Supporting Information

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SI Text

General. All chemicals were purchased from Sigma-Aldrich unless otherwise noted. All cell culture materials were purchased from Gibco (Invitrogen), and cell lines were purchased from American Type Culture Collection (ATCC). For tissue culture, RKO cells were grown in MEM media, containing 10% FBS and 1% antibiotic and antimycotic (ABAM) (Gibco). GIA39 *Escherichia coli* cells (Coli Genetic Stock Center# 5594) were purchased from the *E. coli* genetic stock center. Antibodies for Western blots (yeast cytosine deaminase: Ab35251 and beta-Actin: Ab20272) were purchased from Abcam. The FLAG™ M2 (F3165) and FLAG™-HRP conjugated (A8592) antibodies were purchased from Sigma, and the HIF-1a antibody (BDB 610958) was purchased from BD Biosciences (Fisher Scientific). All antibodies were used according to the manufacturers' instructions.

Plasmids and Genes. Yeast cytosine deaminase was cloned from yeast genomic DNA using the primers: 5′-ttataaggatccatggtgacaggggaatggcaag and 5′-ttataaactagtctactcaccaatatcttcaaaccaatc with the NcoI and SpeI restriction sites underlined, and inserted into the pDIM-C8 plasmid, which contains a *tac* promoter and confers chloramphenicol resistance (1–3). Three thermostabilizing mutations (A23L, V108I, and I140L) (4) were incorporated using QuikChange (Stratagene) following the manufacturer's protocol. This thermostable version of the gene is referred to as "*yCD*" throughout *SI Text* for simplicity and the plasmid is referred to as pDIM-yCD. The DNA encoding the C-TAD domain of HIF-1a (amino acids 786–826) and the CH1 domain of the human p300 protein (amino acids 334–420) were ordered from IDT with *E. coli* codon optimization. The gstHIF-1a plasmid (for gstHIF-1a expression) was constructed using the GST fusion tag from the pGEX-6P-1 plasmid (GE Healthcare) and the *araC* gene and the arabinose promoter from the pBAD plasmid (Invitrogen) and conferred ampicillin resistance. The DNA encoding C-TAD domain of HIF-1a was fused to the DNA encoding GST as described by Freedman et al. (5) to form "gstHIF-1a." The gstHIF-1a fusion is inducible by the addition of arabinose to the media. DNA encoding human codon optimized yCD, Haps3, and Haps59 were purchased from GenScript and cloned into the pcDNA 3.1(+) plasmid with neomycin resistance (Invitrogen).

Library Creation. Purified pDIM-yCD plasmid was digested with dilute concentrations of DNaseI as described (1–3). Singly cut plasmids were isolated using gel electrophoresis and purified using Qiagen's gel extraction kit following the manufacturer's instructions. Isolated singly cut plasmids were repaired and blunted using T4 DNA polymerase (0.5 U/mg) and T4 DNA Ligase (150 U/mg) both from New England Biolabs. The repaired, linear DNA was isolated using gel electrophoresis and used in a ligation reaction with DNA encoding CH1 domain inserts. Three types of CH1 domain inserts were prepared. Two inserts are described as direct inserts and used as an unaltered CH1 gene with appended DNA that encoded peptide linkers. The appended linkers encoded a glycine on the N terminus and either a GGS peptide linker ("3-mer") or a GGGGS ("5-mer") peptide linker on the C terminus. The third CH1 domain insert was prepared using the circular permutation method shown in Fig. S1. The gene encoding the CH1 domain had a piece of DNA appended coding for a $(GSGGG)_3$ linker that joined together the N and C termini of the CH1 domain. The appended *CH1* gene was cyclized and digested with a nonspecific nuclease to create random circular permutations of this gene. To accomplish this, the CH1 DNA was excised from its plasmid using BamHI sites located within the linker region and the ends of the gene ligated together under dilute DNA concentrations to favor intramolecular ligation over intermolecular ligation. A standard cyclization reaction diluted 5 μg of the excised *CH1* gene into 500–600 μL of 1× T4 DNA ligase buffer (New England Biolabs) to a DNA concentration of approximately 8–10 ng/μL. The dilute *CH1* genes were cyclized using T4 DNA ligase (20 units/μL) at room temperature (RT) for 1 h. Cyclized *CH1* genes were isolated using gel electrophoresis. S1 nuclease was added (2.5 U/μg) to purified, cyclized DNA to make a variety of single double-stranded breaks within the CH1 DNA. The singly cut DNA was isolated using gel electrophoresis and repaired as described above in the digestion of pDIM-yCD. To create the yCD-CH1 hybrid library, a ligation reaction was performed with the randomly linearized pDIM-yCD plasmid DNA and a 5-fold higher amount (molar basis) of a 1:1:1 mixture of the three types of CH1 inserts. A typical ligation reaction mixture included 500 ng of plasmid DNA, approximately 200 ng of CH1 inserts, and 5% PEG in 1× T4 ligase buffer. Ligated plasmids were electroporated into DH5α *E. coli* cells and the transformation mixture plated on LB agar containing 50 μg/mL chloramphenicol in a 24.5 × 24.5 cm Bio-Assay dish (Nunc, Thermo Fisher Scientific). The number of transformants was 9.6×10^6 of which approximately 25% contained insert DNA, as estimated by gel electrophoresis of plasmid DNA isolated from the library.

