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Heme overdrive rewires pan-cancer cell metabolism

Swamy R. Adapa[1,7*], Gregory A. Hunter[2*], Narmin E. Amin[3], Christopher Marinescu[1], Andrew Borsky[2], Elizabeth M. Sagatys[4], Said M. Sebti[5], Gary W. Reuther[3], Gloria C. Ferreira[2,6,7], Rays H.Y. Jiang[1,7‡].

[1]USF Genomics Program, Center for Global Health and Infectious Diseases, College of Public Health, University of South Florida, Tampa, FL 33612, USA.

[2]Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA.

[3] Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA.

[4] Department of Pathology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA.

[5]Department of Pharmacology & Toxicology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298, USA

[6]Department Department of Chemistry, College of Arts and Sciences, University of South Florida, Tampa, FL 33620, USA.

[7]Global and Planetary Health, College of Public Health, University of South Florida, Tampa, FL 33612, USA.

*These authors contributed equally to the work

‡Corresponding author: jiang2@usf.edu

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Heme overdrive rewires pan-cancer cell metabolism

Abstract: (125 words)

All cancers reprogram their metabolism. In this study, we report that all cancer cells employ imbalanced and dynamic heme metabolic pathways essential for their oncogenic growth and coined the term 'heme overdrive'. Three key characteristics define heme overdrive, i.e., cancer universality, cancer essentiality and cancer specificity. While heme overdrive is absent in diverse differentiated cells or somatic stem cells, it is present in hundreds of cancers, and is delineated in patient-derived tumor progenitor cells by single cell RNAseq. The only exception to heme overdrive in normal tissues is identified in preimplantation human embryos, where heme overdrive likely links to embryonic omnipotency. Among the major drivers are proteins involved in biosynthesis of heme intermediates (hydroxymethylbilane synthase (HMBS) and uroporphyrinogen decarboxylase (UROD)) and heme trafficking (FLVCR1). CRISPR/Cas9 editing to engineer leukemia cells with impaired heme biosynthesis steps confirmed our whole genomic data analyses that heme overdrive is linked to oncogenic states and cellular differentiation. Finally, we devised a novel "bait-and-kill" strategy to target this cancer metabolic vulnerability.

One-Sentence Summary: (125 characters and spaces)

Heme overdrive re-programs cellular metabolism across diverse types of cancer.

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Main Text (3,000 to 10,000 words)

Introduction

Heme biosynthesis is one of the most efficient metabolic pathways in humans. A total of 270 million molecules of heme are produced for every red blood cell (RBC) [1], at a rate of over 2 million RBCs per second [2]. Heme, a porphyrin ring encaging most of the iron in humans, functions as an essential prosthetic group of numerous proteins with roles ranging from signal sensing, DNA binding, microRNA splicing and processing to enzymatic catalysis [3-21]. To ensure the enormous output of heme biosynthesis, the supply of substrates, intermediates and end-products in the pathway is tightly regulated and precisely balanced [7,22]. Heme is a double-edged sword for cell growth; it is essential in the "right amount"[23] and if not can be toxic [24,25] via unique forms of cell death.

Cancer cells reprogram metabolic pathways to fuel anabolic growth, rapid propagation, and efficient nutrient acquisition [26-28]. Although some studies have implicated heme in carcinogenesis through cytotoxic heme-derived compounds [29,30], lipid peroxidation [31], oxidative damage [32], intestinal flora toxicity [33] and energy production [34], little is known about how heme biosynthesis deregulation and heme trafficking alterations contribute to tumor dependence on heme for survival. Here we describe 'heme overdrive', a novel cancer cell metabolic reprogramming characterized by imbalanced and dynamic heme metabolic pathways essential for cancer cell growth.

"The Warburg effect" [26,35] and "glutamine addiction" [36,37] are two altered forms of metabolism found in cancer cells. Therapeutic intervention based on these altered cancer metabolic pathways are challenging because glycolysis and glutamine metabolism are required in every cell and thus such therapeutic approaches lead to non-specific toxicity. By contrast, heme overdrive is an ideal cancer metabolic pathway for therapeutic targeting as we demonstrate that it is: 1) cancer specific (*i.e.*, it is absent in normal cells), 2) universal (*i.e.*, it is present in all cancers), and 3) cancer cell essential (*i.e.,* cancer cells are addicted to it).

