Supplemental Data Inventory

The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the anti-viral function of ISG15

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Figure S1: Figure S1 shows ISGylation of previously identified, exogenously expressed target proteins in addition to those shown in Figure 2B.

Figure S2: Figure S2 shows ISGylation of human, non-human, and viral exogenously expressed target proteins in addition to those shown in Figure 3A and 3B.

Figure S3: Figure S3 provides another example of ISGylation of non-overlapping fragments in addition to those shown in Figure 3C.

Figure S4: Figure S4A shows that the exogenously expressed mRNAs are more abundant than the corresponding endogenously expressed mRNAs. This suggests that while the exogenously expressed proteins in Figure 4A were not present at a higher steady-state level, they were translated at a higher rate. Figure S4B corresponds to Figure 4C and shows that TAP IQGAP1 and TAP p53 are long-lived proteins.

Figure S5: Figure S5 corresponds to Figure 5B and shows that ISG15 conjugation occurred only in cells that were transfected with the conjugation components.

Figure S6: Figure S6 corresponds to Figure 6D and shows that HA-Herc5-ΔRCC and –Δ100 are enzymatically active, but cannot conjugate ISG15 to target proteins.

Table S1: Table S1 provides a list of all endogenously expressed proteins examined and corresponds to Figures 1B, 1C, and 2A.

Table S2: Table S2 provides a list of exogenously expressed proteins examined and corresponds to Figures 2B, 3A, and 3B.

Table S3: Table S3 is referenced in the experimental procedures section and provides a list of plasmids and antibodies used in this study.

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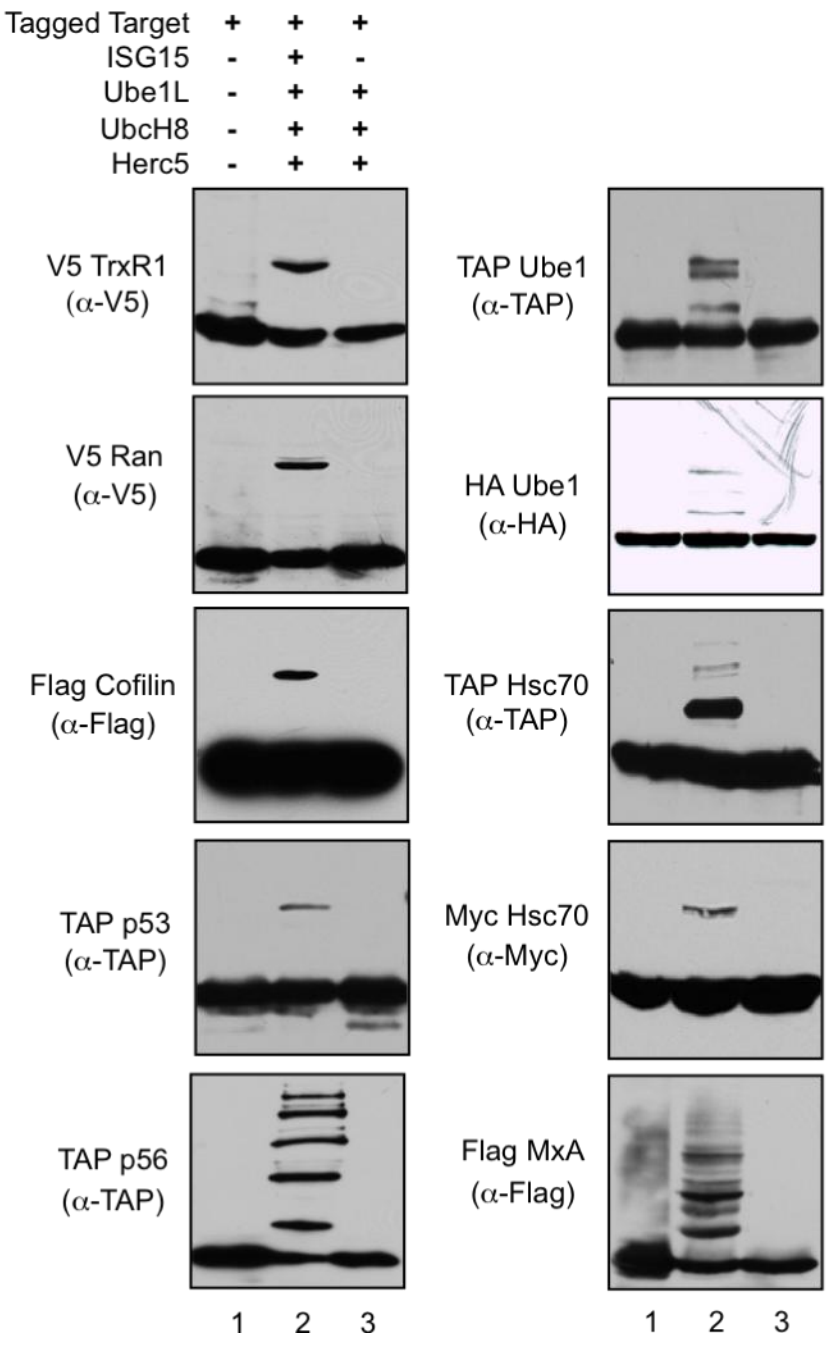

