Title: Protein-programmed accumulation of yeast cytosine deaminase in cancer cells in response to mock-hypoxia

Author List: Tiana D. Warren[1], Krishna Patel[1], James R. Eshleman[2,3], Marc Ostermeier[1*]

[1]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD, 21218, USA

[2]Department of Pathology and [3]Department of Oncology, Johns Hopkins University School of Medicine, 733 N Broadway, Baltimore, MD, 21205, USA

ABSTRACT

One limitation of gene-directed enzyme prodrug therapy (GDEPT) is the difficulty in selectively and efficiently transducing cancer cells with the gene encoding a prodrug-converting enzyme. To circumvent this issue, we sought to move the selectivity from the gene delivery level to the protein level. We developed fusion proteins of the prodrug-activating enzyme yeast cytosine deaminase (yCD) and the oxygen-dependent degradation domain (ODDD) of HIF-1α, a domain that regulates the accumulation of HIF-1α in an oxygen-dependent manner. We called these HOPE fusions for HIF-1α Oxygen-dependent degradation domain/Prodrug-converting Enzyme. The HOPE fusions were designed to selectively accumulate in cells experiencing hypoxia and thus selectively cause conversion of the prodrug 5-fluorocytosine (5-FC) to the chemotherapeutic 5-flourouracil (5-FU) where oxygen levels are low (e.g. at the center of a tumor). Consistent with our hypothesis, HT1080 fibrosarcoma cells transduced with HOPE fusion genes exhibited increased fusion protein accumulation and increased sensitization to 5-FC in mock-hypoxia.

Keywords: protein engineering, enzyme/prodrug therapy, GDEPT, HIF-1α, yeast cytosine deaminase

Gene therapy first emerged in the 1960s and 70s. GDEPT was designed to avoid the major pitfall of chemotherapy: systemic toxicity that causes the death of healthy cells. GDEPT makes chemotherapy more selective by targeting chemotherapy to cancer cells through a two-step process. In the first step, a gene encoding a prodrug-converting enzyme is selectively delivered to cancer cells. In the second step, a prodrug is systemically administered to the patient. Consequently, only in cells that both express the prodrug-converting enzyme and harbor the prodrug, will the prodrug be converted to its toxic form and lead to downstream cell death.

One of the common GDEPT systems is cytosine deaminase with the prodrug 5-fluorocytosine (5-FC). In particular, yeast cytosine deaminase (yCD) is preferred over the *E. coli* CD due to yCD's superior kinetics for 5-FC[1]. Furthermore, a study by Korkegian et al. led to a thermostable triple mutant yCD (A23L/V108I/I140L) exhibiting a half-life of 117 hours at 50°C compared to 4 hours for the wild-type enzyme[2]. Despite having a comparable catalytic efficiency compared to the wild-type enzyme, use of thermostable yCD over the wild-type variant results in increased 5-FC sensitization of *E. coli* and rat glioma cells[3]. The yCD/5-FC system is of particular interest because the toxin generated from 5-FC, 5-fluorouracil (5-FU), is a well-established chemotherapeutic[4]. One of the challenges of GDEPT has been limited transduction efficiency when trying to deliver the gene specifically to cancer cells. To circumvent the issue of limited transduction efficiency, efforts have been made to link the activity or accumulation of prodrug-converting enzymes to the presence of molecular cues specific to cancer cells[5-10], thereby allowing the use of more effective (but non-specific) methods for gene delivery. Here, we use the cellular properties of the oxygen-dependent degradation domain (ODDD) of HIF-1α to link the accumulation of yCD to hypoxia.

Hypoxic cancer cells with increased levels of HIF-1 are a known indicator of poor prognosis[11]. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric, transcription factor that regulates the cell's response to limited oxygen levels. Whereas the HIF-1β subunit is constitutively expressed in specific tissues, HIF-1α exhibits ubiquitous expression in all tissues but its accumulation is dependent on oxygen availability. In normoxia, HIF-1α is hydroxylated at proline residues 402 and 564 in LXXLAP motifs in the oxygen-dependent degradation domain (ODDD) by prolyl hydroxylases 1, 2 and 3. Hydroxylated HIF-1α binds to the Von Hippel Lindau tumor suppressor protein, which then forms an E3-ubiquitin ligase complex with HIF-1α. Attachment of a polyubiquitin tag to HIF-1A marks the protein for degradation by the 26S proteasome. However, this prolyl hydroxylation is prevented by the absence of oxygen (i.e. hypoxia), because oxygen is a direct substrate of the reaction. Therefore, HIF-1α accumulates and is translocated to the nucleus where it dimerizes with HIF-1β to form HIF-1. HIF-1 binds to hypoxia-response elements (HREs) in enhancers that control the expression of genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which promote angiogenesis and erythropoiesis, respectively, to recruit more oxygen to the tumor.

HIF-1α and its HRE binding partners have been combined with GDEPT to create hypoxia-inducible GDEPT systems. In one study, three HREs were incorporated into the PGK and 9-27 promoters to induce expression of bacterial cytosine deaminase in HT1080 cells and 9-3C cells, respectively[8]. Exposure of each of the cell lines to anoxic conditions (lack of oxygen) led to HT1080 cells and 9-3C cells being 1.4 and 5.4 more susceptible to 5-FC , respectively, when compared to normoxic conditions. Shibata et al. developed a recombinant adeno-associated viral vector with five copies of the VEGF HRE, a minimal CMV promoter, and the bacterial nitroreductase gene which increased the sensitivity of

HT1080 cells to CB1954 by 40-50-fold in hypoxia compared to normoxia[9]. Patterson et al. created hypoxia-inducible expression vectors with human cytochrome c P450 reductase which yielded a 30-fold increase in cytotoxicity upon RSU1069 prodrug administration; furthermore, xenografts containing these vectors showed double the survival time in at least half the mice injected with the prodrug[10].

