covalent linkages between E3 and the substrate proteins (Supplementary Fig. 5c)41. Another design is to fuse the F-box proteins with a series of UB associated domain (UBA) and use them as UB ligase traps42, 43. As F-box proteins recruit substrates to SCF E3s, the UB chain extending from the substrate would bind to the UBA repeats with high affinity. Purification of proteins bound to F-box-UBA fusion would enrich the substrates of the F-box protein (Supplementary Fig. 5d). E2-E3 fusions has also been used to identify E3 substrates. Ubc12, the E2 enzyme mediating Nedd8 transfer, was fused to the substrate binding domain of Ring E3 XIAP. The fusion protein, known as a "Neddylator", allows Nedd8 transfer to XIAP substrates so the ubiquitination targets of XIAP could be identified among Nedd8-modified proteins (Supplementary Fig. 5e)44. The development of diverse methods to profile E3 substrates enables the interrogation of E3 function from different perspectives. The substrate profiles generated by various methods could corroborate to reveal the functions of E3s.

Here, we developed a method known as “orthogonal ubiquitin transfer (OUT)” to identify the direct substrates of HECT E3 E6AP (Fig. 4a). In OUT, an affinity-tagged UB variant (xUB) is exclusively transferred through an engineered xE1-xE2-xE3 cascade to the substrates of a specific E3. By purifying xUB-modified proteins from the cell and identifying them by proteomics, we would be able to identify the direct substrates of a E3. In this study, we used OUT to identify 130 potential E6AP substrates, and among them, we confirmed MAPK1, PRMT5, CDK1, CDK4, β-catenin, and UbxD8 are ubiquitinated by E6AP in the HEK293 cells. During the revision of this manuscript, β-catenin was confirmed as an E6AP substrate by another report45. A key advantage of OUT is that it assigns E3 substrates by directly following UB transfer from the E3 to its substrate proteins. Methods based on E3 substrate binding, or change of protein stability upon perturbation of E3 activity, use indirect readouts of substrate ubiquitination to assign E3 substrates. The substrate profiles generated by those methods could be distorted by factors...
such as the binding of adaptor or regulatory proteins to E3, or the attachment of UB chains of non-degradation signals to the substrates. E3 may also regulate the activities of proteasome and other E3s, thus perturbing the activity of one E3 may affect the degradation or ubiquitination levels of the substrates of other E3s. E3 may also regulate the activities of proteasome and attachment of UB chains of non-degradation signals to the substrates by playing important roles in cell regulation. Another limitation of OUT is that co-expression of HBT-xUB and the full xE1–xE3 pair for the OUT cascade. The xUB–xE1 pair we used by OUT is a better reenactor of the wt E3 in transferring xUB to the substrate proteins to enable their identification by OUT.

OUT has a few limitations. First, each E3 would require its own OUT cascade for substrate identification. The xUB–xE1 pair we engineered can be used for the OUT cascade of various E3s. Due to the high sequence homology of the N-terminal helices of the E2s, mutations can be transplanted from xUbc1 to UbcH7 and UbcH5b to generate xE1–xE2 pairs. The great diversity of E3s would require the engineering of individual E3s to assemble xE2–xE3 pairs. Here we used yeast cell surface display to identify HECT mutants of E6AP that can pair with xUbcH7. The helical turn we randomized in the E6AP HECT domain is a common element in many HECT enzymes. It is possible to generate orthogonal xE2–xE3 pairs by transplanting the mutations from xE6AP to other HECTs E3s. If such strategy is not effective, the yeast selection platform for E6AP HECT could be used to engineer other HECTs such as Smurf1,2, Neddy4-1,2, and Huw1, all playing important roles in cell regulation. Another limitation of OUT is that co-expression of HBT-xUB and the full xE1–xE2–xE3 cascade, although successful in HEK293 cells, maybe a challenge in other cell types. The recently developed