5-fluorocytosine (5FC) Sensitivity Assays with GIA39 Cells in Liquid Media. GIA39 cells harboring a pDIM-C8 plasmid for expression of maltose-binding protein (MBP) (negative control), yCD (positive control), Haps3, or Haps59 were cotransformed with either the gstHIF-1a plasmid or an analogous negative control plasmid encoding only GST. These eight GIA39 cell lines were used for 5FC sensitivity experiments. In 96-well format, 1-mL cultures of the eight GIA39 cell lines described above were grown in minimal media at 37 °C with shaking. Minimal media consisted of 1× nitrogen base, 1× yeast synthetic dropout without uracil, 2% glucose, 5FC (varied from 0 to 1 mM), 1.5 μg/mL uracil, 1 mM IPTG, 100 μg/mL of ampicillin, 50 μg/mL chloramphenicol, and with or without 0.15% arabinose (to express gstHIF-1a and GST). The bacteria cells were cultured for 40–48 h at 37 °C after which the OD at 600 nm was measured using a SPECTRAmax Plus 96-well plate reader (Molecular Devices) and analyzed using Softmax Pro software (Molecular Devices). The relative cell densities were calculated using the equation $(A)_{sample}/(A)_{control} \times 100$, in which $(A)_{sample}$ is the absorbance of a sample well containing 5FC and $(A)_{control}$ is the absorbance of the control well lacking 5FC. Experiments performed with GIA39 cell lines expressing yCD and Haps proteins were repeated on three separate days in duplicate ($N = 6$). Control experiments performed with GIA39 cells expressing MBP were repeated twice in duplicate on separate days ($N = 4$). Error was calculated by standard deviation from the mean.

Western Blot for Accumulated yCD and Haps59 in GIA39 Cells. The membrane containing transferred cell lysates was blocked with 3% nonfat milk for 30 min. To observe the expression of Haps59 and yCD, primary antibodies for yCD were diluted into blocking buffer according to the manufacturer's protocol and incubated at RT for 1 h. The membrane was then washed, followed by the addition of sheep-HRP conjugated secondary antibodies (Bethyl Laboratories) using the Snap ID protein detection system

(Millipore) following the manufacturer's protocol. ECL was visualized using a Universal Hood II and QuanityOne software (BioRad).

Purification of yCD, Haps3, and Haps59. After failed attempts to purify the protein switches using a His tag and Ni^{2+} affinity, the genes encoding yCD, Haps3, and Haps59 were cloned into the pGEX-6P-1 plasmid for fusion to the affinity tag GST for purification (GE Healthcare). Separate 500-mL cultures of DH5α bacteria cells harboring each plasmid were grown in the presence of ampicillin at 37 °C until an OD of 0.4–0.6, at which point protein was induced by the addition of 1 mM IPTG for 3 h. Harvested cells were resuspended in lysis buffer (50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 0.1 mM EDTA) containing protease inhibitor cocktail (Sigma). Cells were lysed using a French Press and the insoluble material pelleted by centrifugation at 15,000 × *g* for 45 min at 4 °C. Cleared lysate was loaded onto a 5 mL GSTrap FF column using an AKTA purifier FPLC (GE Healthcare). The GSTrap column was washed with four column volumes of lysis buffer to clear unbounded proteins. The fusion protein was eluted using 50 mM Tris pH 8.0, containing 10 mM reduced glutathione. Alternatively, an in-column cleavage was performed on bound GST fusions using PreScission Protease (GE Healthcare) at 80 U/mg. Storage buffer (25 mM Tris buffer, pH 7.5, containing 50 mM NaCl) was used to elute the cleaved protein from the GSTrap column. Purity was judged to be >95% using SDS-PAGE and protein concentrations were determined using A_{280} and the calculated extinction coefficient for each enzyme. Purification of the gstHIF-1a fusion was performed exactly the same as above, except the fusion was not cleaved with PreScission Protease. GST-HIF-1a was eluted from the GSTrap column in elution buffer (50 mM Tris buffer, pH 8.0, containing 10 mM reduced glutathione) and dialyzed into storage buffer (25 mM Tris buffer, pH 7.5, containing 50 mM NaCl and 10% glycerol) then frozen at −20 °C.

5FC Activity Assay. The cytosine deaminase activity assays for yCD and the Haps proteins were performed on purified samples that were never frozen and within two weeks of purification. The 5FC activity assay was based on similar assays reported by Mahan and coworkers (6, 7). To determine the cytosine deaminase activity, yCD or Haps3 or Haps59 were added to a final concentration of 100 nM in a reaction tube containing 50 mM Tris buffer, pH 7.5. The mixture was incubated at 37 °C for 5 min and then 5FC was added at various concentrations (50 μM–1 mM). For experiments in the presence of 2.5 μM purified gstHIF-1a, both the protein switch and gstHIF-1a were incubated at 37 °C for 5 min before the addition of 5FC. Aliquots (50 μL) were removed from the reaction mixture at various time points (10, 20, 30, 60, 120, 180, and 240 s) and mixed with 0.1 N HCl. Measurements at 290 nm for 5FC and 255 nm for 5-fluorouracil (5FU) were recorded and inserted in the following equations to determine the concentrations of 5FC and 5FU: $[5FC] = 0.119A_{290} - 0.025A_{255}$ and $[5FU] = 0.105A_{255} - 0.049A_{290}$ as described elsewhere (6). Alternatively, CD activity was monitored using the decrease in absorbance at 235 nm, where 5FC absorbs. The production of 5FU was calculated using the decrease in absorbance at 235 nm over time and the extinction coefficient of 5FC. Kinetic parameters were determined using double reciprocal plots. Our calculated catalytic efficiency for triple mutant yCD at 37 °C was $4.98 \times 10^4\ E^{-1}\,M^{-1}\,s^{-1}$, which is consistent with other reports (8, 9). Although both switches exhibited cytosine deaminase activity and produced absorbance spectra changes consistent with the production of 5FU, the measured catalytic activities were inconsistent from purification to purification and decreased markedly over the period of a day. In general, the two protein switches' catalytic activity in the presence of gstHIF-1a ranged from 30–85% of that measured for yCD. The rate of deamination for both protein switches was always the same or higher (up to severalfold) when measured in the presence of purified gstHIF-1a. Analogous experiments with GST tags attached to the protein switches resulted in similar outcomes.