Results

Cancer is characterized by aberrant heme metabolism

Compared to normal cells, cancer cells have enhanced metabolic dependencies [26-28]. Our initial data analyses of genome-scale CRISPR/Cas9-gene loss-of-function from the publicly available project DepMap screens of cell lines derived from metastatic cancers [38] indicated that cancer cells depend on heme synthesis (fig. S1) (Supplemental Table S1). Significantly, these metastatic cancers developed dependencies on heme metabolism-related proteins, such as uroporphyrinogen III decarboxylase (UROD), the enzyme that catalyzes the fifth step in the heme biosynthetic pathway. These cancers also depend on a set of hemoproteins, such as cytochrome c-1 (CYC1), succinate dehydrogenase complex subunit C (SDHC) for cellular respiration, and DGCR8 for microRNA biogenesis [39], each of which uses heme as a co-factor. Unexpectedly, the *FECH* gene that encodes ferrochelatase that catalyzes the final critical step of heme biosynthesis is dispensable in several cancers, suggesting that cancer cell lines are

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capable of bypassing endogenous biosynthesis of heme *in vitro*, yet are still dependent on genes encoding enzymes that mediate intermediate steps of heme biosynthesis. This initial analysis suggests that cancer is dependent on imbalanced heme metabolism (Fig. 1A).

Next, we investigated the genes that encode proteins acting as gatekeepers for heme biosynthesis. The first committed and key regulatory step in mammalian heme biosynthesis is catalyzed by 5-aminolevulinic acid (ALA) synthase (ALAS; EC 2.3.1.37) [22,40]. Two chromosomally distinct genes, *ALAS1* and *ALAS2*, encode the housekeeping and erythroid-specific ALAS isoforms, respectively [41]. While *ALAS1* is expressed in every cell, the expression of *ALAS2* is restricted to developing erythrocytes [40]. We examined the data generated from genome-wide CRISPR-based screens of diverse cancer cell lines, which were designed to provide a complete gene essentiality data set for these cell lines [38,42]. In contrast to *ALAS2*, essentiality of *ALAS1* is a feature of diverse cancer cells independent of cancer type (fig. S2A). Consistent with these forward genetics results, the gene expression patterns in ~10,000 patient tumors from the Genotype-Tissue Expression (GTEx) [43,44] and The Cancer Genome Atlas (TCGA) [45] datasets show that *ALAS2* expression is absent in most tumors except myeloid leukemias (fig. S2B). These results show that, despite ALAS2 being responsible for the majority of heme production (~85%) in humans, many cancer cells, including erythroleukemia of red blood cell lineages (*e.g.* HEL), require ALAS1.

Heme overdrive operates in diverse cancers and early embryos

To better understand the molecular and genetic mechanisms underlying the cancer dependency on heme metabolism, we determined gene essentiality from genome-scale CRISPR/Cas9 loss-of-function screens in over 300 human cancer cell lines covering different cell lineages and estimated gene dependency [38,42]. Then, we focused on the gene dependencies associated with the eight enzymatic steps of the heme biosynthetic pathway by computing the respective pan-cancer essentiality scores, similar to the lethality scores used in the development of a cancer dependency map (DepMap) (42, 43). Gene essentiality was estimated from gene X dependency inferred from CRISPR/Cas9 gRNA gene X knockout. Lower score values indicate larger effect of gene X loss on cell viability, and hence cell lines with lowest and highest scores are most and least gene X-dependent, respectively. Consistent with recent cross-platform studies [46,47], over 2000 genes were defined as pan-cancer essential, based on the distribution of their essentiality scores in over 300 cancer lines derived from 27 of the most prevalent cancer types. The gene essentiality scores were confirmed by using the same method on the total DepMap collection (DepMap release 21Q3) of over 1000 cell lines (Pearson's $R > 0.9$, $p < 0.001$). Strikingly, instead of finding the expected complete and balanced heme biosynthetic pathway, partial heme biosynthetic pathways were found in hundreds of different types of cancers (fig. S3) (Supplemental Table S2 and S3). Specifically, as shown in Fig. 1B and fig. 3S, the pan-cancer gene essentiality is the highest for the genes encoding the enzymes responsible for the fifth and sixth steps of the pathway, UROD and coproporphyrinogen III oxidase (CPOX), respectively. This result was notably surprising, as the genes for the ALAS isoforms, which catalyze the initial and rate-limiting step in the pathway, and the gene for FECH, the final enzyme in the pathway that