Fig. 6 Cellular assays to test the ubiquitination of E6AP substrates identified by OUT. a Inhibition of E6AP expression in HEK293 cells by shE6AP was confirmed with Western blot probed with an antibody against E6AP. b–g Ubiquitination of MAPK1 b, PRMT5 c, CDK1 d, CDK4 e, β-catenin f and UbxD8 g in HEK293 cells was assayed by immunoprecipitation with antibodies against each substrate proteins and probing the ubiquitination levels of the proteins with an anti-UB antibody on the western blots. After 1.5-h treatment of cells with MG132, ubiquitination of each target protein was compared among the blank HEK293 cell (HEK293), HEK293 expressing shE6AP (shE6AP), HEK293 expressing both shE6AP and recombinant E6AP (shE6AP + OE), and HEK293 expressing recombinant E6AP (OE). h Ubiquitination of HHR23A, a known E6AP substrate, was assayed as a control. Rabbit IgG was used as a control for immunoprecipitation in b and e. Mouse IgG was used as a control for immunoprecipitation in c, d and f–h. All blots are representative of at least three independent experiments.
genome editing tools such as CRISPR/Cas9 may provide an opportunity to introduce the OUT cascade into the original genetic background to identify E3 substrates.

We found E6AP expression did not affect the stability of MAPK1. The ubiquitination and degradation of E6AP suggests a role for E6AP in lipid metabolism, since UbxD8, by forming a complex with p97/VCP, regulates lipid droplet size and abundance. Consistently, expression of a dominant-negative E6AP mutant promotes accumulation of lipid droplets, which may involve stabilized UbxD8 due to decreased

**Fig. 7** Effect of E6AP expression on the stability of the substrate proteins. **a** E6AP decreases the steady-state levels of the substrate proteins in the HEK293 cells. Cells were transfected with increasing amount of E6AP plasmid. Levels of the E6AP substrates were assayed with immunoblots of the cell lysate probed with substrate-specific antibodies. Approximately $5 \times 10^6$ cells were used for each transfection of the E6AP plasmid. **b** Quantitative analysis of substrate levels in correlation with E6AP expression. Intensity of the bands in **a** were plotted against the amount of pLenti E6AP plasmid used for transfection assuming 100% of substrate protein when an empty plasmid was used for mock transfection. Results were the average of three repeats. **c** E6AP-dependent degradation of substrate proteins assayed by cycloheximide (CHX) chase. HEK293 cells ($5 \times 10^6$ cells) were transfected with $4 \mu$g of pLenti E6AP plasmid with the same amount of empty pLenti plasmid used in the controls. The cells were treated with 100 mg/ml CHX 48 h after transfection. Cell extracts were collected at 0, 2, 4, and 6 h after incubation with CHX, followed by immunoblotting with substrate-specific antibodies. **d** Quantitative analysis of the levels of the substrate proteins in the cell in the CHX chase experiment. Data are representative of three independent experiments. Besides the new substrates identified by OUT, HHR23A, a known E6AP substrate, was assayed for its degradation regulated by E6AP. The vertical bars in **b** and **d** represent s.e.m. from three independent experiments.
activity of E6AP. The biological significance of E6AP-mediated ubiquitination of CDK1, CDK4 and PRMT5 awaits further investigations. Recent studies have suggested that E6AP is required for cellular senescence, i.e., irreversible exit from the cell cycle, as a physiological response to oxidative stress or oncogene activation. Thus, E6AP-mediated ubiquitination of CDKs in the absence of viral oncoproteins may be involved in the senescence response to various cellular stresses. Indeed, Ingenuity Pathway Analysis showed that many of the potential E6AP substrates identified by OUT are associated with pathways and networks relevant to DNA replication, cell cycle control, oncogenic signaling, cell survival/death and development (Supplementary Data 2 and 3). PRMT5 plays a key role in chromatin regulation by methylating Arg residues in histones. Since various studies have indicated the significance of epigenetic changes in cancers and autism spectrum diseases, it would be interesting to determine how E6AP-mediated PRMT5 ubiquitination is involved in the pathobiology of those diseases.60–62