We have taken the complementary approach of programming hypoxia-dependent prodrug activity into cells at the protein level rather than at the transcription level. Previously, we designed several protein fusions between the CH1 binding domain of the p300/CBP protein and either yCD or herpes simplex virus thymidine kinase[5-7]. These fusion proteins function as switches in which HIF-1α binding to the CH1 domain of the switch causes increased catalytic activity and/or accumulation of the switch. These protein switches confer increased 5-FC sensitivity to *E. coli* [5-7] and mammalian cells[6] in which HIF-1α accumulates. Here, we show that appropriate fusions of yCD to the ODDD of HIF-1α afford low accumulation of the fusion in normoxia and higher accumulation in hypoxia, therefore opening a therapeutic window between normal and cancer cells expressing the fusion protein.

RESULTS AND DISCUSSION

We hypothesized that fusion of the ODDD and yCD would be a mechanism for making cellular yCD activity higher in tumor cells experiencing hypoxia compared to cells experiencing normoxia (**Figure 1a**). We based our hypothesis in part on the observation that a fusion of the ODDD to the Gal4 DNA-binding domain accumulated to lower levels than the Gal4 binding domain alone in normoxic cells[12]. Our envisioned yCD-ODDD fusions would be degraded in healthy normoxic cells but would accumulate in hypoxic tumor cells

and convert 5-FC to 5-FU, thus killing the tumor cells (**Figure 1a**). We named these proteins HOPE fusions for **H**IF-1α **O**DDD **P**rodrug-converting **E**nzymes. Eight different HOPE fusions were designed: four with the ODDD fused to the C-terminus of yCD (HOPE-1, -2, -3 and -4) and four with ODDD fused to the N-terminus of yCD (HOPE-5, -6, -7, and -8) (**Figure 1b**). These fusions differed in the length of the linkers between the two domains and included a fusion without linkers. We tested different length linkers because the protein domains need to be far enough apart such that each domain can fold into its active conformation, but not so far apart that it results in the domains being separated by proteolytic cleavage in the linker (thus uncoupling degradation of the ODDD and the yCD domains).

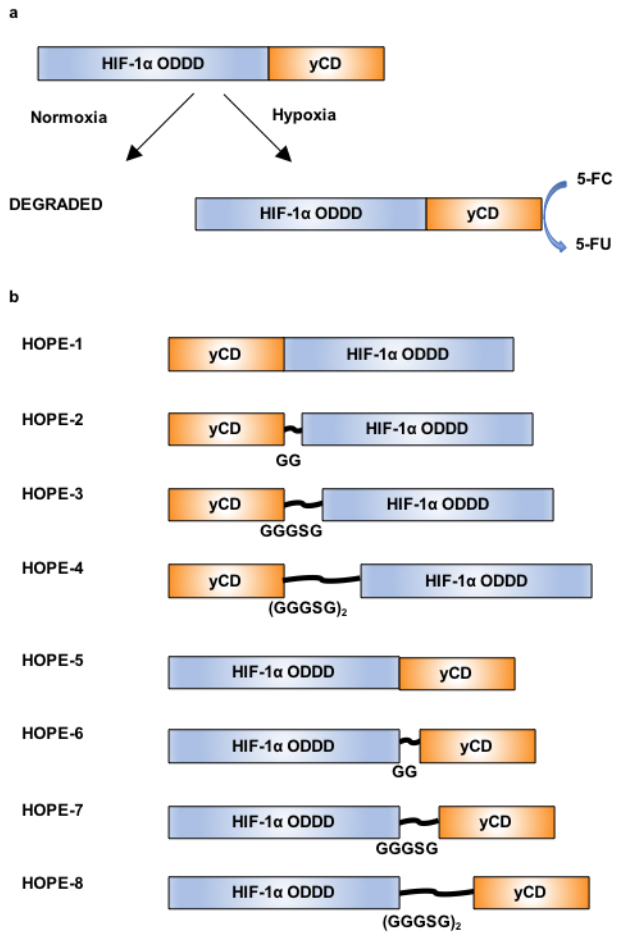

Figure 1: Project objective and HOPE fusions design

(a) Schematic of the desired mechanism of HOPE fusions. In healthy normoxic cells, the presence of the HIF-1α ODDD would mark the fusion to be degraded by the 26S proteasome, like the domain does in HIF-1α . In hypoxic cancer cells, the fusion protein would accumulate and, upon the administration of the prodrug, convert 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and kill the cell. (b) HOPE fusions tested in this study. The HIF-1α ODDD was fused to either the N- or C-terminus of yCD without a linker, and with flexible linkers of different lengths as indicated.

5-FC toxicity assay in E. coli confirms yCD activity. The activity of the yCD domain in each of the HOPE fusions was assessed by testing each fusion for its ability to sensitize an *E. coli* cytosine deaminase knockout strain (GIA39) to 5-FC (**Figure 2**). We plated bacterial cultures expressing each HOPE fusion on minimal media plates

supplemented with uracil and different concentrations of 5-FC. HOPE fusions with the HIF-1α ODDD fused to the C-terminus of yCD caused lower 5-FC sensitivity than the unfused yCD enzyme, but HOPE fusions in the opposite order caused higher sensitivity to 5-FC than yCD. Fusion of the ODDD to the C-terminus of yCD may disrupt folding or function of the yCD for steric reasons, since the C-terminus of yCD is involved in substrate binding and product release[13-14]. This is consistent with the observation that HOPE-4, the fusion of this type with the longest linker, conferred the highest 5-FC toxicity among HOPE fusions 1-4.