Copurification Experiments in *E. coli*. Copurification experiments were performed using GIA39 cells harboring the gstHIF-1a plasmid and either pDIM-Haps3 or pDIM-Haps59 (i.e., versions of the switches lacking the GST tag). A 500-mL culture was incubated at 37 °C until the OD reached 0.4–0.6. At this point, arabinose (0.15%) was added to the culture to express gstHIF-1a, and IPTG (1 mM) was added to express the protein switch. After 3 h at 37 °C, the cells were lysed and passed over a GSTrap column using a AKTA purifier FPLC (GE Healthcare). Unbound proteins were eluted by the passage of four column volumes of lysis buffer over the GSTrap column. Protein complexes were eluted using 50 mM Tris buffer, pH 8.0, containing 10 mM reduced glutathione and separated using SDS-PAGE. Because the switch proteins and gstHIF-1a have similar molecular weights (28 kDa and 31 kDa, respectively), an aliquot was treated with 20 U of PreScission Protease (GE Healthcare) to cleave off the GST tag of gstHIF-1a. The sample was subsequently passed over the GSTrap column again to remove the majority of free GST from the sample (Fig. S4).

5FC Experiments in Parental RKO and MCF7 Cells. RKO and MCF7 cells were acquired from the ATCC and genotyped before use. For controls, parental RKO cells were used to seed 96-well plates, 1,500 cells per well, in 100 μL of MEM media supplemented with 10% fetal bovine serum and 1% ABAM (all from Gibco). Parental MCF7 cells were used to seed 96-well plates, 3,500 cells per well, in 100 μL of DMEM media supplemented with 10% fetal bovine serum and 1% ABAM (all from Gibco). After 24 h, the media was changed with 100 μL of MEM (RKO) or DMEM (MCF7) media containing either 5FC (0–20 mM) or 5FU (0–2 mM). Cells were incubated for 3 to 6 d and then lysed. The cells were lysed by rinsing twice with 100 μL of PBS and then incubated in 100 μL of 0.1% SDS in water. The cells were incubated at 37 °C for 2 h in the SDS solution for complete lysis. To detect dsDNA, SYBR® green (Invitrogen) was added to a final concentration of 0.075% to lysed cells and the fluorescence for each well was recorded at 520 nm after excitation at 485 nm. The percent survival was calculated using the equation $(A)_{sample}/(A)_{control} \times 100$, where $(A)_{sample}$ is the fluorescence of the sample wells with 5FC and $(A)_{control}$ is the absorbance of the control well lacking 5FC and 5FU. Results shown in Fig. S5 *A* and *D* are the mean of experiments performed on three different days. Error bars are the standard deviation from the mean.

Creation of RKO and MCF7 Stable Cell Lines. Genes encoding human codon optimized yCD, Haps3, and Haps59 were cloned into the pcDNA 3.1 (+) plasmid (Invitrogen) under the control of a CMV promoter and containing neomycin resistance. Transfections were performed using Lipofectamine 2000 following the manufacturer's protocol in 6-well plates. A bulk selection using G418 (Geneticin) (Gibco) at a concentration of 0.8 mg/mL was performed 24 h after the transfection. Cells that survived the bulk selection were separated into one cell per well in 96-well plates for further selection in the presence of Geneticin. Single cells that grew into colonies in the presence of Geneticin were examined for their 5FC sensitivity by the addition 800 μM 5FC for yCD clones and 75 μM Co^{2+} and 800 μM 5FC for protein switch clones. Clones of RKO-yCD, RKO-Haps3, and RKO-Haps59 that were sensitive to 5FC containing media (i.e., functionally confirmed) were also confirmed genetically. Genomic DNA isolated from these clones was probed by PCR with primers that annealed outside of the multiple cloning site of the pcDNA3.1 (+) plasmid. Sequencing of the correct size PCR product con-

firmed the successful creation of the desired RKO cell lines. For the MCF7 cells, a gene encoding GFP was linked to the genes encoding yCD and Haps59 via a T2A peptide linker. The T2A linker is self-cleaving, yields two separate proteins (GFP + yCD or GFP + Haps59), with a short C-terminal peptide on GFP and only a single amino acid on the N terminus of the yCD or Haps59 proteins (10). The tandem genes (GFP-T2A-yCD or GFP-T2A-Haps59) were cloned into the pcDNA 3.1 (+) plasmid as well as the gene encoding GFP as an empty vector control. The same transfection protocol used for RKO cells was used for MCF7 cells. A bulk selection using G418 at a concentration of 0.6 mg/mL was performed 24 h after the transfection. Cells that survived the bulk selection were separated into one cell per well in 96-well plates using GFP as a reporter and further selection in the presence of Geneticin. Single cells that grew into colonies were characterized functionally and genetically as described above for RKO cells. Sequencing of MCF7-yCD and MCF7-Haps59 clones confirmed the successful creation of the stable cell lines.