**Methods**

**Reagents.** XL1 Blue cells were from Agilent Technologies (Santa Clara, CA, USA). pET-12b and pET-28a plasmids for protein expression were from Novagen (Madison, WI, USA). pCTCON2 plasmid and the yeast strain EBY100 were from K. Dane Wittrup of Massachusetts Institute of Technology. The plasmid with the human Uba1 gene was from Wade Harper of Harvard Medical School. The plasmid was obtained from a mammalian expression plasmid by the transfer of biotin from yeast selection, the genes of the mutant E6AP HECT from yeast selection, the genes of the potential E6AP substrates were digested with Nhel and XhoI and cloned into pCTCON2 plasmid to generate pCTCON2-E6AP HECT. To generate E6AP library in pCTCON2 plasmid, the E6AP HECT domain gene was PCR amplified with WY13 and WY14 to incorporate randomized codons at residues 651, 652, 653, 654, and 656. The PCR fragment amplified with WY13 and WY14 was combined with fragment amplified with primers WY7 and WY8 and cloned into the pET28a vector between restriction sites SacI and NotI. The CDK1 and CDK4 genes were PCR amplified from their cDNA in human genes with primers Bo13 and Bo73. PCR fragment was digested by restriction enzymes SacI and NotI, and cloned into pET28. The two PCR fragments had mutations Q608R, S621R and D623R incorporated into the adenylation(A) domain of Uba1. The two PCR fragments were assembled by overlapping PCR and cloned into the pET-wt Uba1 vector between restriction sites SacI and NotI. The two PCR fragments had mutations in both the adenylation and the UFD domains. pET-UbcH7 plasmid was digested with Nhel and XhoI, and cloned into the pET28a. For the expression of full-length E6AP, PCR primers WY21 and WY8 were used to amplify the full-length gene from pGEX4-wt E6AP and cloned into the pET28a-Flag vector between restriction sites SacI and NotI. For the expression of full-length xE6AP with mutated HECT domains, mutant HECT genes were amplified with primers WY4 and WY8 and cloned into the pET28a-Flag vector between restriction sites PstI and NotI. The CDK1 and CDK4 genes were PCR amplified from their cDNA in human genes with primers Bo13 and Bo73. PCR fragment was digested with Nhel and XhoI, and cloned into the pET28a. The β-catenin gene was cloned into pGEX plasmid. The pET or PGEX plasmids were transformed into BL21(DE3) pLysS chemical competent cells (Invitrogen) for protein expression.

**Construction of the E6AP library.** The gene of the E6AP HECT domain was PCR amplified from pET-E6AP plasmids with primers WY11 and WY12. The amplified fragment was double-digested with Nhel and XhoI, and cloned into pCTCON2 plasmid to generate pCTCON2-E6AP HECT. To generate E6AP library in pCTCON2 plasmid, the E6AP HECT domain gene was PCR amplified with WY13 and WY14 to incorporate randomized codons at residues 651, 652, 653, 654, and 656. The PCR fragment amplified with WY13 and WY14 was combined with fragment amplified with primers WY7 and WY8 and cloned into the pET28a vector between restriction sites SacI and NotI. The CDK1 and CDK4 genes were PCR amplified from their cDNA in human genes with primers Bo13 and Bo73. PCR fragment was digested by restriction enzymes SacI and NotI, and cloned into pET28a. The Uba1 gene was amplified from a mammalian expression plasmid with primers WY13 and WY14 to incorporate randomized codons at residues 651, 652, 653, 654, and 656. The PCR fragment amplified with WY13 and WY14 was combined with fragment amplified with primers WY7 and WY8 and cloned into the pET28a vector between restriction sites PstI and NotI. The CDK1 and CDK4 genes were PCR amplified from their cDNA in human genes with primers Bo13 and Bo73. PCR fragment was digested by restriction enzymes SacI and NotI, and cloned into pET28a.

**Yeast display of the E6AP library.** The E6AP library in pCTCON2 plasmid was chemically transformed into EBY100 yeast cells. Briefly, yeast cells were first cultured at 30 °C in 200 mL YPD (20 g dextrose, 20 g yeast, and 10 g extract in 1 L deionized water, sterilized by filtration) to an optical density at 600 nm (OD600) around 0.5. The cells were then pelleted at 1,000 × g for 5 min. Cells were washed by 20 mL TE (100 mM Tris base, 10 mM EDTA, pH 8.0) and 20 mL LiOAc-TE (100 mM LiOAc in TE) before resuspension in approximately 800 μL LiOAc-TE. A typical transformation reaction contained a mixture of 1 μg pCTCON2 plasmid DNA, 2 μL denatured single-stranded carrier DNA from salmon testes (Sigma-Aldrich), 25 μL resuspended yeast competent cells, and 300 μL polyethylene glycol (PEG) solution (40% (w/v) PEG 3350 in LiOAc-TE). To achieve a library size of 10^8, yeast transformants were diluted in parallel. Control was also prepared in which the pCTCON2 plasmid was excluded. Both the transformation reactions and the control were incubated at 30 °C for 1 h and then at 42 °C for 20 min. Cells in each transformation were pelleted by centrifuging at 1,000 × g for 30 s and re-suspended in 20 mL SDCAA medium (2% (w/v) dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 50 mM sodium citrate, and 20 mM citric acid monohydrate in 1 L deionized water, sterilized by filtration). Yeast cells were re-suspended, pooled together into 1 L SDCAA medium, and allowed to grow at 30 °C over a 2-day period to an OD600 above 5. For long-term storage of the yeast library, 20 mL of the yeast culture was aliquoted in 15% glycerol stock and stored at −80 °C. To titrate the transformation efficiency, 10 μL of the re-suspended yeast transformants was serially diluted in SDCAA medium and plated on Trp−plates (20 g agar, 20 g dextrose, 5 g (NH4)2SO4, 1.7 g Difco yeast nitrogen base without amino acids, 1.3 g drop-out mix excluding Trp in 1 L deionized water, and autoclaved). Yeast cells transformed with pCTCON2 plasmids would appear within 2 days of incubation at 30 °C.