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catalyzes heme production, had lower essentiality values and were dispensable in several cancers (Fig. 1B). Though the heme biosynthesis genetic dependences of 27 major forms of human cancer vary, the estimated patterns of essentiality across the cancer lines clearly revealed a range of heme and heme precursor requirements. These results uncovered the "imbalanced" nature of heme biosynthesis in cancer, with many cancers surviving without functional first and terminal enzymatic steps, but dependent on the intermediate steps (fig. S3 and fig. S4). The gene essentiality analysis also highlighted the importance of heme trafficking (e.g., heme importer FLVCR2) and key hemoproteins (e.g., CYC1) in cancer cells survival (Fig. 1B and fig. S3).

To examine cancer heme metabolism *in vivo*, we analyzed the *in vivo* mouse CRISPR/CAS9 loss-of-function and metabolic essentiality data from pancreatic and lung cancer models [48]. The genetic screen [48] indicates that the majority of metabolic gene essentialities were similar between the *in vitro* and *in vivo* settings, with the exception of the heightened requirement of the intermediate steps of heme biosynthesis *in vivo*. Strikingly, out of the ~2900 examined metabolic genes[48], the genetic dependencies of both murine pancreatic and lung cancers were only increased for the enzymes responsible for the intermediate steps of the heme biosynthesis pathway (fig. S5) (Supplemental Table S4). The essentiality extent of either *ALAS* or *FECH*, which code for the first and terminal enzymes of the heme biosynthesis pathway, respectively, did not differ significantly between the *in vivo* murine cancer models. This analysis of the CRISPR/Cas 9 loss-of-function screens performed in mouse model systems revealed that aberrant heme metabolism has increased importance *in vivo* for cancer cell survival (fig. S5). Furthermore, this effect is independent of tumor origin or tissue environment in pancreatic and lung cancers.

To assess heme biosynthesis in patient tumor samples, we analyzed and compared the gene expression patterns in ~10,000 tumors *vs*. those in normal tissues available from the GTEx project [43,44] and TCGA program [45]. We used methods designed for pairwise comparative gene expression of GTEx and TCGA datasets [49]. In over 80% of all tumors, the genes for hydroxymethylbilane synthase (HMBS), the third enzyme of the heme biosynthetic pathway, and the heme exporter FLVCR1 were among the most upregulated (fig. S6A,B)(Supplemental Table S5). By the same criteria, the onco-signaling genes *AURKA*, *KRAS* and *MYC* were upregulated in over 90%, 60% and 50% of all tumor types, respectively. In contrast, expression of the genes encoding the second and terminal enzymes of the heme biosynthetic pathway, aminolevulinate dehydratase (ALAD, a.k.a. porphobilinogen synthase) and ferrochelatase (FECH), respectively, are down-regulated suggesting buildup of intermediate heme precursors in tumors. *HPX*, the gene for the heme-binding and scavenger hemopexin [50], was also downregulated in most of the tumors (fig. S6C). This finding, which corroborates the previously reported role of hemopexin as a key player for the checkpoint in cancer growth and metastases [51], leads us to suggest that heme content is high in the tumor microenvironment [52]. *ALAS2* belongs to the 10% of genes that were not expressed in most of the large tumor collections, except myeloid leukemias (<2% of total tumors). Overall, our comparative analysis of the differential gene expression in tumor tissues, spanning 31 cancer types, indicated that only a few of the nine heme biosynthesis enzyme-encoding genes were upregulated in cancer cells. The unbalanced expression of the genes for heme biosynthesis and heme transporters likely elicits a lack of heme homeostasis in cancer cells, where the

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insufficient heme supply from the dedicated biosynthetic pathway is compensated with an enhanced heme flux.