5FU Production in RKO Cells. We confirmed that RKO cells expressing Haps59 generate 5FU and that HIF-1a accumulation increased the amount of 5FU produced. We used an activity assays similar to that used by others to demonstrate the conversion of 5FC to 5FU by yCD in human cell lysates (11, 12). RKO cells expressing an empty vector, yCD or Haps59, were grown to confluency in four T-25 flasks in MEM media, with two flasks growing RKO-Haps59 cells. One RKO-Haps59 flask was exposed to 100 μM Co^{2+} for >24 h. Cells in all four flasks were collected and resuspended in 400 μL of PBS, pH 7.4 and then lysed using two freeze–thaw cycles. RKO-Haps59 cells that had been exposed to 100 μM Co^{2+} were resuspended in PBS containing 100 μM Co^{2+}. After the lysis, cell debris was pelleted by centrifugation of samples for 5 min (20,000 × *g*, 4 °C) and protein concentrations were determined using a DC protein assay (BioRad). Cleared lysates (to a final concentration of 0.2 mg/mL) were added to a PBS solution containing 50 μM 5FC and incubated at 37 °C for 16–18 h.

The Analytical Pharmacology Core at Johns Hopkins University School of Medicine performed analysis of these samples. 5FU concentrations were quantified using an analytic assay based on reversed-phase HPLC with tandem mass spectrometric detection (13). The calibration curve and quality control samples were prepared in PBS, pH 7.4. Samples were quantified over the assay range of 50 to 5,000 ng/mL. Results can be seen in Fig. S6.

Western Blots for the Effect of Co^{2+} on HIF-1a (Fig. 4 *A* and *D*) and Haps59 (Fig. S7*A*) Accumulation. Mammalian cell whole cell lysates were prepared after incubation with or without 150 μM $CoCl_2$ for 4 h. After exposure, cell cultures were incubated at 4 °C for 30 min and then treated with radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease inhibitor cocktail (Sigma). Cells were scrapped from the flask and placed in a 1.5-mL tube and incubated on ice for 30 min. Cell debris was removed by centrifugation (20,000 × *g* for 30 min) and the supernatant was transferred to a fresh 1.5-mL tube. Protein concentrations of the whole cell lysates were determined using the DC protein assay (BioRad). A total of 50 μg of each lysate were separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen) and then transferred to PVDF membrane (BioRad). The membrane was blocked with 3% nonfat milk for 30 min.

Primary antibodies for HIF-1a were diluted into blocking buffer according to the manufacturer's protocol and incubated at 4 °C overnight. The membrane was then washed, followed by the addition of mouse-HRP conjugated secondary antibodies (BioRad) using the Snap ID protein detection system (Millipore) following the manufacturer's protocol. ECL was visualized using a Universal Hood II and QuanityOne software (BioRad). After detection of HIF-1a, the membrane was stripped and then reprobed with beta-actin-HRP conjugated antibodies (Abcam) to verify protein-loading levels.

To observe the expression of Haps59 and yCD in these same RKO lysates, primary antibodies for yCD were diluted into blocking buffer according to the manufacturer's protocol and incubated at RT for 1 h. The membrane was then washed, followed by the addition of sheep-HRP conjugated secondary antibodies (Bethyl Laboratories) using the Snap ID protein detection system (Millipore) following the manufacturer's protocol. ECL was visualized using a Universal Hood II and QuanityOne software (BioRad). After initial protein screen, the membrane was stripped and then reprobed with beta-actin-HRP conjugated antibodies (Abcam) to verify protein-loading levels (Fig. S7).

Coimmunoprecipitation (coIP) Experiments. yCD antibodies were shown to cross-react with many mammalian proteins (Fig. S7*A*). To circumvent this problem, a FLAG tag (Sigma-Aldrich) was added to the N terminus of Haps59 for coIP experiments. CoIP experiments were performed on lysates of RKO cells transiently expressing an empty vector, yCD or FLAG-Haps59 (Haps59 with an N-terminal FLAG tag) after overnight incubation with 150 μM Co^{2+}. These cells were lysed using RIPA buffer (Sigma) containing protease inhibitor cocktail (Sigma), and then primary antibodies (anti-HIF-1a or FLAG-M2) were added at a concentration of 1.5 μg antibody per 100 μL cell lysates. The mixture was incubated at 4 °C overnight with light mixing. After incubation with the primary antibody, the coIP complexes were purified using PureProteome Protein G Magnetic Beads (Millipore) following the manufacturer's protocol. Protein samples were eluted in 70 μL of denaturing sample buffer. An additional negative control was performed using the TATA-binding protein (TBP) antibody, which should have no affinity for HIF-1a or FLAG-Haps59. The TBP antibody (Abcam) was added to the FLAG-Haps59 lysate, and this sample was treated exactly the same as the other samples.

For Western blot analysis, 20 μL of each eluted complex sample was separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen) and then transferred to PVDF membrane (BioRad). The membrane was blocked with 3% nonfat milk for 30 min. Primary antibodies against yCD or FLAG-M2 were diluted according to the manufacturer's protocol and incubated at room temperature for 1 h. Primary antibodies for HIF-1a were diluted into blocking buffer according to the manufacturer's protocol and incubated at 4 °C overnight. The membrane was then washed followed by the addition of mouse-HRP conjugated secondary antibody (FLAG-M2) or sheep-HRP conjugated secondary (yCD) using the Snap ID protein detection system (Millipore) following the manufacturer's protocol. ECL was visualized using a Universal Hood II and QuanityOne software (BioRad). To confirm the identity of the FLAG-Haps59 band, the membrane initially probed with yCD antibodies was stripped and then reprobed with anti-FLAG antibodies as described above.