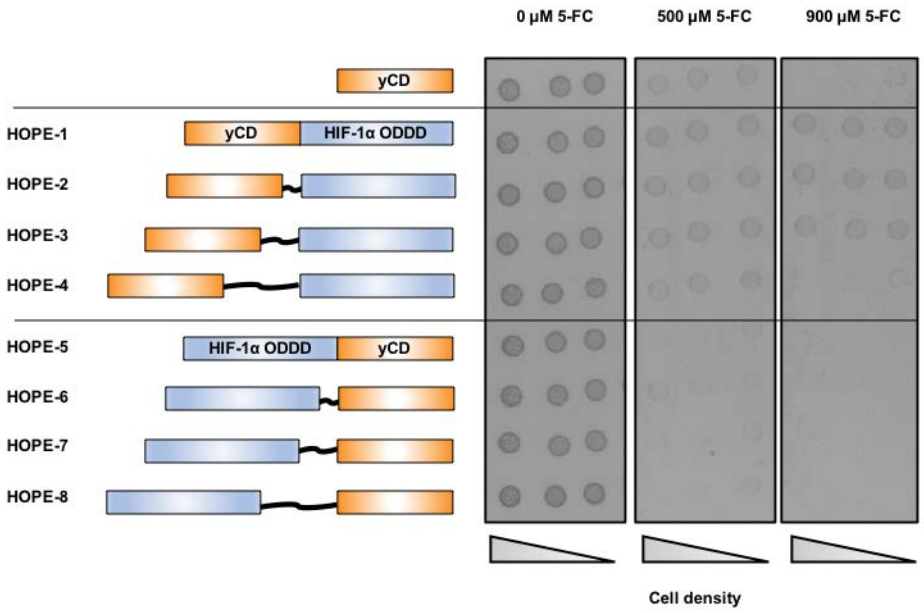

Figure 2: 5-FC toxicity assay of HOPE fusions in *E. coli*

Different dilutions of cultures of *E. coli* GIA39 cells expressing the indicated proteins were spotted on minimal media plates containing uracil and the indicated concentration of 5-FC. Plates were incubated for 36 hours at 37°C and imaged for growth.

Accumulation of HIF-1α in HT1080 cells in mock-hypoxia using cobalt chloride. We next sought to test the HOPE fusions in HT1080 fibrosarcoma cells, but

wanted to first confirm that HIF1α accumulated under mock hypoxia in these cells as expected. Cobalt chloride ($CoCl_2$) is a hypoxia mimetic that induces HIF-1α accumulation by inhibiting the prolyl hydroxylases responsible for marking the protein for degradation[15]. We confirmed that $CoCl_2$ caused HIF-1α accumulation in wild-type HT1080 cells (**Figure 3a**). We chose 100 μM $CoCl_2$ for all subsequent mock-hypoxia experiments since it was the lowest concentration that clearly caused HIF-1α accumulation, and higher levels of $CoCl_2$ can have toxic effects.

Generation of HT1080 stable cell lines by lentiviral transduction. We created stable cell lines to ensure continuous expression of each of HOPE fusion throughout the course of the experiments. We excluded HOPE-2 and HOPE-6 (the fusions with the GG linker) from our human cell studies since screening in *E. coli* suggested their activity would be no different than their corresponding fusions with the GGGSG linker. We cloned each of the selected HOPE fusion genes, yCD, ODDD, and an empty vector control into a third-generation lentiviral vector system that afforded bicistronic expression of EGFP from an internal ribosome entry site (IRES) (**Figure 3b**). After performing the lentiviral transduction, the transduced cells were passaged three times and then sorted into EGFP-positive single cells. We isolated genomic DNA from each stable line and performed nested PCR on the region containing the HOPE fusions or yCD. We confirmed insertion of the correct full-length gene by Sanger sequencing.

