Supporting Information

Buti et al. 10.1073/pnas.1106200108

SI Materials and Methods

Cells. T-REx 293 (Invitrogen), adenocarcinoma cell line (AGS, CRL1739; American Type Culture Collection), HCT116 (CCL-247; American Type Culture Collection), HEK293T (CRL-11268; American Type Culture Collection), HCT11p53$^{-/-}$ (obtained from Bert Vogelstein, Howard Hughes Medical Institute for Cancer Research at the John Hopkins Kimmel Cancer Center, Baltimore, MD), and Madin-Darby canine kidney cells were cultured in DMEM supplemented with 10% FBS and the appropriate antibiotics.

Bacterial Strains and Infections. *Helicobacter pylori* (Hp) strain G27 and the isogenic mutants ΔVirb10, Δcytotoxin-associated gene A (CagA), and EPISA were described previously (1, 2). Strains were cultured on blood agar plates or in *Brucella* broth medium supplemented with selective antibiotics and 10% calf serum and grown in 5% CO_2 at 37 °C. Before infection, cells were washed two times, incubated in DMEM without antibiotics, and supplemented with 5% calf serum. Cells were infected with the specific Hp strains at a multiplicity of infection (moi) of 1:100 for 7 h unless indicated otherwise.

Plasmids. A list of the primers used is in Table S1. Apoptosis-stimulating protein of p53 (ASPP2) cDNA was a gift from X. Lu (Ludwig Institute for Cancer Research, Oxford, UK) and used as a template to generate the following constructs: FLAG ASPP2, FLAG 1- to 330-aa ASPP2, FLAG 1- to 875-aa ASPP2, FLAG Ct ASPP2 (920–1128 aa), and cherry-ASPP2, which were all cloned in retroviral tetracycline-regulated expression (pREV-TRE) retroviral vector (Clontech) according to standard procedures. CagA constructs were amplified from the *cagA* sequence of strain G27(1) and introduced in pcDNA5FRT/TO (Invitrogen) along with the sequence to encode the acceptor peptide (AP) or AP* tag. BirA and GFP-CagA constructs were cloned into the plasmid HIV-I Alex Gustavo George enhanced vector (3).

Lentiviral and Retroviral Transduction. BirA lentivirus was produced as described by Mostoslavsky et al. (3). Human ASPP2 and control (GFP) shRNA constructs were obtained from the TRC Consortium at the Broad Institute of Massachusetts Institute of Technology and Harvard University. Lentivirus was produced using the instructions provided by the Broad Institute (http://www.broadinstitute.org/rnai/public/resources/protocols). Retrovirus production was described previously (4). T-REx 293, AGS, or HCT116 cells were infected in a six-well plate with 100–500 μL viral preparation supplemented with 6 μg/mL Polybrene (Sigma). The medium was replaced after 6 h, and after 24 h, cells were placed under puromycin selection (0.4 μg/mL for AGS and 1 μg/mL for HCT116) or hygromycinB (0.4 μg/mL for HCT116; Roche).

Transfection. All transfections were carried out using Lipofectamine 2000 (Invitrogen) using the instructions provided by the manufacturer.

Reagents. Rabbit polyclonal antiserum CagA was described previously (5). Mouse anti-ASPP2 (39–7000) was purchased from Invitrogen, and rabbit anti-ASPP2 (ab36004) was purchased from Abcam. Anti-p53 (DO-1; sc-126) and anti-CagA (sc-b300) were purchased from Santa Cruz Biotechnologies. Anti-p53 (9282), p21 (2947), Bax (2772), Lyn (2732), Histone H4 (2592), and cleaved Caspase 3 (CC3; 9661) were purchased from Cell Signaling. Anti-FLAG M2 (F1804) was purchased from Sigma. Anti-p97 (10r-P104a) was purchased from Fitzgerald Industries International. Doxorubicin (Dox) was purchased from Sigma and used at a final concentration as indicated in the figures. Treatment of cells with the proteasome inhibitor ZL_3VS (working concentration of 50 μM) has been previously described (6).

Cell Fractionation. The procedure to separate membrane/cytoplasmic from nuclear proteins was adapted from Li et al. (7).

Protein Interaction Studies. ***Large-scale purification and MS.*** The procedure was adapted from Lilley and Ploegh (8). Briefly, 1 billion T-REx 293 stable transfectants treated overnight with 2 μg/mL Dox (Sigma) to express an AP followed by a TEV cleavage site placed N-terminally of full-length (AP-CagA), N terminus (AP-Nt CagA), or C terminus of CagA (AP-Ct CagA) and a biotin-resistant version of the acceptor peptide fused to full-length CagA (AP*-CagA) together with BirA were lysed in Brij-containing buffer and treated as described above. After the tobacco etch virus (TEV) cleavage, the eluates were resolved by SDS/PAGE and revealed by silver staining. The bands of interest were excised, digested with trypsin, and analyzed by LC-MS/MS as described in Lilley and Ploegh (8).