Amino Acid Sequence for Haps Proteins. ***Haps3.*** MVTGGMASGDPEKRKLIQQQLVLLLHAHKCQRREQANGEVRQCNLPHCRTMKNVLNHMTHCQSGKSCQVAHCASSRQIISHWKNCTRHDCPVCLPLKNAGGSKWDQKGMDIAYEEALLGYKEGGVPIGGCLINNKDGSVLGRGHNMRFQKGSATLHGEISTLENCGRLEGKVYKDTTLYTTLSPCDMCTGAIIMYGIPRCVIGENVNFKSKGEKYLQTRGHEVVVVDDERCKKLMKQFIDERPQDWFEDIGE

Haps59. MVTGGMASDPEKRKLIQQQLVLLLHAHKCQRREQANGEVRQCNLPHCRTMKNVLNHMTHCQSGKSCQVAHCASSRQIISHWKNCTRHDCPVCLPLKNAGGWDQKGMDIAYEEALLGYKEGGVPIGGCLINNKDGSVLGRGHNMRFQKGSATLHGEISTLENCGRLEGKVYKDTTLYTTLSPCD-

MCTGAIIMYGIPRCVIGENVNFKSKGEKYLQTR-GHEVVVVDDERCKKLMKQFIDERPQDWFEDIGE

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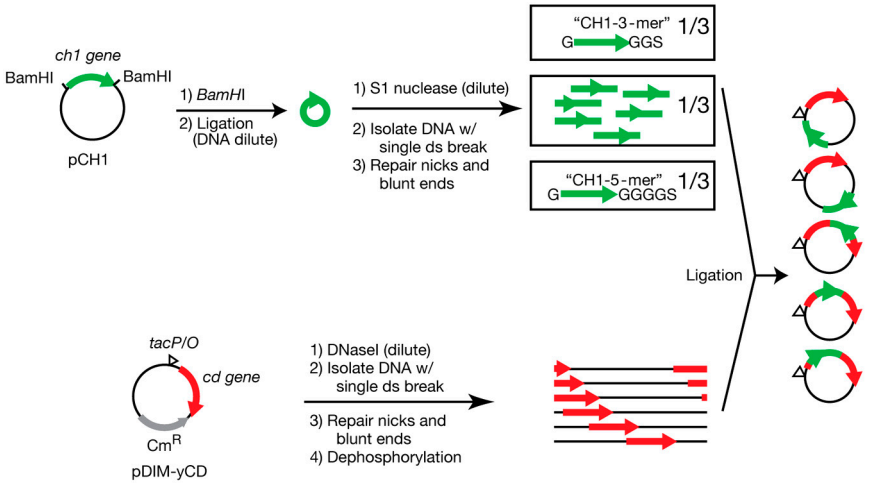

Fig. S1. Schematic showing domain insertion method used to create the yCD-CH1 hybrid library. The CH1 domain inserts (cpCH1, CH1-3-mer, and CH1-5-mer) were mixed in an equimolar ratio before they were used in the ligation mixture with singly cut pDIM-yCD plasmids.