Based on the gene essentiality results both *in vitro* (Fig. 1B, fig. S3 and fig. S4) and *in vivo* (fig. S5) settings, the pan-cancer gene expression patterns (fig. S6), the detected porphyrin accumulation in a wide range of tumors [30], and the determined elevated heme flux in diverse cancer cells [32,34,53], we defined the salient heme overdrive-associated features. They are: 1) heme biosynthesis enzymes with aberrantly increased (HMBS, UROS and UROD) and suppressed (ALAD and FECH) activities, 2) accumulation of heme precursors (porphyrins), and 3) enhanced heme flux (Fig. 1, A and B). This cellular status contrasts with the tightly regulated steady-state or homeostasis, defined by a flawless enzyme-catalyzed channeling of substrates to products along the heme biosynthetic pathway and circumventing the toxicity and instability of the pathway intermediates, while ensuring that the cell heme requirements are met [7]. With heme overdrive, the production of heme precursor molecules exceeds that of heme, porphyrin intermediates accumulate, and “imbalanced” heme biosynthesis of increased porphyrin intermediates and decreased end-product arises. Likely, to compensate for the reduced amount of heme synthesized, heme flux is increased [30]. Cancer cells appear to have adopted a dependence on this seemingly “inefficient” metabolic pathway designed to produce aberrant levels of heme intermediates with unknown biological functions to date.

Early embryonic stem cells represent a special metabolic state that bears similarities with that of cancer cells [54,55]. Therefore, we also studied the expression of the heme biosynthetic enzyme-encoding genes in human preimplantation embryos and embryonic stem cells. Specifically, we explored a high-resolution data set generated upon single-cell RNA-sequencing in human early embryonic cells and embryonic stem cells [56,57]. In the human zygote and early preimplantation embryos, the level of *ALAS1* expression is 100-fold higher than that of *FECH* (Fig. 1C). This large difference in gene expression for two key enzymes in heme biosynthesis was observed in all 17 early human embryos examined (Supplemental Table S6). The large difference in expression levels is restricted to very early embryos because it rapidly narrows, via an increase in *FECH* expression and a decrease in *ALAS1* expression after initial rounds of embryonic cell division. *ALAS2* expression is not detected in any of the early human embryos, which is congruent with the non-essentiality of *ALAS2* in all examined cancers. As a control, the genes *AURKA* and *SOX4* show expected expression patterns during early embryogenesis [58,59]. The similarity of these gene expression data with those observed in cancer cells supports the possibility that a form of heme overdrive operates in preimplantation human embryos.

Heme overdrive is absent in normal cells

To evaluate if heme overdrive is specific to cancer cells, we investigated and compared heme biosynthesis in diverse types of non-cancerous cells, *i.e.,* normal differentiated cells, replicating fibroblasts, human primary hepatocytes, and human primary hematopoietic stem cells. We looked for evidence of heme overdrive, defined by a non-homeostatic, imbalanced heme biosynthetic pathways and heightened accumulation of heme pathway intermediates. Using published datasets, we found that heme overdrive, observed in cancer cells, is absent in normal differentiated cells and somatic stem cells (Fig. 2A) First and consistent with this finding, normal human peripheral blood

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mononuclear cells (PBMCs) do not accumulate heme intermediates even upon ALA-induction [60]. Second, colon cancer cells incubated with ALA to induce protoporphyrin IX (PPIX) production do accumulate PPIX, while no significant PPIX accumulation is observed in replicating human colon-derived fibroblasts (stromal cells) previously incubated with ALA [61]. Further, the increased HMBS activity in colon cancer cells relative to stromal cells [61] is consistent with a buildup of heme intermediates including PPIX. Third, with our previously described primary human hepatocyte system [62], we demonstrate that normal, metabolically active, primary hepatocytes do not accumulate porphyrins as indicated by the absence of the PPIX fluorescence (Fig. 2B). By contrast, >99% of liver cancer cells produced and accrued PPIX upon induction with ALA, one of the canonical features of heme overdrive.