Metabolic labeling. T-REx 293 stable transfectants were treated overnight with 2 μg/mL Dox (Sigma) to express AP-CagA, AP*-CagA, AP-Nt CagA, or AP-Ct CagA together with BirA. Cells were starved for 1 h in medium lacking methionine and cysteine (starvation medium) and subsequently, were labeled for 3 h with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine (0.1 mCi/nL; Perkin-Elmer) in starvation medium containing dialyzed-inactivated FCS. Cells were lysed using different buffer conditions: SDS-containing buffer (1% SDS, 20 mM Hepes, pH 7.8, 50 mM KCl, 5 mM EDTA, 5% Glycerol, 0.05% Nonidet P-40), Brij-containing buffer (1% Brij58, 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$), or Nonidet P-40–containing buffer (0.05% Nonidet P-40, 20 mM Hepes, pH 7.8, 50 mM KCl, 5 mM EDTA, 5% Glycerol). All buffers were supplemented with complete protease inhibitor (Roche). Lysates were cleared by centrifugation of 13,500 × *g* for 15 min at 4 °C, and biotinylated proteins were immunoprecipitated with Streptavidin-agarose beads (Sigma). Immunoprecipitated material was eluted overnight by incubation with 100 U TEV protease (Invitrogen) at 4 °C. The cleaved material was separated by SDS/PAGE, and polypeptides were visualized by autoradiography.

1. Xiang Z, et al. (1995) Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* 63:94–98.
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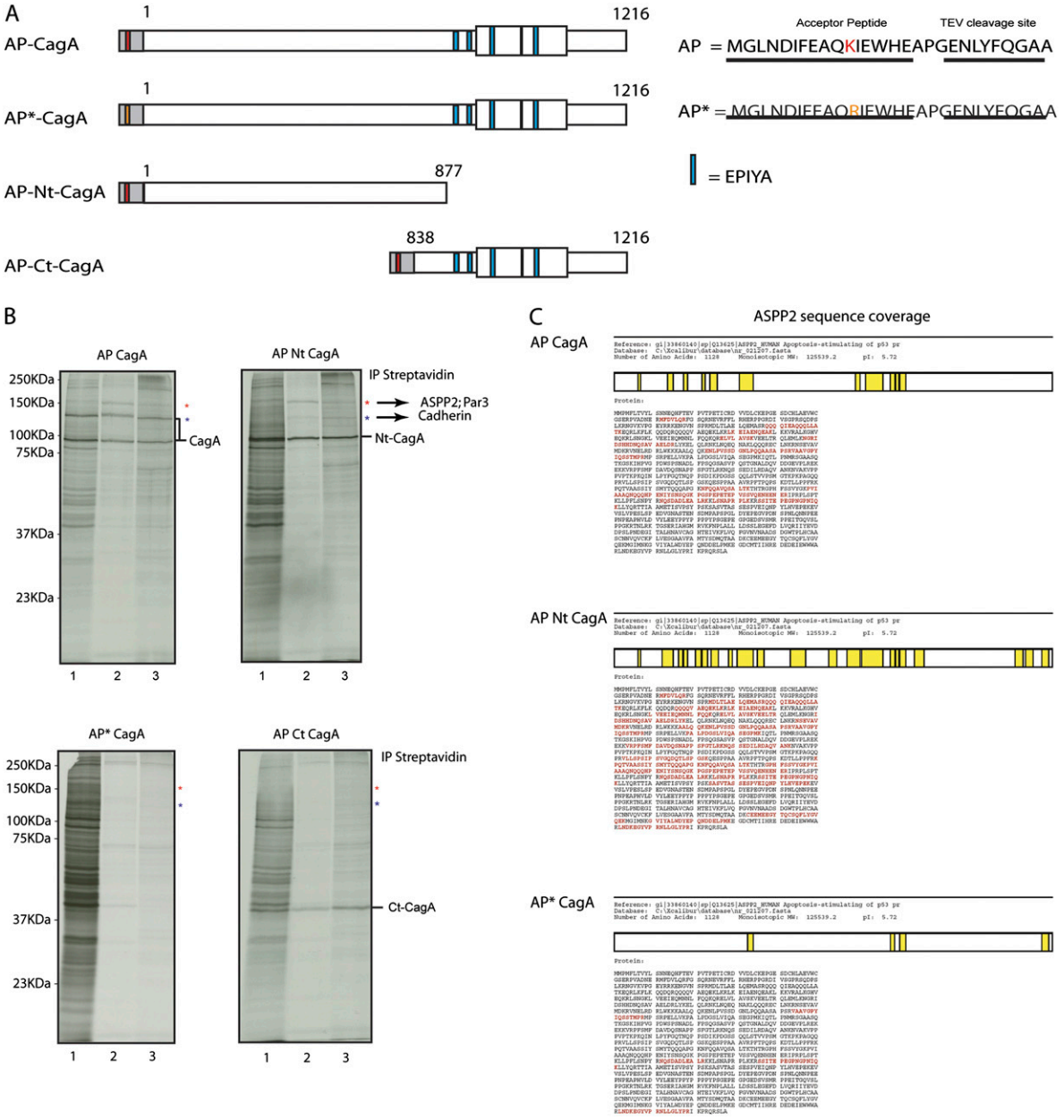
4. Park B, et al. (2008) Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol* 9:1407–1414.
5. Bagnoli F, Buti L, Tompkins L, Covacci A, Amieva MR (2005) Helicobacter pylori CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci USA* 102:16339–16344.
6. Shamu CE, Story CM, Rapoport TA, Ploegh HL (1999) The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J Cell Biol* 147:45–58.
7. Li Y, et al. (2006) An intron with a constitutive transport element is retained in a Tap messenger RNA. *Nature* 443:234–237.
8. Lilley BN, Ploegh HL (2004) A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429:834–840.
9. Amieva MR, Salama NR, Tompkins LS, Falkow S (2002) Helicobacter pylori enter and survive within multivesicular vacuoles of epithelial cells. *Cell Microbiol* 4:677–690.