**Model selection of yeast cells displaying E6AP HECT domain.** Yeast cell EBY100 was transformed with pCTCON2−wt E6AP HECT and streaked on a Trp−plate. After two days of incubation at 30 °C, cells were scraped from the Trp−plate to inoculate in a 5 mL SDCAA culture that was allowed to shake at 30 °C to reach an initial OD600 of 0.5. Cells were centrifuged at 1000 × g for 5 min and induced for E6AP HECT expression by resuspension in 5 mL SDCAA (2% (w/v) galactose, 6.7 g Difco yeast nitrogen base without amino acids, 5 g Bacto casamino acids, 38 mM Na2HPO4 and 62 mM NaH2PO4, in 1 L deionized water, sterilized by filtration). The yeast culture was shaken at 20 °C for 16–24 h. For analysis of E6AP display on the surface of yeast cells, 10^6 cells were re-suspended in 0.1 mL Tris−acetate buffer (150 mM NaCl, 10 mM Tris−acetate, pH 7.5 at 30 °C) and stained with 0.1% (w/v) calcofluor white in 1 mL of 1% (w/v) BSA solution. The cells were then washed with 1 mL 0.1% (w/v) calcofluor white in 1 mL of 1% (w/v) BSA solution. The cells were then washed with 1 mL 0.1% (w/v) calcofluor white in 1 mL of 1% (w/v) BSA solution.
containing 10 mM MgCl₂ and 50 mM Tris-HCl (pH 7.5). The reaction was incubated at 4 °C for 2 h at 30 °C, and then mixed with 100 μL 3% BSA. A mouse anti-HA antibody (Santa Cruz Biotechnology, sc-7392) was added to the reaction mixture to extract the pCTCON2 plasmid DNA. The plasmid was transformed into XL1 blue (Stratagene). The transformed cells were allowed to grow at 30 °C to an OD₆₀₀ between 1 and 2. Glycerol (-1600 μL) were loaded onto a 25 cm × 75 μm internal diameter fused silica column (New Objective, Woburn, MA) self-packed with 1.9 μm C18 resin (Dr. Maisch, Germany). Separation was carried out over a 2-hour gradient by a Dionex Ultimate 3000 RSLc nano system at a flowrate of 350 nL/min. The gradient ranged from 3% to 80% (v/v) buffer B (buffer A: 0% (v/v) formic acid in water, buffer B: 0.1% (v/v) formic acid in ACN). In each cycle, the mass spectrometer performed a full MS scan followed by as many tandem MS/MS scans allowed within the 3-second time window (top speed mode). Full MS scans were collected in profile mode at 120,000 resolution at m/z 200 with an automatic gain control (AGC) of 200,000 and a maximum ion injection time of 50 ms. The full mass range was set from 400-1600 m/z. Tandem MS/MS scans were collected in the ion trap after higher-energy collisional dissociation (HCD) activation of the entire ion population. The product ions were collected with the AGC set for 10,000 and the maximum ion injection time set to 20 s using the dynamic exclusion parameters. The product ions were collected with the AGC set for 10,000 and the maximum ion injection time set to 20 s using the dynamic exclusion parameters.
+57.021465 Da was set for carboxidimethyl cysteine. The Percoll column within Proteome Discoverer was used to filter the peptide spectral match (PSM) false discovery rate to 1%35.