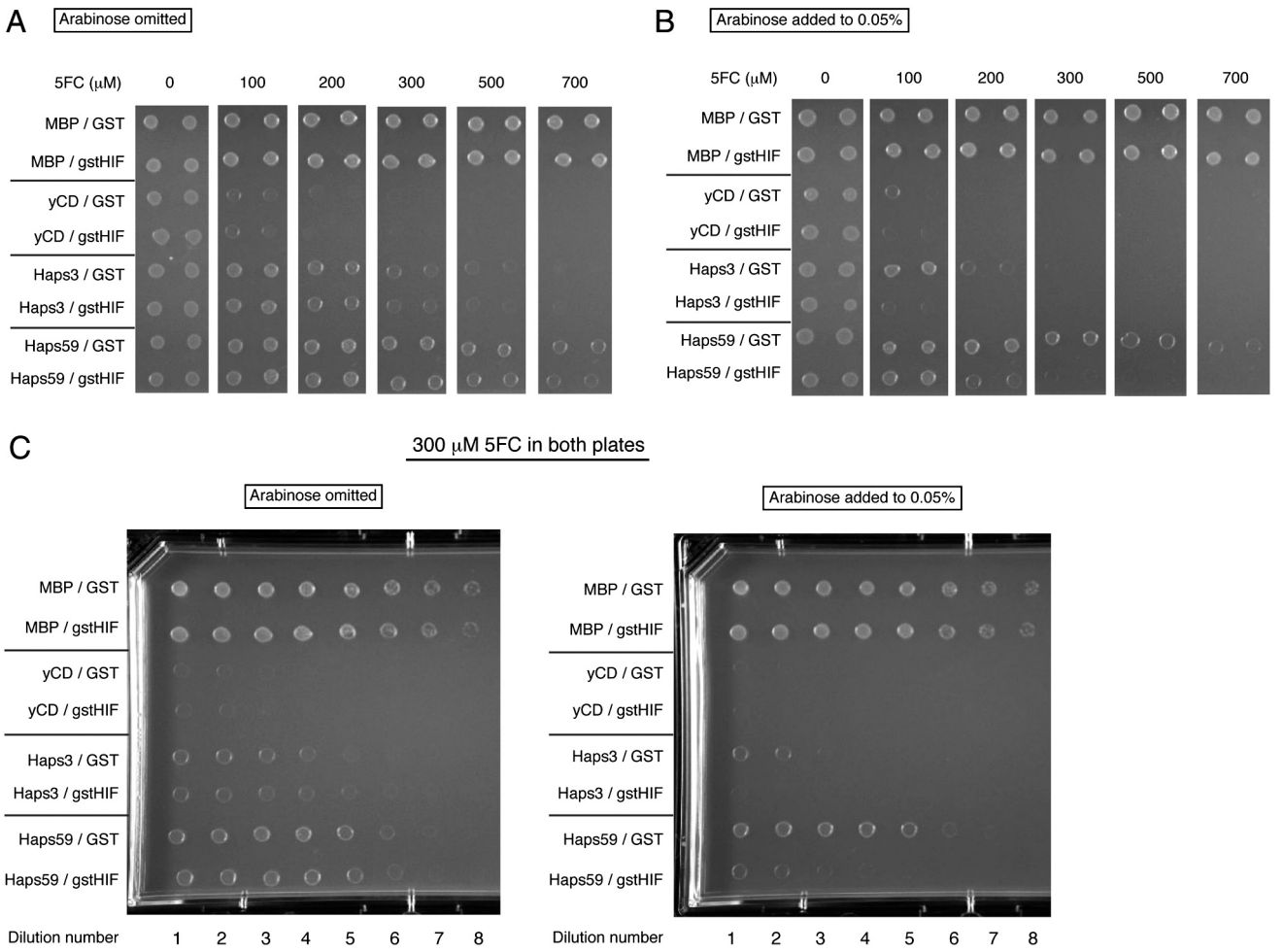

Fig. S2. Growth of GIA39 cells expressing MBP, yCD, or Haps59 and either GST or gstHIF-1a on minimal media agar plates as a function of 5FC concentration. *A* is in the absence of arabinose and *B* is in the presence of 0.05% arabinose to express GST or gstHIF-1a. (*C*) Example "full" plates showing all dilutions that exhibited growth. Both plates contained 300 μM 5FC and either omitted arabinose (*Left*) or contained 0.05% arabinose (*Right*). Threefold less arabinose was used in these experiments compared to the liquid media experiments to lessen the burden of high expression from the arabinose promoter. Dilutions 3 and 4 from all plates were used to create Fig. 3*B* and *A* and *B* of this figure. The control lines (MBP and yCD) were unaffected by the expression of either GST or gstHIF-1a. 5FC toxicity in cells expressing Haps3 increased more with gstHIF-1a than GST, whereas only gstHIF-1a increased the 5FC toxicity for cells expressing Haps59.

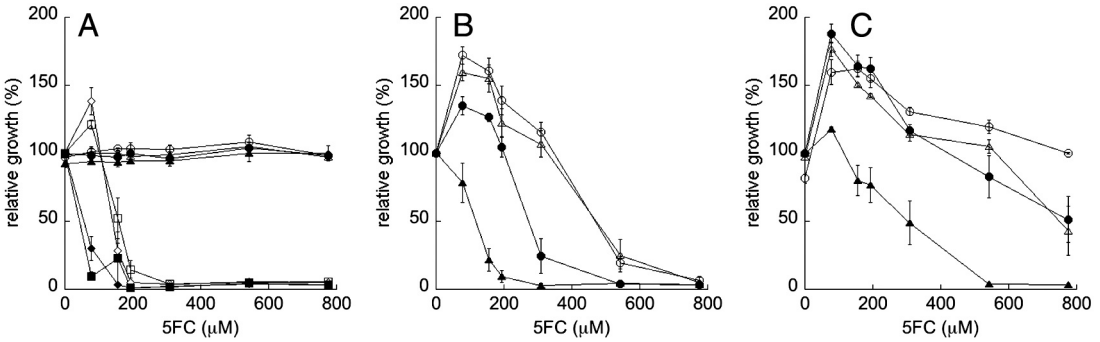

Fig. S3. Growth of GIA39 cells expressing MBP, yCD, or Haps59 and either GST or gstHIF-1a in minimal liquid media as a function of 5FC concentration. Media either omitted (open symbols) or contained (solid symbols) 0.15% arabinose to compare the effects of coexpressing GST or gstHIF-1a. (*A*) GIA39 cells expressing control proteins (MBP + GST, circles; MBP + gstHIF-1a, triangles; yCD + GST, diamonds; yCD + gstHIF-1a, squares). GIA39 cells expressing (*B*) Haps3 or (*C*) Haps59 with either GST (circles) or gstHIF-1a (triangles) demonstrate that gstHIF-1a increases the 5FC toxicity of cells expressing these switches. For all graphs, error bars, SD ($N = 6$). Experiments with yCD (*A*) revealed that both GST and gstHIF-1a induction by arabinose caused a small but equal increase in 5FC toxicity. This effect likely arises from the added burden of high expression from the arabinose promoter to cells that are coping with near lethal levels of 5FC. In contrast, gstHIF-1a expression increased the 5FC toxicity of cells expressing Haps3 (*B*) and Haps59 (*C*) to a much greater extent than did GST expression. We attribute the increase in cell density observed at sublethal 5FC concentrations for cells expressing yCD, Haps3, or Haps59 to a stress response that allows growth to higher densities in the minimal media because the increase is not observed when MBP is expressed.