D

Tot. number of peptides retrieved

	AP* CagA	AP Ct CagA	AP Nt CagA	AP CagA
Apoptosis stimulating of p53	4	0	36	18
Partitioning defective protein-3 (Par-3)	0	0	8	0
N-Cadherin	0	0	2	2

Fig. S1. Characterization of CagA-ASPP2 interaction. (*A*) WT CagA (1–1216 aa), N-terminal CagA (1–877 aa), and C-terminal CagA (840–1,216 aa) were fused at the N terminus to a biotin AP followed by a TEV protease cleavage site. We generated a biotin-resistant AP (AP*) where the Lys, recognized and biotinylated by BirA, was replaced with an Arg. The AP* tag was followed by a TEV cleavage site and fused to the N terminus of WT CagA, which was used as a negative control. The lysine to arginine substitution in the biotin acceptor peptide (AP*-CagA) prevents biotinylation of the fusion protein by BirA. Phosphorylation sites of CagA (EPIYA motifs) are shown in blue. (*B*) T-REx 293 stable transfectants treated overnight with Dox to induce the expression of the indicated constructs together with the biotin ligase BirA were metabolically labeled with [^{35}S]-methionine/cysteine for 3 h and lysed in 1% SDS (1), 1%-Brij58 (2), or 1% Nonidet P-40 lysis buffer (3). Biotinylated proteins were recovered using streptavidin-conjugated beads and resolved by SDS/PAGE. Specific proteins were coimmunoprecipitated with AP-CagA and AP-Nt CagA. (*C*) MS analysis of ASPP2 peptides retrieved from samples that were treated as described in *A* and analyzed by LC-MS/MS. (*D*) Number of total peptides identified by LC-MS/MS in T-REx 293-expressing BirA and the indicated constructs and processed as indicated in *Materials and Methods*.