Bioinformatics analysis. Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com) was used to map and identify the biological networks and molecular interactions with a significant proportion of genes having E6AP ubiquitination targets. Fisher exact test in Ingenuity Pathway Analysis software was used to calculate p-values for pathways and networks. The level of statistical significance was set at a p-value < 0.05. IPA was also used to visualize the identified biological networks. Proteins identified by the OUT screen were also analyzed by the CRAPome database (http://www.crapome.org)36. CRAPome is based on data from interactome studies that carried out affinity purification under denaturing conditions. In contrast, the tandem purification for OUT screens was performed under more stringent denaturing conditions.

Lentiviral silencing of E6AP. Lentiviral GP2 plasmids encoding shRNAs against E6AP (6 different shRNAs) were obtained from GE Dharmacon (Lafayette, CO), and lentiviruses were produced using the manufacturer’s lentivirus packaging system and 293FT cells. HEK293 cells were infected with each lentivirus, followed by selection with puromycin for stable cell populations. The efficiency of gene silencing in each shRNA group was determined by immunoblotting using stable cell populations. For functional restoration, HEK293 cell population stably expressing anti-E6AP shRNA #1 was infected with the lentivirus packaged with pLentis-Myc-wt E6AP.

In vitro assay to confirm the substrates of E6AP. All assays were set up in 30 µL TBS supplemented with 10 mM MgCl2 and 1.5 mM ATP. In each UB transfer reaction, 5 µM of potential substrates (MAPK1, CDK1, CDK4, PRMT5, β-catenin, and Ubx28) were incubated with 1 µM wt Ubα1, 5 µM wt Ubch7, 10 µM E6AP, and 20 µM wt Ub for 2 h at 30°C. The reactions were quenched by boiling in Laemmli buffer with BME, and analyzed by Western blotting probed with substrate-specific antibodies.

Co-immunoprecipitation and to confirm E6AP substrates. Transfection of pLenti-E6AP into the HEK293 cells was conducted with the Lipofectamine® 2000 according to the manufacturer’s protocol. To immunoprecipitate substrate proteins, cells were treated with 10 µM MG132 (American Peptide, Sunnyvale, CA) for 90 min at 72-h post-transfection. HEK293 cells were infected with each lentivirus, followed by selection with puromycin for stable cell populations. The efficiency of gene silencing in each shRNA group was determined by immunoblotting using stable cell populations. For functional restoration, HEK293 cell population stably expressing anti-E6AP shRNA #1 was infected with the lentivirus packaged with pLentis-Myc-wt E6AP.

E6AP induced protein degradation. To examine the effects of E6AP on steady-state levels of the substrates, HEK293 cells (5 × 106 cells) were transiently transfected with 0.5, 1, 2, and 4 µg pLenti-wt E6AP with Lipofectamine 2000. Cells were harvested at 48-hour post-transfection and the amount of substrate proteins in the cell lysate was assayed by immunoblotting with substrate-specific antibodies. For cycloheximide (CHX) chase assays, HEK293 cells (3 × 106 cells) were transiently transfected with 4 µg empty pLenti or pLenti-E6AP plasmids. After 48 h, cells were treated with 100 µg/ml CHX to block de novo protein synthesis and the cells were harvested after variable length of incubation time with CHX. The amount of substrate proteins in the cell were assayed by immunoblotting with antibodies against each substrate proteins. Protein levels were normalized to tubulin. Alternatively, CHX chase assays were performed on HEK293 cells stably expressing anti-E6AP shRNA to measure the effect of decreased expression of E6AP on substrate stability. Uncropped scans of the Western blots are presented in Supplementary Figs. 6–11.

Data availability. The proteomics data supporting the findings of this study have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the identifier PXD005584 (http://proteomecentral.proteomeexchange.org). All other data are available from the corresponding authors upon reasonable request.

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Author contributions
J.Y. and H.K. conceived the idea, Y.W. conducted most experiments, X.L. developed the scheme for cell line construction, tandem affinity purification and target validation, L.Z. contributed to substrate verification, D.D. performed proteomic analysis, K.B., B.Z., and J.Y. performed bioinformatics analysis, K.B., B.Z., and J.Y. wrote the manuscript with Y.W., X.L. and H.K.; CA112282 to H.K.), the National Science Foundation (1420193 and 1710460 to J.Y.), the Chicago Biomedical Consortium (Catalyst-026 to H.K. and J.Y., PDR-010 to X.L.), Project 985 startup grant (WF220417001 and WF114117001/004 to B. Z.), the Lynn Sage Breast Cancer Research Foundation, and the Department of Pharmacology at Northwestern University.

Additional information
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