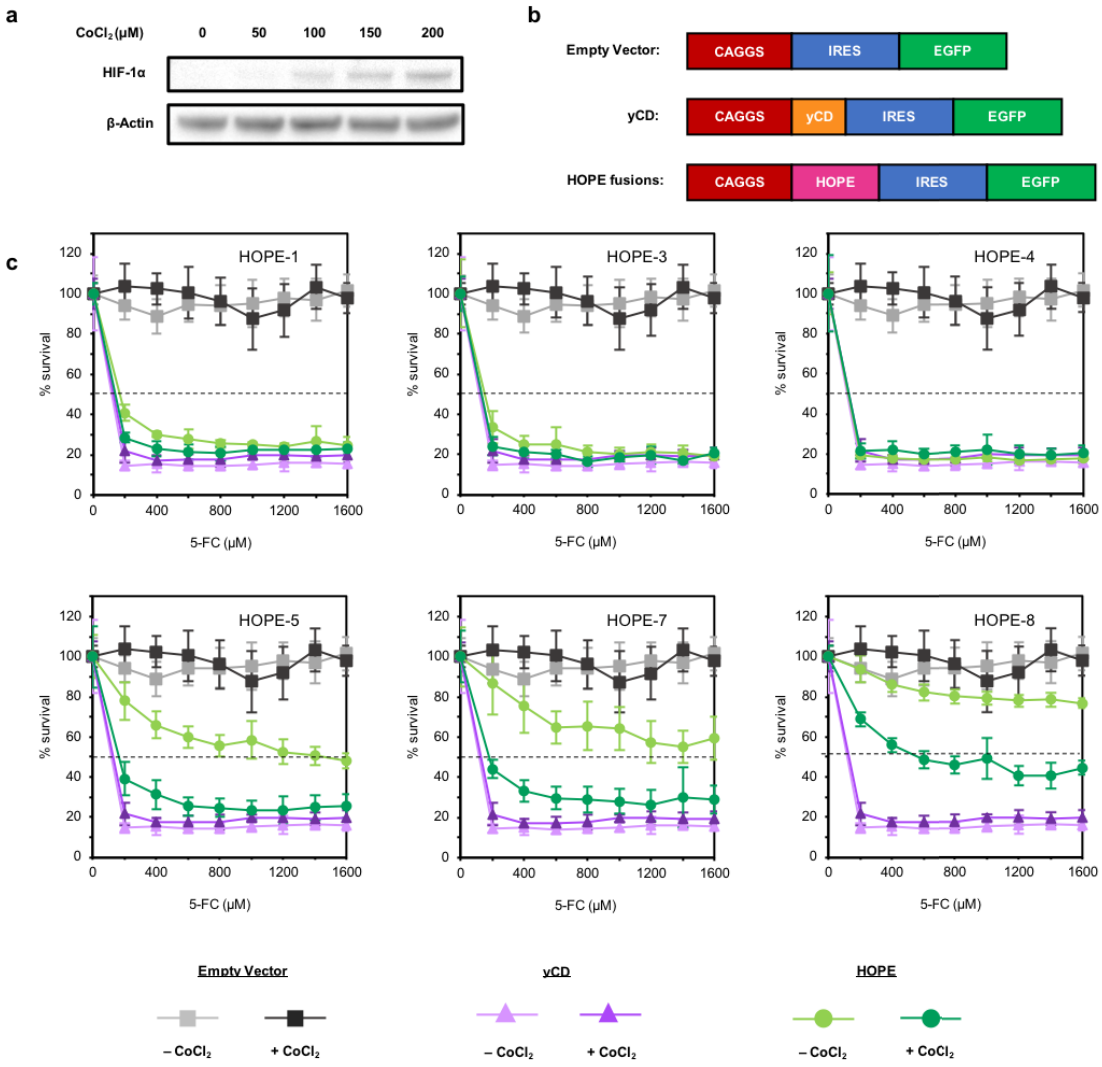

Figure 3: 5-FC toxicity in HT1080 fibrosarcoma cells expressing HOPE fusions

(a) Western blot using anti-HIF-1α antibodies showing the accumulation of HIF-1α in HT1080 cells at different concentrations of $CoCl_2$, a hypoxia-mimetic. (b) Lentiviral insertion cassettes for stable expression of yCD or HOPE fusion. Bicistronic expression of EGFP allowed the identification of successfully transduced cell lines. (c) 5-FC toxicity assay for stable HT1080 cell lines expressing a HOPE fusion, yCD, or empty vector (EV) control and grown in the absence or presence of $CoCl_2$. Identical empty vector and yCD curves are provided in each graph for reference. Each curve is from a representative single experiment. Two replica experiments showing the same trends are provided as Supplementary Figure 1. Error bars reflect the propagated error in the cell viability assay in that experiment (n=6).

Mock-hypoxia increases 5-FC toxicity in HT1080 fibrosarcoma cells expressing HOPE fusions. We next tested whether the fusion proteins resulted in increased sensitization to 5-FC. We seeded each of the HT1080 stable cell lines in 96-well plates and challenged the cell lines to grow at various 5-FC concentrations with or without the addition of 100 μM $CoCl_2$ to induce mock-hypoxia. 5-FC toxicity was assessed via cell survival as measured by the alamarBlue assay. We performed five replicas (four for HOPE-5). A replica with an extended range of 5-FC concentrations is shown in **Figure 3c**. We focused on lower concentrations of 5-FC in the other four replicas to get better estimates of 5-FC LC_{50} values by linear interpolation. Two examples of these replicas are shown in **Supplementary Figure 1.** LC_{50} values are tabulated in **Supplementary Table 1**, from which we calculated a fold-difference in LC_{50} between with and without $CoCl_2$ (**Figure 4**). As expected, $CoCl_2$ did not alter the 5-FC toxicity of cell lines expressing yCD or the empty vector control (**Figure 3c**, **Supplementary Table 1**). None of the HOPE fusions equaled the 5-FC toxicity caused by yCD; however, to different degrees, all HOPE fusions exhibited $CoCl_2$-dependent differences in 5-FC toxicity (**Figure 4**). While HOPE-1, -3, and -4 caused marginal differences in LC50 (on the order of 2.5-fold or less), HOPE-5 and -7 caused a much greater $CoCl_2$-dependent increase in 5-FC toxicity. However, this came at the expense of decreased 5-FC toxicity overall. For HOPE-5 and HOPE-7, 5-FC became at least 7.8- and 6.3-fold more toxic in the presence of $CoCl_2$, respectively. HOPE-8 also appears to have a significant $CoCl_2$ dependence.

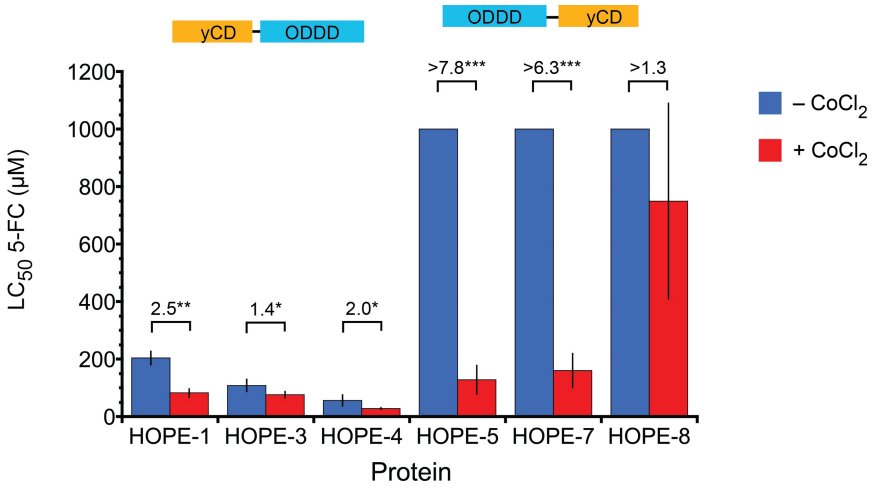

Figure 4. $CoCl_2$-dependent toxicity conferred by HOPE fusions in HT1080 fibrosarcoma cells. The LC_{50} values (tabulated in **Supplementary Table 1)** are the mean and the error bars are the standard deviation (n=4 except HOPE-5, which is n=3). For HOPE-5, -7, and -8, the value in the absence of $CoCl_2$ (1000 μM) is a lower bound, which was the highest 5-FC concentration tested. The numbers above each pair of data are the LC_{50} ratio. Statistical significance of these ratios was evaluated by a paired, one-tail Student's *t*-test. (*<0.05, **<0.01, ***<0.001).