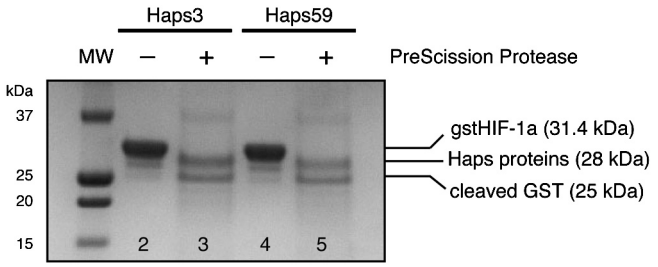

Fig. S4. Copurification of Haps3 and Haps59 with gstHIF-1a as visualized with Coomassie blue stain. GIA39 cells harboring the gstHIF-1a plasmid were used to express gstHIF-1a and either Haps3 or Haps59. The lysates were passed over a GSTrap column. Lanes 2 and 4 show the eluted proteins purified from cells expressing gstHIF-1a and either Haps3 (lane 2) or Haps59 (lane 4). In both of these lanes a heavy band corresponding to the molecular weight of gstHIF-1a (which is present in excess) and lighter, faster migrating band corresponding to the Haps protein was observed. To remove the majority of the gstHIF-1a, an aliquot of each purified protein mixture was treated with PreScission protease to cleave the GST tag off of gstHIF-1a, then passed over a GSTtrap column to remove most of the cleaved GST protein. The flow through from this experiment was loaded in lane 3 (Haps3) and lane 5 (Haps59).

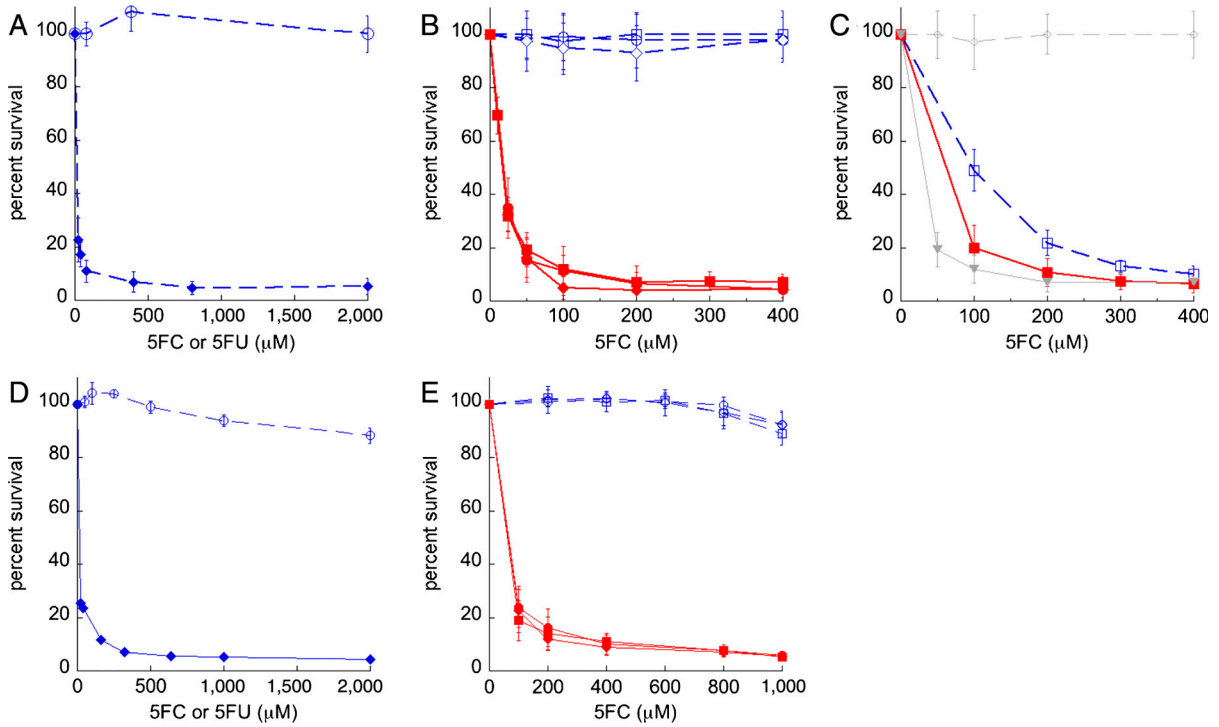

Fig. S5. 5FC and 5FU sensitivity experiments in RKO and MCF7 cells. (*A*) Parental RKO cells treated with 5FU (solid blue diamonds; ◆) or 5FC (blue circles; ○). Each point represents the mean of six experiments performed on three separate days. Error bars, SD ($N = 6$). (*B*) Positive and negative control experiments in stable RKO cell lines. The 5FC sensitivity of RKO cells stably expressing yCD (red, solid symbols) or an empty vector control (blue, open symbols) that were incubated in normoxic conditions in the absence (squares) or presence (circles) of 100 μM Co^{2+} or in 1% O_2 (diamonds). Error bars, SD ($N = 6$). (*C*) Haps3-expressing RKO cells incubated in normoxic conditions show a small increase in sensitivity to 5FC in the presence of 100 μM Co^{2+} (solid red squares, ■) over cells in the absence Co^{2+} (open blue squares, □). Error bars, SD ($N = 4$). Controls from RKO cells expressing yCD or EV (*B*) are shown in gray for comparison. (*D*) Parental MCF7 cells treated with 5FU (solid blue diamonds; ◆) or 5FC (blue circles; ○). Each point represents the mean of nine experiments performed on three separate days. Error bars, SD ($N = 9$). (*E*) Positive and negative control experiments in stable MCF7 cell lines. The 5FC sensitivity of MCF7 cells stably expressing yCD (red, solid symbols) or an empty vector control (blue, open symbols) that were incubated in normoxic conditions in the absence (squares) or presence (circles) of 100 μM Co^{2+} or in 1% O_2 (diamonds). Error bars, SD ($N = 9$).