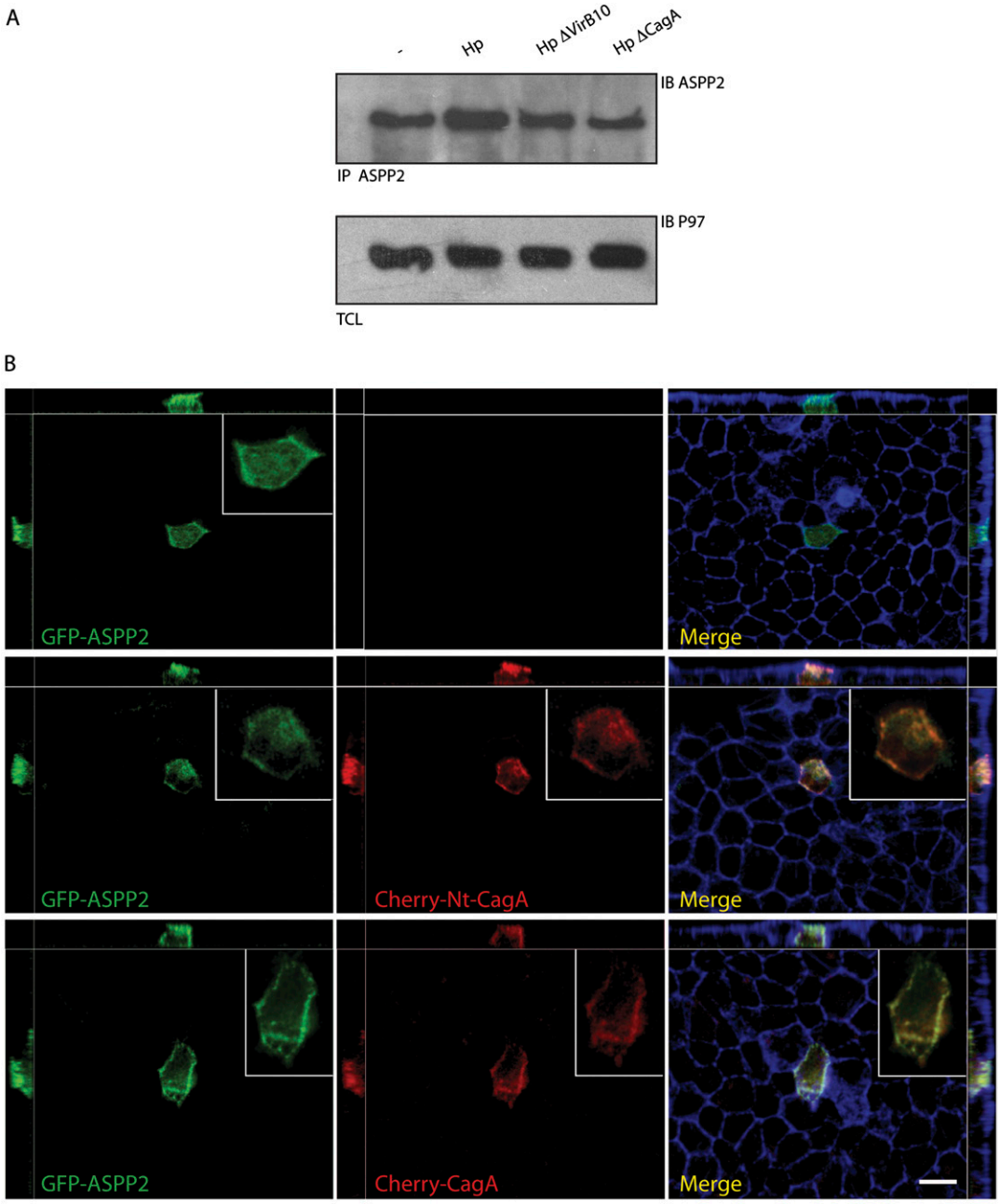


Fig. S2. CagA affects ASPP2 cellular distribution. (*A*) AGS cells were infected for 7 h with the indicated Hp strains (moi = 1:100) or left uninfected and lysed in 1% SDS-containing buffer. ASPP2 was recovered by immunoprecipitation (IP) and detected by immunoblotting (IB) with an anti-ASPP2 antibody; p97 serves as loading control. (*B*) Polarized MDCK monolayer was transfected with GFP-ASPP2 alone or together with either Cherry-Nt-CagA or Cherry-CagA, fixed and counterstained for F-actin (blue). (*Insets*) Optical section of cell expressing the different constructs to show the subcellular localization of the proteins. (Scale bar: 10 μm.)