HOPE fusions accumulate in mock-hypoxia. We next examined whether the HOPE fusions accumulation increased under mock-hypoxia in HT1080 cells. Initial experiments on stable pools of HT1080 cells expressing yCD, ODDD, and HOPE-2, -3, -5, -6 and -8 from the CAGGS promoter provided evidence that ODDD (not fused to yCD) and the HOPE fusions accumulated when $CoCl_2$ was present, as detected by western blots on protein extracts using an anti-HIF-1α antibody (**Supplementary Figure 2**). We next grew cultures from single cells to substantiate this result. Two T25 flasks for each stable line were seeded with 150,000-200,000 cells. After 2 days of growth, we incubated each stable line with and without $CoCl_2$. As expected, western blots on protein extracts using an anti-HIF-1α antibody showed that $CoCl_2$ caused increased levels of endogenous HIF-1α for all of the stable cell lines including the empty vector and yCD controls (**Figure 5**). More

importantly, $CoCl_2$ caused a marked increase in a band appearing at the expected molecular weight for the HOPE fusions (42 kDa). No such band was apparent under normoxic or mock-hypoxic conditions for the empty vector and yCD controls.

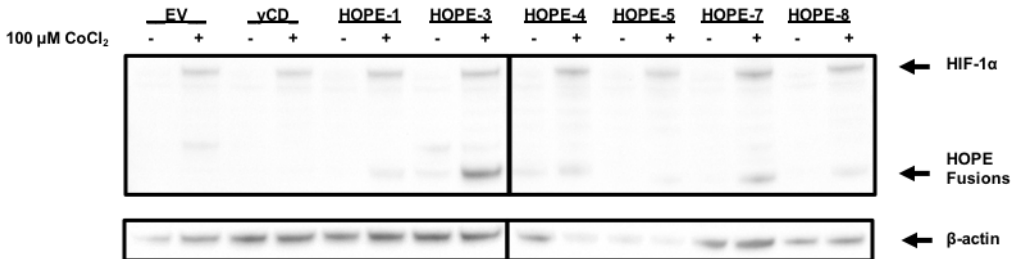

Figure 5: Accumulation of HOPE fusions in HT1080 experiencing mock-hypoxia

HT1080 stable cell lines expressing yCD or a HOPE fusion were grown in the presence or absence of 100 μM $CoCl_2$. Soluble proteins were analyzed by western blot using anti-HIF-1α antibodies. A western blot for beta-actin was used as a loading control. Western blots of replica experiments are provided as **Supplementary Figure 3**.

In the yCD-ODDD orientation, toxicity increases with linker length (**Figure 4**). This matches results in *E. coli* where the fusion with the longest linker (HOPE-4) caused the highest 5-FC toxicity among the fusions with the yCD-ODDD orientation. Surprisingly, the HOPE fusions in the yCD-ODDD orientation caused higher 5-FC sensitivity in HT1080 than those in the ODDD-yCD orientation – the opposite of what was observed in *E. coli*. This likely reflects cellular differences between *E. coli* and HT1080 cells in the translation rates, folding, and proteolytic environment.

At this juncture, we can only speculate as to the reason fusions in the yCD-ODDD orientation cause higher 5-FC toxicity but little $CoCl_2$ dependence, whereas fusions in the ODDD-yCD orientation generally cause lower 5-FC toxicity but better $CoCl_2$ dependence. One might imagine that HOPE fusion accumulation levels in HT1080 might predict 5-FC sensitization. We observed no obvious correlation between LC_{50} values and HOPE accumulation. For example, HOPE-3 repeatedly showed marked $CoCl_2$-dependent increases in HOPE accumulation (**Figure 4** and **Supplementary Figure 3**) but little to no difference

in LC_{50} values. However, to adequately examine this question we would also need to probe for yCD, since it is possible that proteolyzed HOPE fusions in the absence of $CoCl_2$ might result in a stable, free yCD domain. However, we were unable to detect the HOPE fusions on western blots with anti-yCD antibodies in HT1080 protein extracts. This likely results from the sensitivity limitations of the anti-yCD antibodies.

However, we can offer a hypothesis for why fusion orientation could result in differences in the degradation of the HOPE fusions. Previous studies on substrate degradation by the ubiquitin-proteasome pathway show that stable domains located near the ubiquitin signal on the substrate can prevent degradation of the full fusion protein and results in a cleaved substrate[16]. Proline 402 of the HIF-1α ODDD is located 2, 7, and 12 residues from the yCD domain for HOPE-1, -3, and 4, respectively. Degradation can progress in the N- to C- or C- to N-terminus direction depending on the location of the ubiquitin tag and unfolded region at which proteosomal degradation initiates[16]. We expect the HOPE fusions to be degraded by the proteosome starting with the ODDD. Thus, we expect that the ODDD-yCD fusions are degraded from the N-terminus and the yCD-ODDD fusions are degraded from the C-terminus. The 10-amino acid N-terminus of yCD lacks secondary structure[17]. In the ODDD-yCD orientation, the yCD domain is at least 35 residues away from proline 564 in the ODDD and the unstructured N-terminus of yCD is directly fused to the ODDD, and thus the yCD domain may be more amenable to proteosomal degradation in normoxia. This could be why the three fusions in the ODDD-yCD orientation showed a bigger difference in 5-FC toxicity between the normoxic and hypoxic conditions than those fusions in the yCD-ODDD orientation. In the yCD-ODDD orientation of HOPE-1, -3, and 4, degradation from the C-terminal end of the fusions might

result in release of unfused, fully-active yCD enzyme in normoxia because of the C-terminus of yCD is structured (and the N-terminus is not).

CONCLUSIONS

We showed that fusing HIF-1α 's ODDD to the N-terminus of yCD yields HOPE proteins that cause higher 5-FC sensitivity in mock hypoxia than in normoxia. These results will need to be confirmed in hypoxic cells. Further engineering of these fusions so that they cause equivalent 5-FC sensitivity to that caused by yCD, without compromising the hypoxia dependence, will be necessary for these fusions to be a viable alternative to yCD for use in GDEPT.