Figure S1: ISGylation of previously identified, exogenously expressed proteins (related to Figure 2B). HEK293T cells were transfected with a plasmid expressing an epitope-tagged target protein and either no additional plasmids (lane 1), or with plasmids expressing Ube1L, UbcH8, Herc5, and ISG15 (lane 2), or plasmids expressing Ube1L, UbcH8, and Herc5 (lane 3). Cell extracts were prepared 48 hours post-transfection and analyzed by immunoblotting using antibodies against the indicated epitope tag.

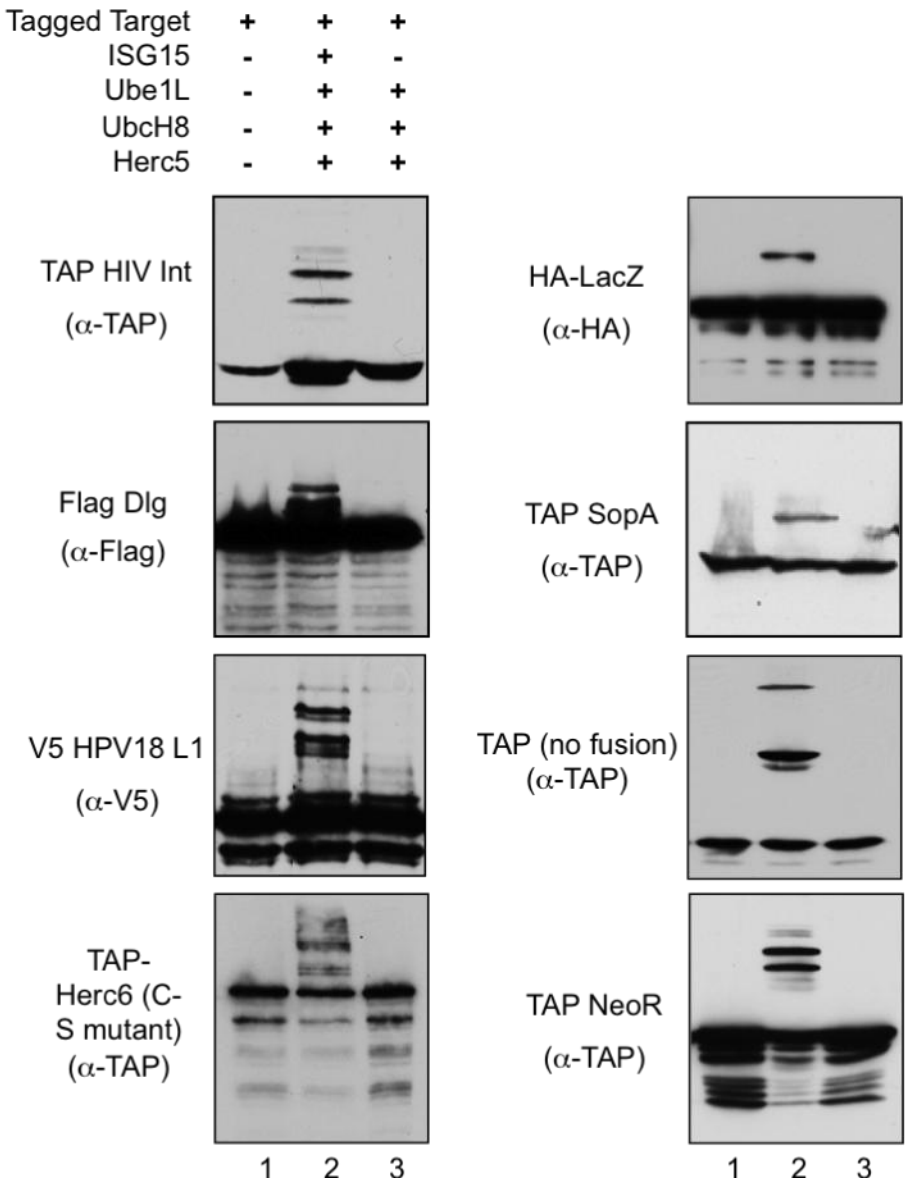

Figure S2: ISGylation of additional human, non-human, and viral exogenously expressed proteins (Related to Figure 3A and 3B). HEK293T cells were transfected with a plasmid expressing an epitope-tagged target protein and either no additional plasmids (lane 1), or with plasmids expressing Ube1L, UbcH8, Herc5, and ISG15 (lane 2), or plasmids expressing Ube1L, UbcH8, and Herc5 (lane 3). Cell extracts were prepared 48 hours post-transfection and analyzed by immunoblotting using antibodies against the indicated epitope tag.

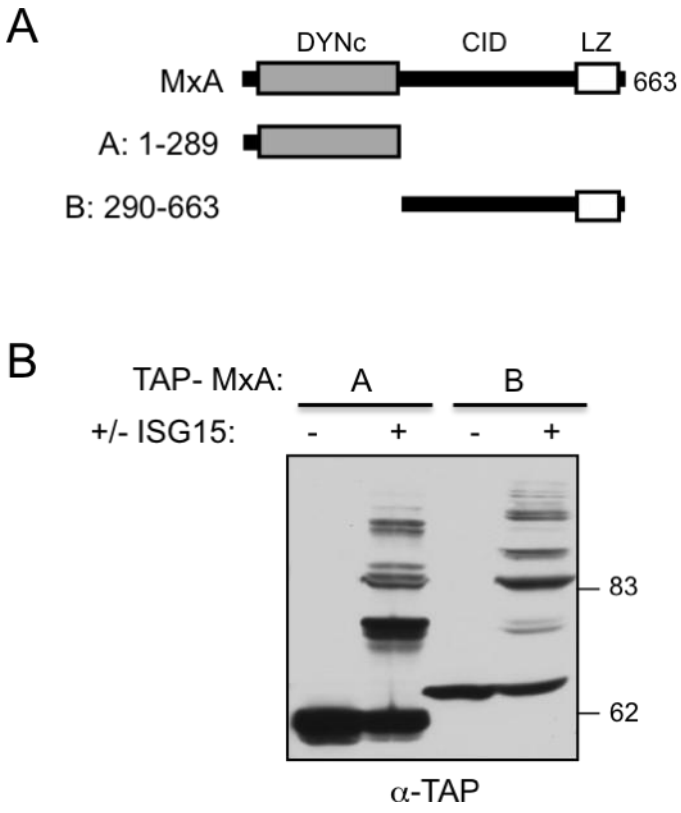

Figure S3: ISGylation of non-overlapping fragments of MxA (Related to Figure 3C). (A). A schematic of MxA is shown, along with two non-overlapping fragments (A-B, with numbering of amino acids shown). DYNc, Dynamin domain; CID, central interactive domain; LZ, leucine zipper motifs. **(B).** Plasmids expressing TAP-tagged fragments A and B were transfected into HEK293T cells along with plasmids expressing Ube1L, UbcH8, Herc5, with or without ISG15. Cell extracts were prepared 48 hours post-transfection and analyzed by immunoblotting with an antibody against the TAP tag.