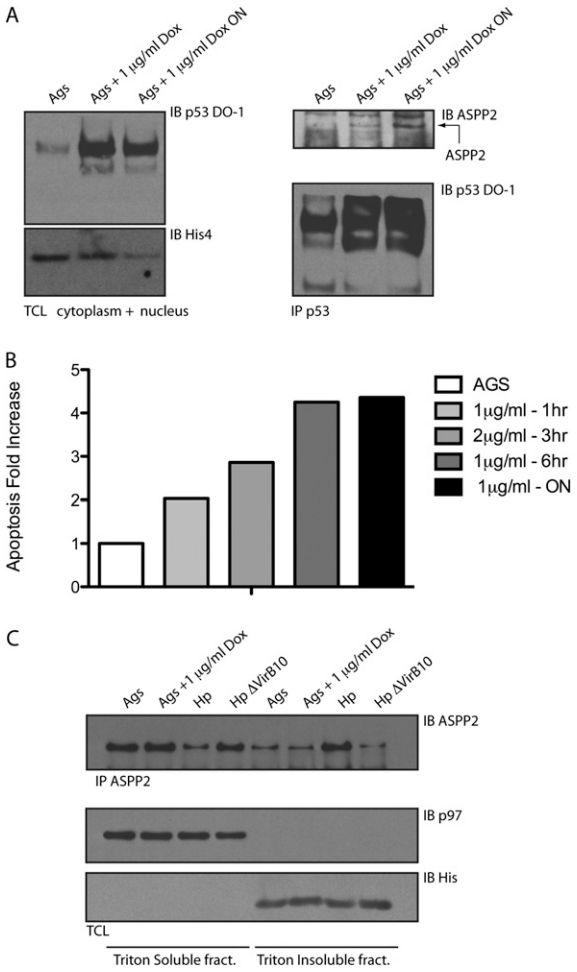


Fig. S3. Dox treatment of AGS cells induces ASPP2-p53 interaction and stimulates the apoptotic response. (*A*) AGS was treated with 1 μg/mL Dox for 3 h or overnight or was left untreated. Cells were harvested, and both cytoplasm and the nuclear fraction were collected after sonication in Nonidet P-40–containing buffer. Endogenous p53 was IP with the indicated antibody. Total cell lysates (TCLs) and immunoprecipitates were IB with the indicated antibodies. His4 serves as a loading control as well as a nuclear marker. (*B*) Levels of CC3 assayed by flow cytometry of AGS cells were treated with different concentrations of Dox for the indicated time periods or left untreated. (*C*) AGS cells were infected for 7 h with the indicated Hp strains (moi = 1:100), left uninfected, or treated with 1 μg/mL Dox for 3 h and lysed in Triton-X100 (T-X100)-containing buffer. The T-X100 insoluble fraction was then extracted in SDS buffer. Fractions were IB with the indicated antibodies or IP with anti-ASPP2 and IB with anti-ASPP2. p97 serves as a cytoplasmic marker, and His4 serves as a nuclear marker as well as a loading control.