METHODS

Construction of HOPE fusions. The HOPE fusions were initially assembled on a version of pcDNA5/FRT (Invitrogen) in which the CMV promoter was replaced with the CAG promoter. To prepare the ODDD and yCD for fusion with the desired linkers, the DNA encoding the ODDD was PCR-amplified from p3XFLAG-HIF1 (a gift from Gregg Semenza), and a human codon-optimized version of yCD was PCR amplified from a previous construct in our lab.[6] The primers for amplifying the ODDD contained DNA encoding the linkers, The ODDD and yCD blunt PCR products were ligated together and then PCR amplified to obtain the desired HOPE gene cassettes with the ODD and yCD fused in the right orientation with the proper linker in between. These PCR products were then cloned into pcDNA5-FRT downstream from the CAG promoter between the HindIII and BamHI sites. The correct sequence confirmed by Sanger sequencing. The CAG-HOPE cassettes were flanked by a SpeI site and a BamHI site, which were used to clone the

cassettes into a modified pHIV-EGFP vector (Addgene #21373) (see below). For bacteria experiments, each HOPE gene was PCR-amplified with suitable flanking restriction sites and cloned into pSkunk[5] downstream from the *tac* promoter.

5-FC toxicity assay in *E. coli*. All experiments in *E. coli* were conducted in GIA39 cells, which lack a functional bacterial cytosine deaminase. The HOPE fusions and yCD were expressed from the IPTG-inducible *tac* promoter in pSkunk[5] (*p15a* origin, *f1* origin, *aadA* streptomycin/spectinomycin resistance). The genes for HOPE fusions and yCD contained the human codons for the ODDD and human-optimized codons for the yCD domain. GIA39 *E. coli* cells expressing yCD or the HOPE fusions were cultured in 1 mL minimal media supplemented with 50 μg/mL streptomycin and 45 μM uracil. After 18 hr of growth, each culture was diluted to 0.03 OD_{600} then serially diluted 1:5 five times. A total of 1.5 μl of select dilutions was pipetted onto minimal media plates supplemented with 45 mM uracil and different 5-FC concentrations. Plates were incubated for 36 hours at 37°C.

Construction of pHIV-EGFP Vectors containing the CAG-HOPE fusion cassette. The pHIV-EGFP vector (Addgene #21373) was digested with PstI-HF to remove the EF-1α promoter. The NEB Quick Blunting kit was used to create blunt ends on the digested DNA. This linear DNA was digested with BamHI-HF to prepare the pHIV-EGFP vector for receiving the CAG-HOPE fusion cassettes. The pcDNA5/FRT vectors containing the CAG-HOPE fusion cassettes were digested with SpeI-HF. The NEB Quick Blunting kit was used to create blunt ends. This linear DNA was then digested with BamHI-HF to separate the CAG-HOPE fusion cassettes from the pcDNA5/FRT vector backbone. This DNA was ligated to the previously prepared pHIV-EGFP vector backbone DNA to create the HOPE plasmids used in the HT1080 cell experiments. The correct sequences were verified by Sanger sequencing.

Lentiviral transduction to create stable lines in HT1080 cells. The following plasmids were purchased from Addgene: pMD2.G (#12259), pMDLg/pRRE (#12251), and pRSV-Rev (#12253). HT1080 cells (CCL-121) and 293T cells (CRL-3216) were purchased from ATCC. 293T cells were cultured in DMEM, 10% heat inactivated FBS and 1% penicillin/streptomycin. Approximately 3.1 x 10^6 cells were seeded into a T25 flask. The next day, the cells were transfected with 8 μg of DNA (2 μg each of a pHIV-HOPE vector, pMD2.G, pMDLg/pRRE, and pRSV-Rev) using Lipofectamine 3000. The media was changed 6 hr after transfection. On the same day as the transfection, 1 x 10^6 HT1080 cells were seeded to a T25 flask to prepare for transduction. The viral media was collected 24 hr after transfection, filtered using a 0.22 μm filter and diluted 1:1 with complete media. 0.5X diluted viral media was added to HT1080 cells for 24 hrs, then washed with 1X PBS and replaced with 1X complete media. The cells were passaged three times then sorted for EGFP positive cells using a SONY SH800 cell sorter. Stable integration for each insert was confirmed by harvesting the genomic DNA, performing nested PCR, and sending the amplicon for Sanger sequencing.

Accumulation of HIF-1 by addition of cobalt chloride. HT1080s cells were grown in 150 cm petri dishes to 90% confluency. The media was changed and $CoCl_2$ was added at final concentrations to various final concentrations between 0 and 200 μM. The cells were incubated 4 hours at 37°C and 5% CO_2. After incubation, the cells were lysed and the proteins were harvested by adding 300 μL of RIPA buffer (Sigma) with 1% v/v protease inhibitors (Sigma P8340), shaking for 30 min at 4°C and centrifugation for 20 min at 15,000xg and 4°C. Soluble proteins were collected in the supernatant. The protein samples were analyzed via western blot as described below.

Prodrug toxicity assay on HT1080 stable lines in normoxia and mock-hypoxia. Five hundred cells were seeded to the 60 inner wells of two black bottom 96-well plate in a final volume of 50 µL. The next day, 50 µL of media was added with different concentrations of 5-FC and a final concentration of 0 or 100 µM $CoCl_2$ (6 wells per 5-FC concentration). To account for background fluorescence, 6 wells contained only 100 µL of media without cells. For the 100% survival condition, 6 wells contained 500 cells with media lacking 5-FC and containing 0 or 100 µM $CoCl_2$. After incubating the plates for 72 hr at 37°C and 5% CO_2, cell survival was measure using the alamarBlue assay. Briefly, 10 µL of alamarBlue was added to each of the inner 60 wells, the plate were incubated for 4 hr at 37°C and 5% CO_2 and the fluorescence of each well was measured with a Spectramax Gemini M3 spectrophotometer at an excitation wavelength of 562 nm and emission wavelength of 585 nm with a cutoff at 570 nm. Percent survival was calculated as a ratio of the fluorescence of the test condition to the fluorescence of the condition lacking 5-FC from the same plate.

$CoCl_2$-induced accumulation of HOPE fusions. Two T25 flasks were seeded with 150,000-200,000 cells for each HOPE fusion stable line. After two days, the media in each flask was removed, then one flask received fresh media lacking $CoCl_2$ and the other flask received fresh media at 100 µM $CoCl_2$. The cells were incubated for another 24 hrs and the proteins were harvested using RIPA buffer. Briefly, the flasks were incubated on ice for 30 min, rinsed twice with ice-cold 1X PBS, then scraped off using 50 µL RIPA buffer with 1% v/v mammalian protease inhibitors. This lysate was agitated for 30 min then centrifuged for 20 min at 15,000xg at 4°C. The supernatant containing the soluble proteins was transferred to a fresh microcentrifuge tube and the total protein concentration was determined using the Bio-Rad DC assay.