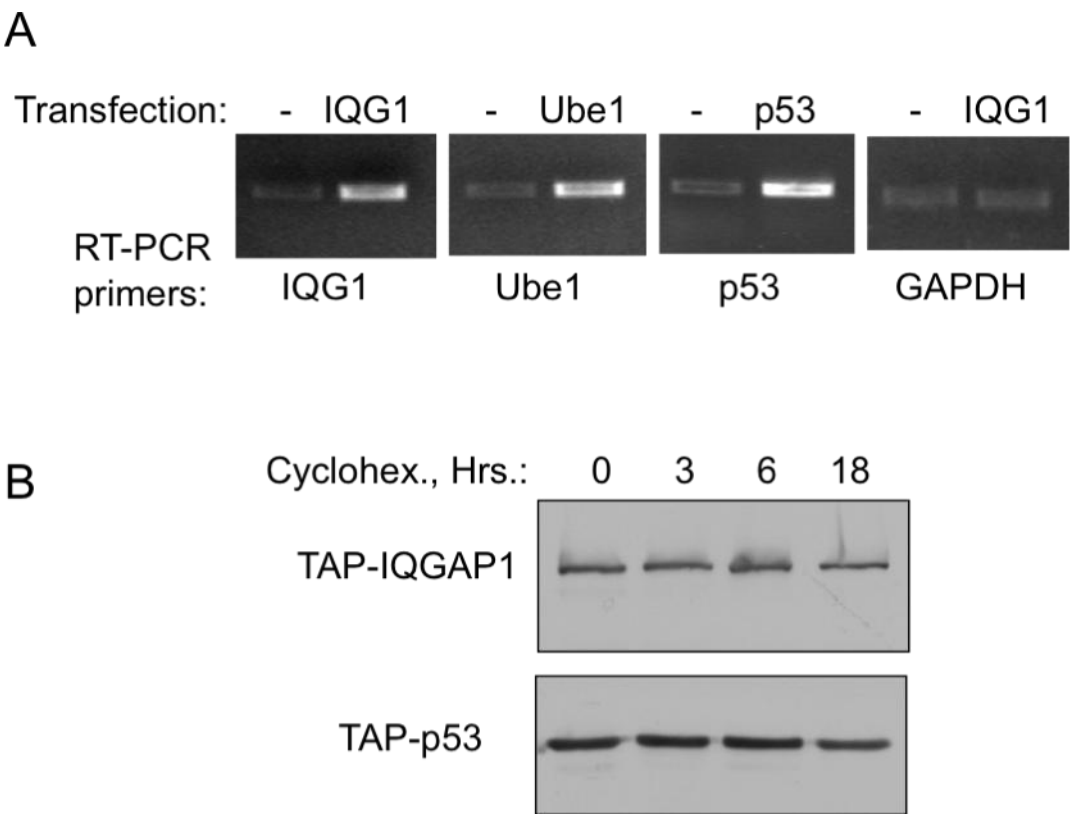

Figure S4: (A) Exogenously expressed mRNAs are more abundant than the corresponding endogenously expressed mRNAs (Related to Figure 4A). Total mRNA was isolated from untransfected HEK293T cells (-) or cells transfected with TAP-IQGAP1 (IQG1), TAP-Ube1 (Ube1), or TAP-53 (p53) expression plasmids for 24 hours. After DNase treatment, cDNA was prepared with and without reverse transcriptase. RT-PCR reactions were performed using primer sets that detect both the endogenously and exogenously expressed mRNAs. A GAPDH primer set was used as a control for equal RNA in the each set of samples and is shown for the IQGAP1-transfected samples. **(B) TAP-IQGAP1 and TAP-p53 are long-lived proteins (Related to Figure 4C).** Plasmids expressing TAP-IQGAP1 or TAP-p53 were transfected into HEK293T cells. Twenty-four hours after transfection, cells were treated with cycloheximide at a concentration of 40 μg/ml. Extracts were then prepared immediately, or 3, 6 hours, or 18 hours later. Immunoblots with anti-TAP antibody indicated that the protein levels did not decline more than 2-fold over 18 hours