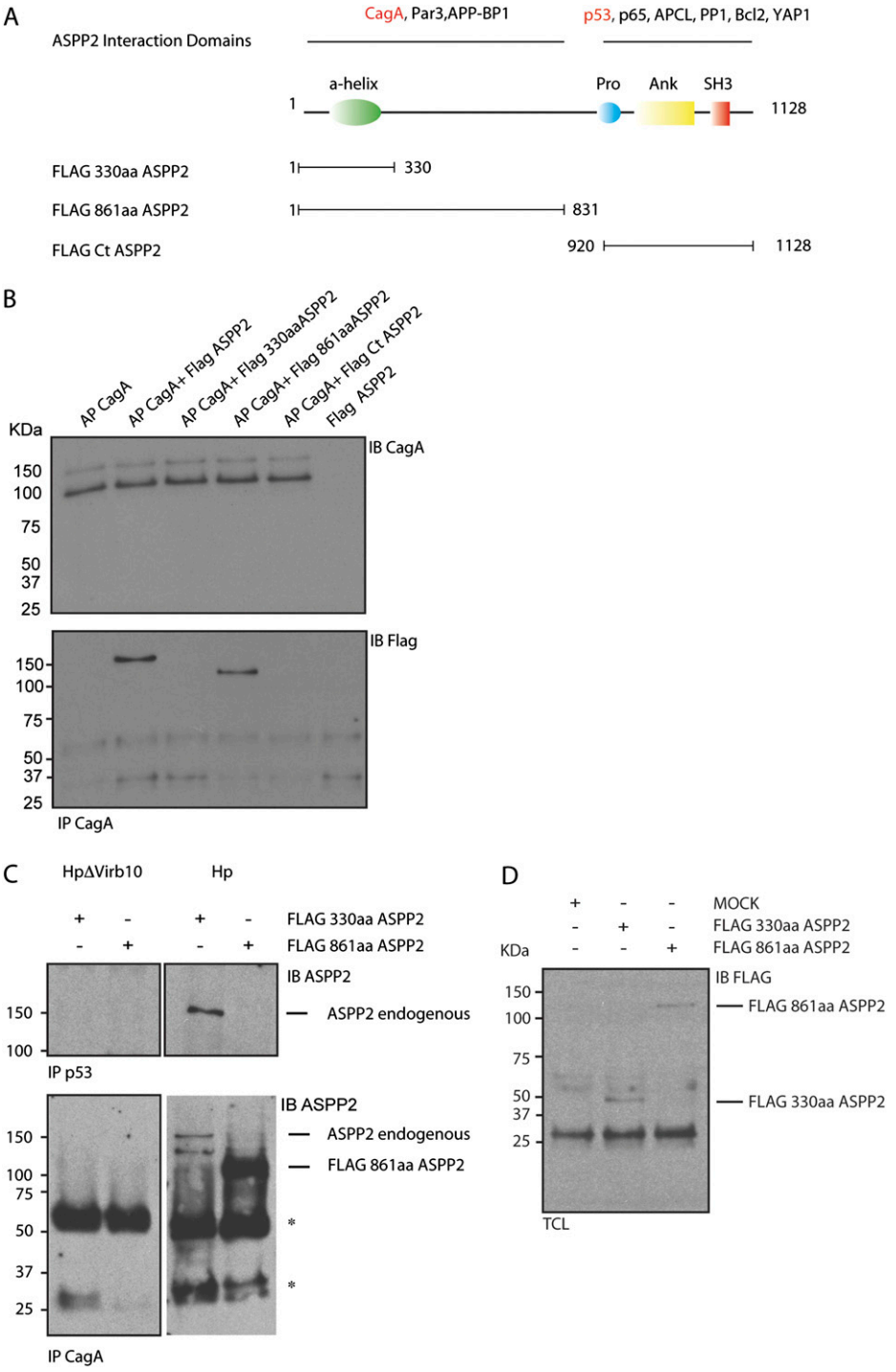


Fig. S4. Overexpression of the N-terminal 1- to 861-aa ASPP2 fragment interferes with binding of endogenous ASPP2 to CagA and p53 in a dominant negative fashion. (*A*) Domain organization of full-length ASPP2 and the truncated (N-terminally FLAG-tagged) fragments used in this study. The N-terminal domain mediates the association of ASPP2 with Par3, APP-BP1, and CagA (from this study). Its C-terminal domain instead mediates the interaction with p53, p63, APCL, PP1, Bcl2, and YAP1. In green is the predicted α-helix domain (α-helix), in blue is the proline-rich domain (Pro), in yellow is the four ankyrin domains (Ank), and in red is the SH3 domain (SH3); the region that binds p53 is highlighted in gray. (*B*) HEK293T cells were transfected with the indicated constructs and lysed in 1% Brij lysis buffer. CagA was retrieved by IP with an anti-CagA antibody. Immunoprecipitates were IB with anti-CagA (*Upper*) or anti-FLAG antibody (*Lower*). (*C*) HCT116*p53*$^{+/+}$ cells stably transduced with the indicated constructs were infected with the indicated Hp strains (1:50 moi) for 7 h or left uninfected and lysed in Nonidet P-40–containing buffer. The samples were split in two parts, and endogenous p53 or CagA was IP. Immunoprecipitates were IB with the indicated antibodies. Endogenous ASPP2 was coimmunoprecipitated together with p53 only in the Hp-infected cells expressing FLAG 330-aa ASPP2 construct. *Non-specific bands recognized by the mouse ASPP2 antibody. This antibody is direct against the human ASPP2 691–1,128 aa, and it recognizes only endogenous ASPP2 and the FLAG 861-aa ASPP2 construct. (*D*) HCT116*p53*$^{+/+}$ cells stably transduced with the indicated constructs were lysated in SDS-containing buffer and IB with a FLAG antibody.