To uncover the role of heme metabolism in normal human stem cells, we analyzed the erythropoiesis data generated by RNAi-based knockdown of gene expression in human hematopoietic progenitor cells [63]. By mapping normal stem cell gene essentiality following the methods described in Egan *et al*. [63], we show that erythropoiesis is heme-dependent in both early progenitor (undifferentiated) cells and differentiated cells from normal human bone marrow-derived hematopoietic stem cells. In contrast to cancer cells, where *ALAS2* is dispensable, normal human hematopoietic stem cells depend on *ALAS2* for survival (Fig. 2C). The differential gene essentiality profiles between cancer and stem cells are consistent with the molecular basis for specific *ex-vivo* purging of cancer hematopoietic progenitor cells as a way to eradicate malignant cells that may contaminate an autologous graft and cause relapse after transplant [64,65]. Taken together, the distinct genetic essentialities and the markedly different porphyrin accumulation modes between cancer and non-cancerous cells indicate that heme overdrive is absent in somatic stem cells.

CRISPR/Cas9-mediated knockout of *ALAS2* and *FECH* validate abnormal heme metabolism underlying cell proliferation and oncogenesis

We modified chronic myeloid leukemia K562 cells with CRISPR/Cas9-mediated knockout (KO) of the genes encoding FECH and ALAS2 (Fig. 2D). Under a heme homeostasis model, a cell line without *FECH* should not survive, because *FECH* is a single copy gene in humans and with no functional substitute [66-68]. Indeed, knockdown (KD) of *FECH* in human stem cells led to cell death [63]. However, under heme overdrive conditions, we predicted that cancer cells rely on heme trafficking and are addicted to the porphyrin intermediates, and thus do not depend on the final step of heme biosynthesis for survival. As hypothesized, K562 cells with the *FECH* edited out (K562-FECH KO) (Fig. 2D) had normal growth (Fig. 2E) under standard culture conditions. The unimpeded growth of the K562-FECH KO cells presumably indicates that at least some cancer cell lines can metabolically function as heme auxotrophs.

As predicted, K562 cells with the *ALAS2* deleted (K562-ALAS2 KO) (Fig. 2D) lost their differentiation capacity although their growth was not hindered (Fig. 2E, F). Given the proposal that oxidation stress, with the involvement of reactive oxygen species (ROS), is the first step in erythroid differentiation of K562 cells [69], we used the organic peroxide tert-butyl hydroperoxide (t-BHP) to trigger ROS-mediated erythroid differentiation. As early as 30 minutes following t-BHP-mediated ROS induction, the parental K562 cells started synthesizing heme, an early step in cellular differentiation [69], while the K562-ALAS2 KO

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cells failed to initiate this metabolic step, indicated by the lack of heme production (Fig. 2F). [69]. Further, at 48 hours, the parental K562 cells readily differentiated into erythroid cells, but the K562-ALAS2 KO cells continued to proliferate and failed to differentiate, as expected because ALAS2 is required for erythroid differentiation. Conceivably, in the absence of endogenous production of heme destined to hemoglobin, the erythroid lineage commitment is blocked, forcing the K562-ALAS2 KO cells to an obligatory heme overdrive path and permanent arrest in a non-differentiable/proliferation or cancerous state.