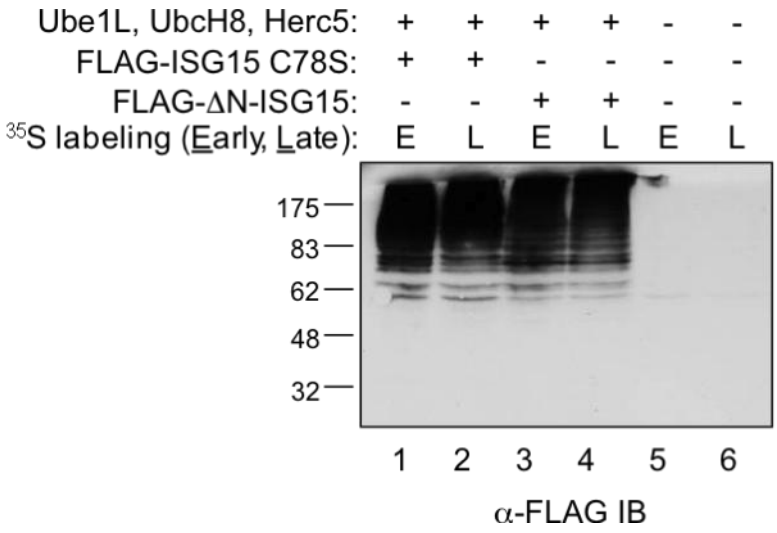

Figure S5: ISG15 conjugation in ^{35}S labeled extracts. Extracts correspond to those used in immunoprecipitations in Figure 5B. This demonstrates that ISG15 conjugation occurred in all cells that were transfected with the conjugation components (lanes 1-4) and not in untransfected cells (lanes 5-6).