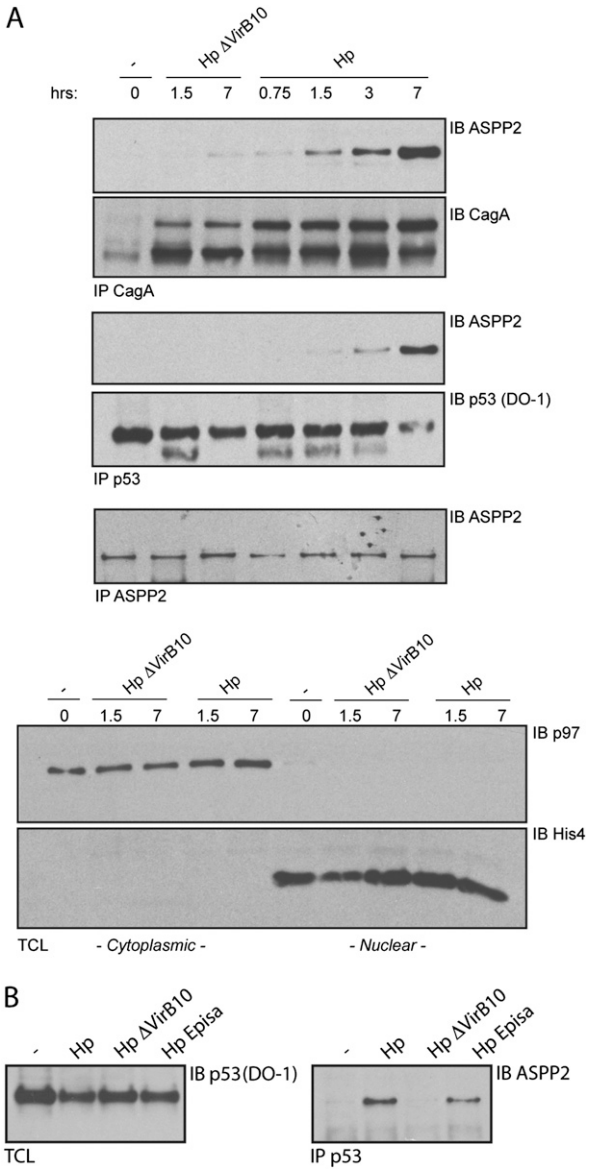


Fig. S5. Phosphorylation of CagA is not required for the interaction between ASPP2 and p53. (*A*) AGS cells were infected with the indicated Hp strains (1:100 moi) or not infected, harvested at different time points, and processed as described in Fig. 3. Briefly, cells were lysed in Nonidet P-40–containing buffer, and p53 was IP. TCLs and immunoprecipitates were IB with the indicated antibodies, and p97 served as a loading control as well as a cytoplasmic marker. His4 serves as a nuclear marker. (*B*) AGS cells were infected with the indicated Hp strains (1:100 moi) for 7 h or left uninfected and sonicated in Nonidet P-40–containing buffer, and cytoplasmic p53 was IP. TCLs and immunoprecipitates were IB with the indicated antibodies.

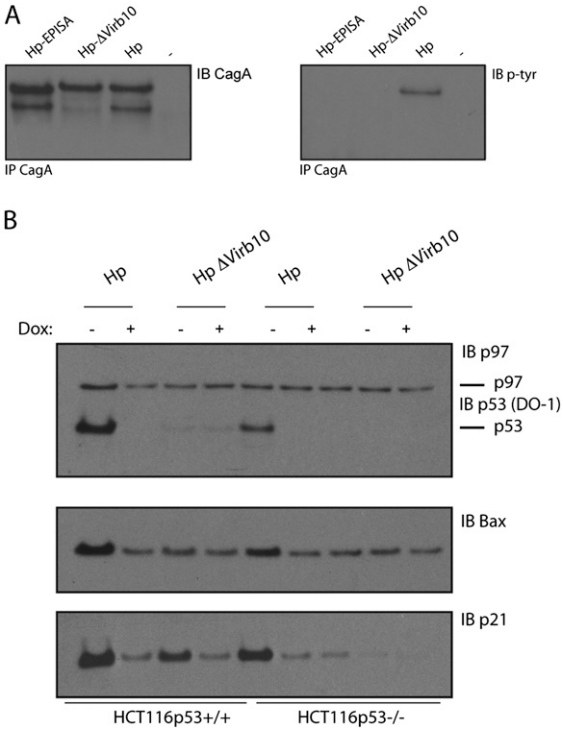


Fig. S6. CagA inhibits the accumulation and transcriptional activity of p53 in response to DNA damage. (*A*) HCT116*p53*$^{+/+}$ cells were infected for 24 h with the indicated Hp strains (moi = 1:50) or left uninfected and lysed in 1% SDS-containing lysis buffer. CagA was recovered by IP with an anti-CagA antibody and IB with the indicated antibodies. (*B*) HCT116*p53*$^{+/+}$ or HCT116*p53*$^{-/-}$ cells were infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected. Where indicated, cells were treated 5 h postinfection with 1 μg/mL Dox for 1.5 h. SDS-TCLs were IB with the indicated antibodies, with p97 serving as loading control.

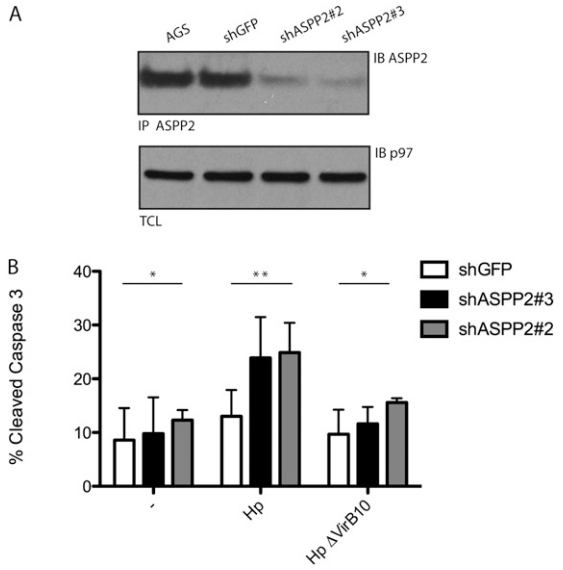


Fig. S7. Depletion of ASPP2 in AGS cells. (*A*) Efficiency of ASPP2 knockdown in AGS cells transduced with a lentiviral GFP (control) or ASPP2 shRNA constructs. Cells were lysed in 1% SDS-containing buffer, and ASPP2 was retrieved by IP and detected by IB with an anti-ASPP2 antibody. p97 was used as loading control. (*B*) Levels of CC3 assayed by flow cytometry in shGFP (white bars), shASPP2#3 (black bars), or shASPP2#2 (gray bars) AGS cells infected for 24 h with the indicated Hp strains (1:50 moi) or left uninfected. Error bars ± SEM (n = 5 for shGFP/shASPP2#3 and n = 3 for shASPP2#2). *P = ns; **$P < 0.05$. Significance was tested using two-way ANOVA Bonferroni multiple-comparison test.