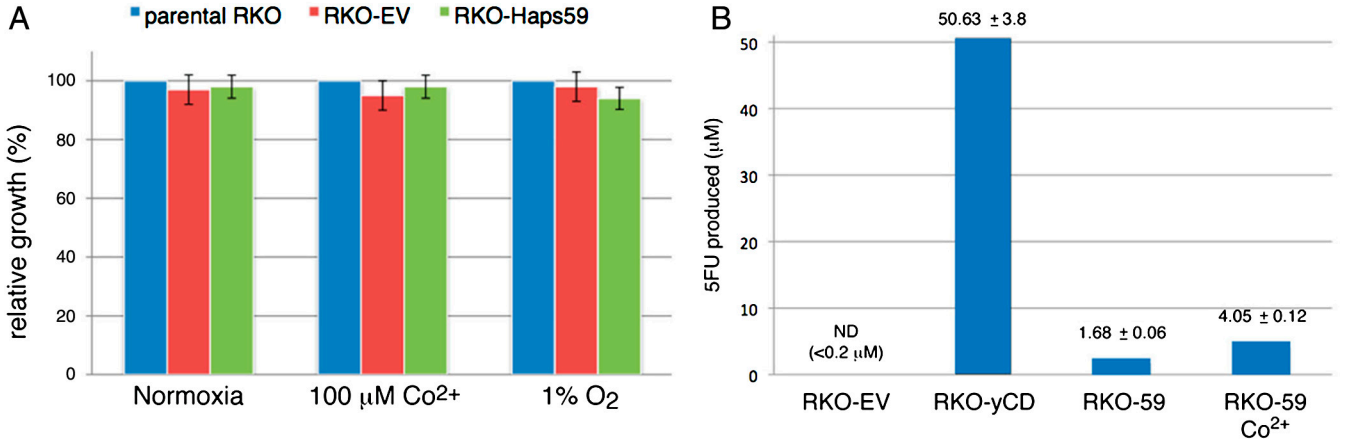

Fig. S6. (*A*) The relative growth of various RKO cells under experimental conditions. The presence of Co^{2+} or 1% O_2 did not have an effect on the growth of RKO cells expressing Haps59 or RKO cells expressing an EV when compared to the parental RKO cells. (*B*) 5FU production by RKO cells. Lysates from RKO cells expressing an empty vector (RKO-EV) or Haps59 (RKO-59; either with or without Co^{2+}) were added to a solution containing 5FC. 5FU production was measured by using an analytic assay based on reversed-phase HPLC with tandem mass spectrometric detection (9). Expression of yCD instead of Haps59 resulted in 10-fold more 5FU production, which corresponds to complete conversion 5FC. We speculate that the instability of Haps59, especially after cell lysis, could contribute to the large difference in production of 5FU compared to yCD lysates.

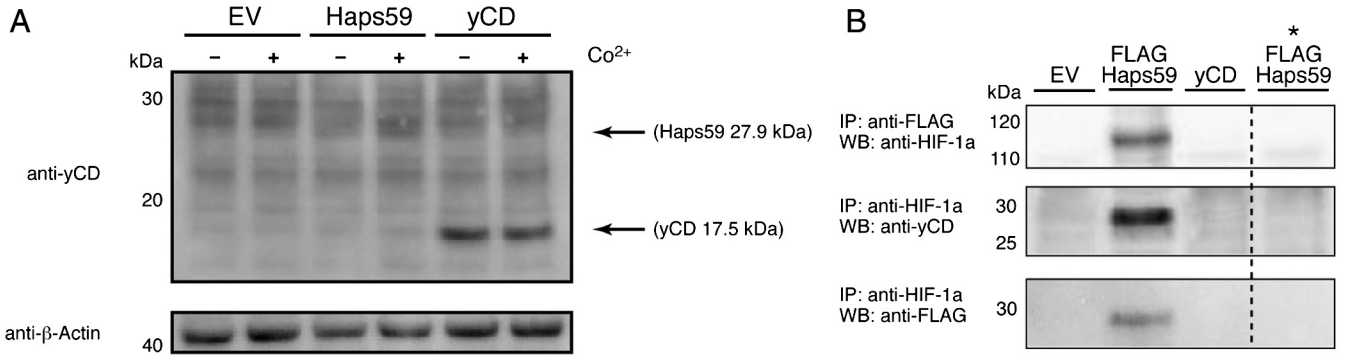

Fig. S7. (*A*) The effect of Co^{2+} on Haps59 and yCD accumulation in RKO cells stably expressing these proteins. Haps59 and yCD were detected by Western blot using anti-yCD antibodies. An EV was used as a negative control. The cross-reaction of the yCD antibodies with many human proteins complicated Haps59 detection, but the results suggest that Haps59 may accumulate to a higher level when the cells were grown under conditions that cause HIF-1a to accumulate. yCD accumulation was unaffected by HIF-1a accumulation. (*B*) Haps59 and HIF-1a interact in RKO cells. In these experiments, a FLAG epitope was appended to the N terminus of Haps59 (FLAG-Haps59) for switch immunoprecipitation and detection. Western blot (WB) analysis of coIP reactions with lysates of RKO cells expressing FLAG-Haps59, yCD, or an EV control show that HIF-1a is precipitated with anti-FLAG antibodies only in FLAG-Haps59-expressing cells (*Top*) and that FLAG-Haps59 is precipitated by anti-HIF-1a antibodies (*Bottom*). The far right lane (*) shows that coimmunoprecipitations of lysates of FLAG-Haps59-expressing cells using TATA-binding protein antibody [a negative control instead of the IP antibodies (*Left*)] did not result in detection of HIF-1a or FLAG-Haps59.