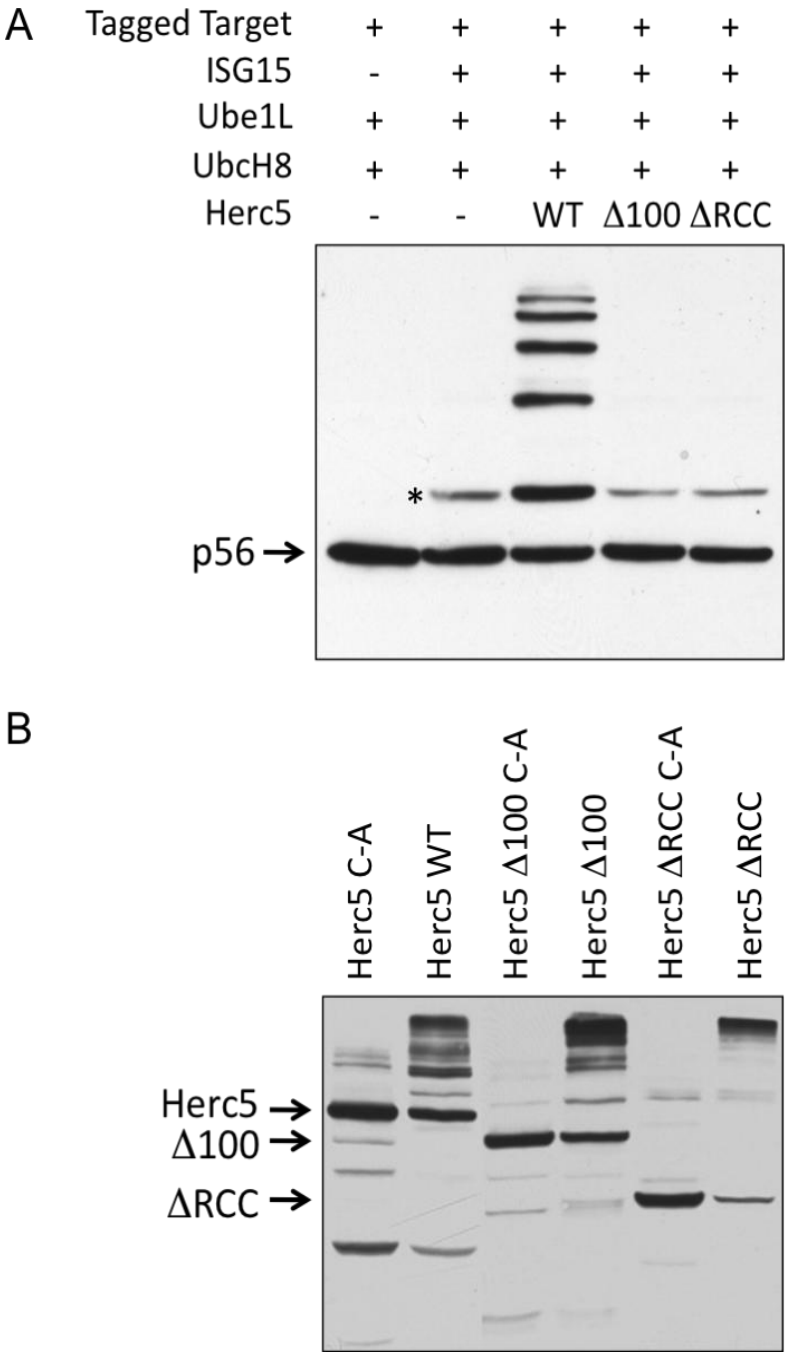

Figure S6: TAP-Herc5-ΔRCC and -Δ100 cannot conjugate ISG15 to target proteins, but are enzymatically active (Related to Figure 6D). (A) Plasmids expressing FLAG-p56 were transfected into HEK293T cells along with plasmids expressing Ube1L, UbcH8, ISG15, and NTAP-Herc5 or the indicated Herc5 mutants. Cell extracts were prepared 48 hours post-transfection and ISGylation of p56 was determined by immunoblotting with anti-FLAG antibody. (B) HEK293T cells were transfected with ISG15, Ube1L, UbcH8, and the indicated TAP-tagged Herc5 expression plasmids. Herc5 proteins and ISGylated forms of Herc5 were detected with anti-TAP antibody.

Table S1. Endogenously expressed proteins

Target protein	Previously Identified Target?	Interferon-induced?	Detectable ISGylation in IFN-treated HeLa cells?	Detectable ISGylation in 4-Plasmid Transfection?
Ube1	yes[1, 2, 3]	no	yes	yes
IQGAP1	yes[1]	no	yes	yes
Hsc70	yes[1, 2, 3,4]	no	no	no
Moesin	yes[1]	no	no	no
Tubulin	yes [3, 4]	no	no	no
Matrin 3	yes[1]	no	no	no
Enolase	yes[1, 2, 3, 4]	no	no	no
MxA	yes[1, 3]	yes	yes[1]	na
RIG-I	yes[1]	yes	yes[1]	na
p56	yes[1, 3]	yes	yes[1]	na
PKR	yes[1]	yes	no	na