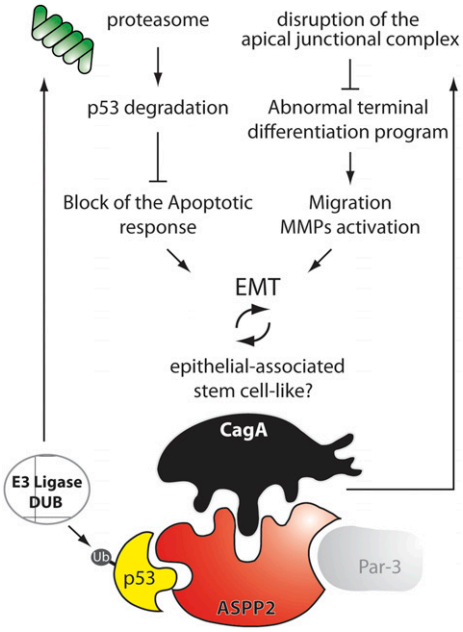


Fig. S8. Proposed model of the effects induced by the CagA-ASPP2 interaction. CagA targets ASPP2, and through this interaction, p53 is recruited and sent to the proteasome for its degradation. Thus, epithelial cells that have received CagA acquire the ability to resist apoptosis in a manner that is dependent on the initial ASPP2-CagA association. In epithelial cells, ASPP2 also has a role in establishing polarity and maintaining epithelial integrity. CagA may also hijack this second function of ASPP2 and thereby, cause the target cells to migrate, invade, and induce an abnormal terminal differentiation program reminiscent of an epithelial to mesenchymal transition (EMT) phenotype.

Table S1. List of primers

	Primer
BirA For	AAACCATGGCCAATGAAGGATAAC
BirA Rev	AAAGGATCCTCAGTGGTGGTG
AP-TEV-For	GCGGCCGCACCATGGGCCTGAACGACATCTTCGAGGCTCAGAAAATCGAA TGGCACGAAGCCCCGGGAGAAAATCTGTACTTTCAAGGCGCAGCTCGAG
AP-TEV-Rev	CTCGAGCTGCGCCTTGAAAGTACAGATTTTCTCCCGGGGCTTCGTGCCATTCG ATTTTCTGAGCCTCGAAGATGTCGTTCAGGCCCATGGTGCGGCCGC
AP*-TEV-For	GCGGCCGCACCATGGGCCTGAACGACATCTTCGAGGCTCAGCGTATCGAATGG CACGAAGCCCCGGGAGAAAATCTGTACTTTCAAGGCGCAGCTCGAG
AP*-TEV-Rev	CTCGAGCTGCGCCTTGAAAGTACAGATTTTCTCCCGGGGCTTCGTGCCATTCGATA CGCTGAGCCTCGAAGATGTCGTTCAGGCCCATGGTGCGGCCGC
CagA FL For	AAACTCGAGCAATGACTAACGAAACCA
CagA FL Rev	AAAGGATCCTTAAGATTTTTGGAAACC
CagA NT Rev	AAAGGATCCTTATCCAAGTTTTGCATTCAAC
CagA CT For	AAACTCGAGCACAATCCGTTAAGAATG
ASPP2 Flag For	AAAGTCGACATGGATTATAAAGATGATGATGATAAAATGATGCCGATGTTTCTTACC
ASPP2 Rev	AAAATCGATTCAGGCCAAGCTCCTTTGTCTTG
ASPP2 331 Rev	AAAATCGATTCATCCATCAGATGAAACTGGTAG
ASPP2 2583 Rev	AAAATCGATTCACGAGGTGTACACGAACTACAC
ASPP2 2760 For	AAAGTCGACATGGATTATAAAGATGATGATGATAAAAGGGTGAAATTCAACC
CagA Fw	AAACTCGAGGCATGACTAACGAAACCATT
CagA Rev	AAAGGATCCTCAAGATTTTTGGAAACCAC
Cherry For	AAAGGCGGCCGCCATGGTGAGCAAGGGC
Cherry Rev	AAACTCGAGCCTTGTACAGCTCGTCCATG