Western Blots. Approximately 40 μg of total soluble proteins was separated on a 4-12% bis-tris for 45 min at 200 V. The proteins were transferred to a PVDF membrane using a semi-dry Trans blot at 15 V for 40 min. The membrane was blocked with 5% milk overnight, probed with 1:1,000 rabbit anti-HIF-1α (Abcam ab2185) for 90 min, then probed with 1:3,000 goat anti-rabbit HRP conjugate. The proteins were visualized using the Bio-Rad Clarity ECL substrate. For the loading control, the membrane was stripped after visualization and reprobed for β-actin or a second gel using the samples and volumes was probed for β-actin with 1:10,000 anti-β-actin antibody (Abcam 6276).

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Supporting Information

Supplementary Figures 1-3. Supplementary Table 1.

AUTHOR INFORMATION

Corresponding Author

*Email: oster@jhu.edu.

Author Contributions

T.D.W., J.R.E. and M.O. designed experiments. T.D.W and K.P. conducted experiments. T.D.W. and M.O. wrote the manuscript.

Notes

The authors declare no competing interest.

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Supporting Information

Protein-programmed accumulation of yeast cytosine deaminase in cancer cells in response to mock-hypoxia

Tiana D. Warren[1], Krishna Patel[1], James R. Eshleman[2,3], Marc Ostermeier[1]

[1]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD, 21218, USA

[2]Department of Pathology and [3]Department of Oncology, Johns Hopkins University School of Medicine, 733 N Broadway, Baltimore, MD, 21205, USA

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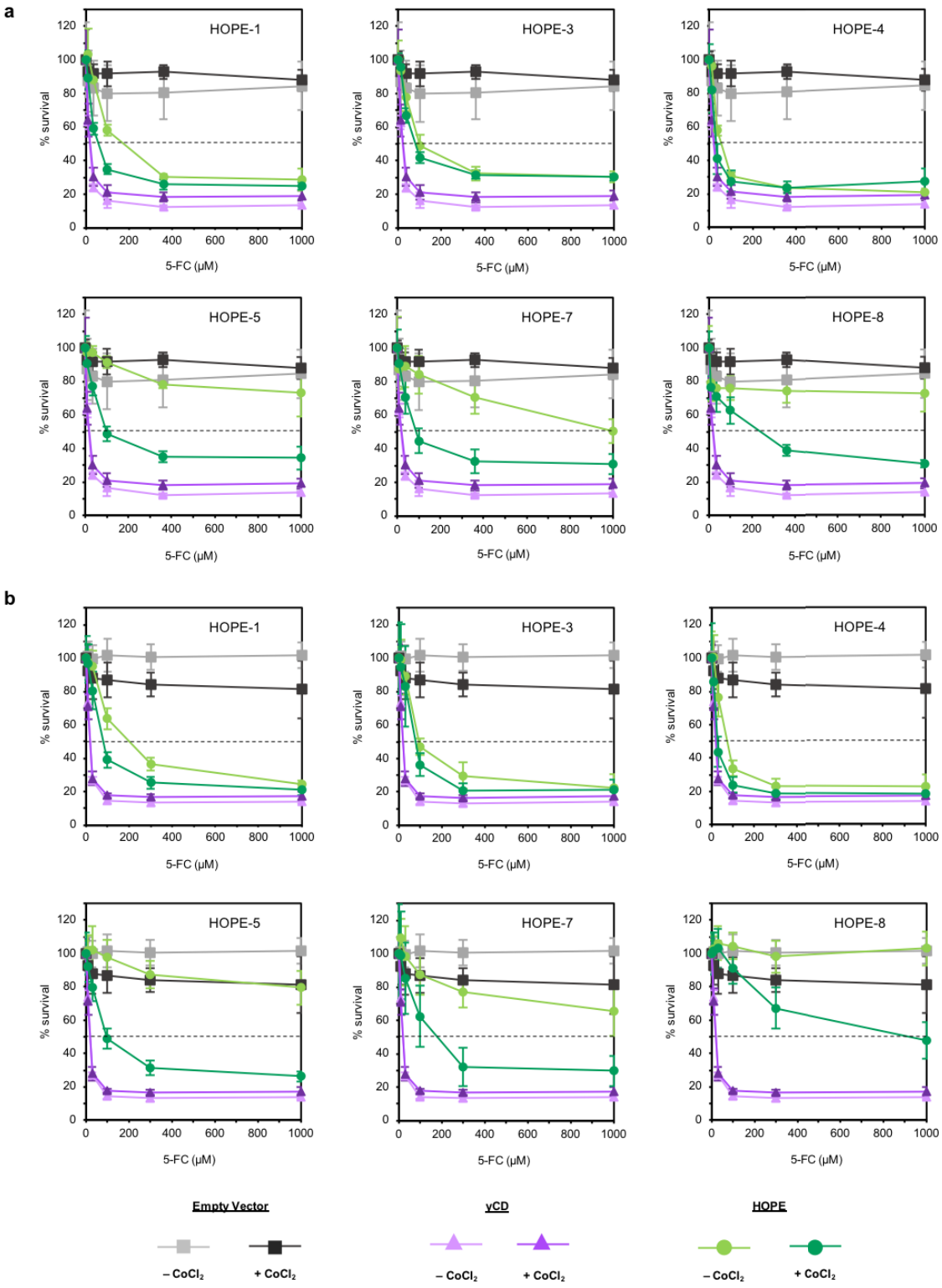
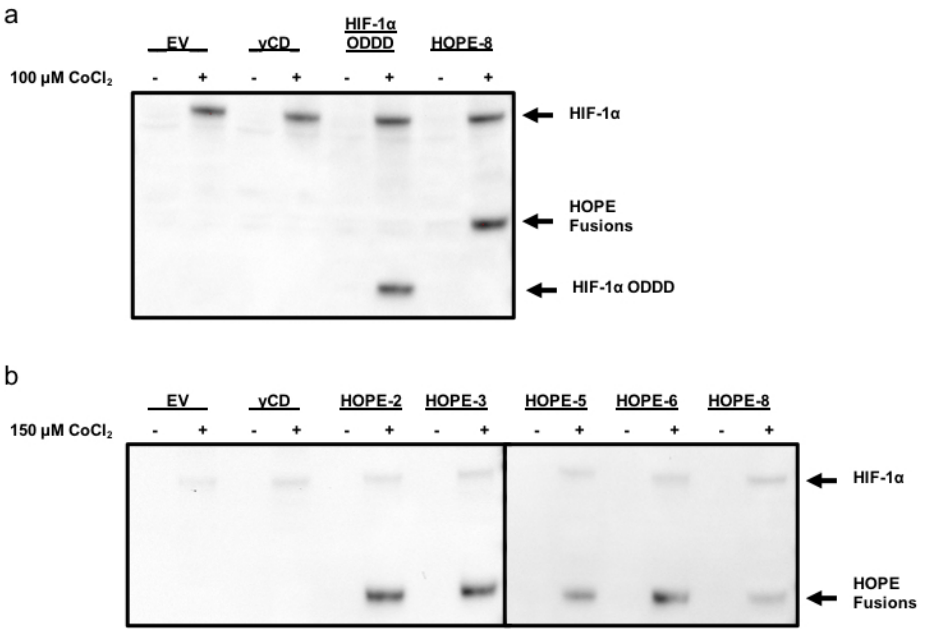
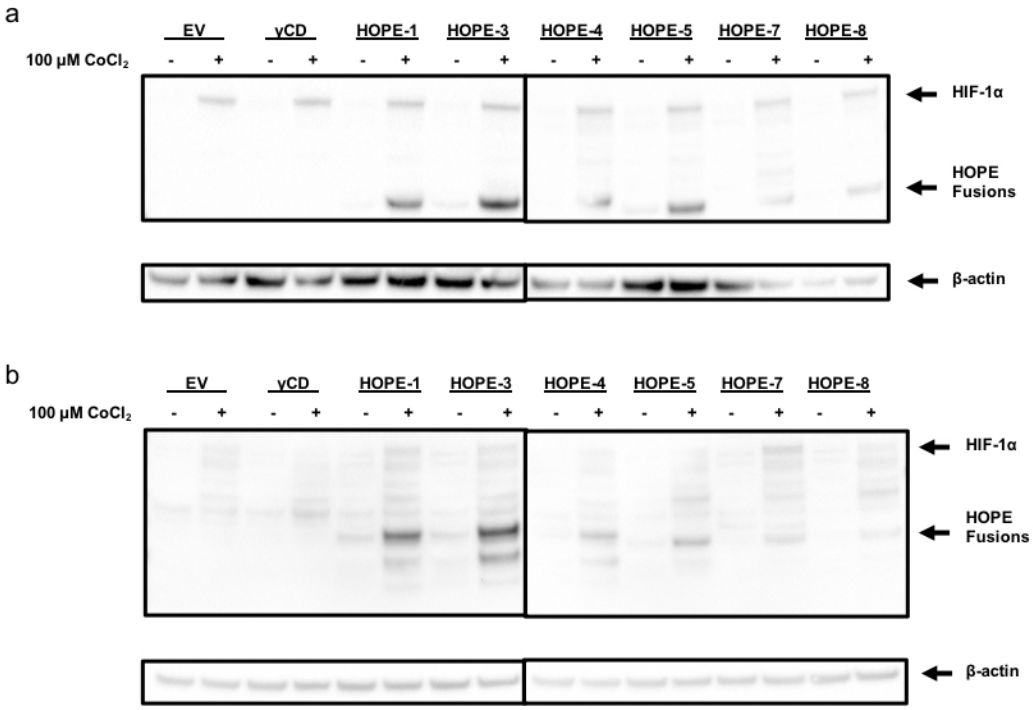