RIG-G	yes[1]	yes	no	na
OAS1	no	yes	no	na
PLSCR1	no	yes	no	na
E6AP	no	no	no	no
hnRNPk	no	no	no	no
Nucleophosmin	no	no	no	no

[1]Zhao, et al., 2005
[2]Giannakopoulos et al, 2005
[3]Wong, et al., 2006
[4]Takeuchi, et al., 2006

Table S2. Proteins expressed by plasmid transfection

Target Protein	Previously Identified Target?	Detectable ISGylation in 5-Plasmid Transfection?
IQGAP1 (TAP, FLAG)	yes[1]	yes
Moesin (V5)	yes[1]	yes
Ube1 (V5, TAP, HA)	yes[1, 2, 3]	yes
TrxR1 (V5)	yes[1, 3]	yes
Ran (V5)	yes[1, 3]	yes
Cofilin (FLAG)	yes[1, 3]	yes
p56 (TAP)	yes[1, 3]	yes
Hsc70 (TAP, Myc)	yes[1, 2, 3, 4]	yes
MxA (FLAG)	yes[1, 3]	yes
p53 (HA, TAP, FLAG)	no	yes
E6AP (C-A mutant; TAP)	no	yes
Herc6 (C-S mutant HA, TAP)	no	yes
Herc4 (TAP)	no	yes
Dlg (FLAG)	no	yes
Paxillin (C-term.; FLAG)	no	no
Wbp2 (FLAG)	no	no
Shigella OSPG (TAP)	na	yes
Salmonella SopA (TAP)	na	yes
TAP epitope, no fusion	na	yes
S. cerevisia Rsp5 (untagged)	na	yes
E. coli Neo^R protein (TAP)	na	yes
E. coli β-gal. (TAP, HA)	na	yes
HPV16 L1 protein (untagged) and HPV18L1 (V5)	na	yes
HIV Integrase (TAP)	na	yes
GFP	na	no

[1]Zhao, et al., 2005
[2]Giannakopoulos et al, 2005
[3]Wong, et al., 2006
[4]Takeuchi, et al., 2006

Table S3. Plasmids and antibodies used in this study

TARGET PLASMIDS	NOTES
pcTAP	Previously described (Dastur et al., 2006)
pcTAP IQGAP1, A, B, C	The cDNA of IQGAP1 was previously described (Ho et al., 1999).
pcMV10 IQGAP1	Same as above, but IQGAP1 was cloned into the pcMV10 vector.
pcTAP Ube1	Ube1 was previously described (Beaudenon and Huibregtse, 2005; Huibregtse et al., 1995), and was subcloned into the pcTAP vector.
pcV5 Ube1	Same as above, but Ube1 was cloned into the pcV5 vector.
pcHA Ube1	Same as above, but Ube1 was cloned into the pcHA vector.
pcV5 Moesin	Previously described (Zhao et al., 2005)
pcV5 TrxR1	Previously described (Zhao et al., 2005)
pcV5 Ran	Previously described (Zhao et al., 2005)
pcFLAG Cofilin	Cofilin cDNA was cloned into the pcFLAG vector.
pcTAP Hsc70	pcTAP Hsc70 was provided by R. Krug.
pcTAP E6AP (C-A mutant)	The cDNA of E6AP C-A mutant was previously described (Huibregtse et al., 1995), and was subcloned into the pcTAP vector.
pcHA p53	The cDNA of p53 was previously described (Huibregtse et al., 1991), and was subcloned into the pcHA vector.
pcTAP p53	Same as above, but p53 was subcloned into the pcTAP vector.
pcHA β-Gal	β-Gal was amplified from pSVβgal (Promega) and cloned into the pcHA vector.
pcTAP Herc6 (C-A mutant)	Herc6 was amplified from cDNA, cloned into the pcTAP vector, and the active site cysteine mutated (C985A).
pcTAP p56	His-3X FLAG-p56 was provided by Dr. Robert Krug (UT Austin, Austin, TX), and was subcloned into the pcTAP vector.
pcTAP MxA, A, B	pcTAP MxA was provided by Dr. Robert Krug (UT Austin, Austin, TX), and was used to generate the TAP fusion proteins A and B.
pcMV10 MxA	Same as above, but MxA was cloned into the pcMV10 vector.
pcTAP HIV Integrase	HIV-1 Integrase cDNA was subcloned into the pcTAP vector.
pcTAP SopA	The cDNA of SopA was subcloned from a plasmid provided by J. Chen (Purdue University) into the pcTAP vector.
p16shell, p18shell	Provided by John Schiller (NCI, Bethesda, MD)
pcV5-HPV18L1	HPV18 L1 ORF was cloned into the pcV5 vector
pcTAP OSPG	OSPG was amplified from Shigella genomic DNA provided by S. Payne, and cloned into the pcTAP vector.
pcMyc Hsc70	pcMyc Hsc70 was provided by Dr. D. Manor.
pcFLAG Dlg	Dlg cDNA (Lee et al., 1997), and was cloned into pcFLAG vector.
pcMV10 GFP	GFP was amplified from pEGFP-C1 (Clontech) and cloned into the pcMV10 vector (Sigma)

PLASMIDS FOR ISG15 CONJUGATION	NOTES
pc3XFLAG ISG15 & pcHA ISG15	Previously described (Zhao et al., 2005)
pcFLAG-ISG15 & pcFLAG-ΔN-ISG15	Plasmids were made by introducing a sequence encoding an N-terminal FLAG epitope (DYKDDDDK) into a pcDNA3.1 plasmid containing either full length human ISG15 or residues 80-158 of ISG15 (ΔN)
pcDNA Ube1L	Previously described (Zhao et al., 2005)
pcDNA Ube1LΔUFD	Previously described (Durfee et al., 2008)
pcDNA UbcH8	Previously described (Zhao et al., 2005)
pcDNA UbcH8 F62A	Previously described (Dastur et al., 2006)
pCS2+MT (Myc)-Herc5	M. Ohtsubo of Hiroshima University
pcTAP Herc5 pcTAP Herc5 C994A	Previously described (Dastur et al., 2006)
pcHA Herc5 and pcHA Herc5ΔRCC	Herc5 and Herc5ΔRCC were subcloned from the pcTAP vector into the pcHA vector (Dastur et al., 2006)

ANTIBODIES	NOTES
Anti-Hsc70	Santa Cruz Technologies
Anti-HPV16 L1	Santa Cruz Technologies
Anti-IQGAP1	BD Biosciences
Anti-Ube1	Abcam
Anti-Moesin	Cell Signaling Technology
Anti-Flag M2	Sigma Aldrich
Anti-V5	AbD Serotec
Anti-HA	Covance
Anti-C-Myc	Covance
Anti-TAP (Peroxidase-anti-peroxidase)	Rockland Immunochemicals for Research
Anti-p56	Kindly provided by Ganes Sen (Cleveland Clinic, Cleveland, OH)
Anti-Herc5	Kindly provided by Enzo Life Sciences
Anti-Puromycin	Kindly provided by Peter Walter (UCSF, San Francisco, CA)
Anti-E6AP	Prepared as described (Talis et al., 1998)

References for Supplementary Tables and Figures

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