Supplementary Figure 1: Representative replicate 5-FC toxicity assays for the HOPE fusions. (**a**) and (**b**) are two replica experiments of the experiment in Figure 3c (see its legend for details) but with different 5-FC concentrations tested. These two replicas plus two more replica experiments were used to determine the LC_{50} values in Figure 4 and Supplementary Table 1.



Supplementary Figure 2: Accumulation of HOPE fusions in stable pools of HT1080 experiencing mock-hypoxia HT1080 stable cell lines expressing yCD, the HIF-1α ODDD, or a HOPE fusion were grown in the presence or absence of 100 or 150 μM $CoCl_2$. Soluble proteins were analyzed by western blot using anti-HIF-1α antibodies.



Supplementary Figure 3: Replicates showing the **accumulation of HOPE fusions in HT1080 experiencing mock-hypoxia** HT1080 stable cell lines expressing yCD or a HOPE fusion were grown in the presence or absence of 100 µM $CoCl_2$. Soluble proteins were analyzed by western blot using anti-HIF-1α antibodies. A western blot for beta-actin was used as a loading control.

Supplementary Table 1: LC_{50} values for 5-FC of HT1080 cells grown in the presence and absence of $CoCl_2$

Protein	Mean LC_{50} for 5-FC (µM)[a]	
	without $CoCl_2$	with $CoCl_2$
yCD	19 ± 3	20 ± 0
HOPE-1	204 ± 23	83 ± 14
HOPE-3	109 ± 21	76 ± 11
HOPE-4	56 ± 19	29 ± 3
HOPE-5	>1000[b]	128 ± 49
HOPE-7	>1000[b]	160 ±58
HOPE-8	>1000[b]	750 ± 340

[a] LC50s were measured in the experiments of Supplementary Figure 1 and two additional biological replicates. LC50 values from Figure 3c were not included. LC50 values could not be accurately estimated for some proteins in the experiment of Figure 3c because the LC50 values were significantly less than the lowest 5-FC concentration tested. Errors are the standard deviation (n=4 except HOPE-5, which is n=3).
[b]minimum value (highest concentration tested)