Single-cell transcriptomics reveal heme overdrive in AML patient biopsies

To study heme overdrive in primary cancer cells, we performed single-cell RNAseq on biopsies from acute myeloid leukemia (AML) patients (Supplemental Table S7 and S8). For each patient, we obtained around 2000 single-cell transcriptomes, and we analyzed cells of various differentiation stages and cell types. Interestingly, in 2 patients, only a minority of cells (5-10% of total cells) were identified as cancer progenitors with hyper proliferation features (Fig. 3A), and they exhibited hybrid features of mature erythroid cells (*e.g*. expressing the genes *HBB* and *GYPA* for hemoglobin β and glycophorin A, respectively) and stem cells (*e.g*. expressing *SOX4*). The cancer progenitor cells (Fig. 3B) had both mature cell features, *e.g. HBB* expression, and early progenitor properties, *e.g.* proliferative phenotype. Specifically, they were characterized by elevated iron and heme metabolic gene expression, cell proliferation markers, *KRAS* and anabolism process genes, yet they had reduced normal stem cell progenitor marker gene expression *e.g.*, *SOX4* and *JUN* (Fig. 3C) [70,71]. Additionally, cancer/AML progenitor cells exhibited imbalanced heme biosynthesis, exemplified by elevated gene expression for enzymes that catalyze the intermediate steps of heme biosynthesis, *e.g.* HMBS and UROD (Fig. 3D and E). The cancer progenitor cells also had a higher expression level of genes related to heme trafficking such as *FLVCR1* and *DNM2* (Fig. 3C). Our results show that these cancer progenitor cells exhibit features of heme overdrive, consistent with the abnormal heme anabolism and endogenous PPIX accumulation upon ALA administration in leukemia cells compared to normal PBMCs [60]. Notably, in two solid tumors (Fig. 3F) with independently generated single cell transcriptomes [72,73], the genes encoding the enzymes for the intermediate steps of heme biosynthesis were similarly overexpressed in the cancer progenitor cells (Fig. 3F).

From our single cell transcriptome results, we searched for clues of specialized oncogenic microenvironments that support enhanced substrate trafficking and molecular interactions. Interestingly, only the cancer progenitor cells highly express cell-to-cell interaction-related genes, *e.g.*, *ICAM4*, *MAEA*, *ITGA4* (fig. S7A), which are critical in establishing the stem cell niche for erythropoiesis [74,75]. The specific expression of these niche interaction genes suggest that the cancer progenitor cells exist in a specialized niche with intimate contact with neighboring cells and extracellular matrix.

Expression was remarkably enhanced for genes encoding proteins involved in metabolic substrate trafficking processes in the cancer progenitor cells, including genes for key protein players in lipid import, purine and pyrimidine salvage pathways (fig. S7B). These specific metabolic flux related genes, such as *DNM2* (endocytosis), *APOC1* (lipid transport), *MRI1* (L-methionine salvage), *MTAP* (NAD salvage), and *SLC29A1*

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(nucleoside import) indicate that the cancer progenitor cells represent hubs of metabolic trafficking activities. In particular, the lipid import gene *APOC1* is expressed in cancer progenitor cells, but not in the other cell populations from the same patient samples (Fig. S8, A and B). The metastatin S100 family members linked to tumor niche construction [76] are also enriched in the progenitors. These potential dynamic metabolic trafficking patterns inferred from the abundant expression of metabolites import genes in cancer early progenitors (Fig. S7C) are reminiscent of 'metabolic parasitism' [77], a bioenergetically favorable process [78] that utilizes energy-rich and pre-existing macromolecules to fuel robust cell proliferation.

Targeting cancer heme overdrive with a bait-and-kill strategy

Two aspects of cancer heme overdrive offer opportunities for therapeutic intervention. First, an imbalanced heme biosynthetic pathway (*i.e.*, with aberrantly increased and suppressed enzyme activities) is absent in normal cells. Second, heme overdrive is inducible hundreds to thousands-fold in cancer cells, but not in non-cancerous cells. In fact, photodynamic therapy (PDT), which uses ALA to induce biosynthesis and accumulation of photosensitizing porphyrins, exemplifying the inducible principle of heme overdrive, has been used for imaging and treatment for decades [79-81]. Thus, heme overdrive may be a unique metabolic reprogramming feature of cancer cells that can be therapeutically exploited. Since an imbalanced heme flux is fundamental to heme overdrive, cancer cells may be made particularly vulnerable to PPIX-induced cytotoxicity. Selective cancer cell death could possibly be achieved by activating PPIX-induced cytotoxicity with a two-step "bait-and-kill" strategy.

Knowing that cancer cells readily take up the heme precursor ALA and bypass the first and rate-limiting step of porphyrin biosynthesis catalyzed by ALAS, we set to assess our engineered two-step process for cancer metabolic death. This two-step process involves first, to 'bait' with ALA to induce PPIX accumulation up to a few hundred-to-thousand-fold higher concentration as compared to control cells (Fig. 4A and B), and second, to 'kill' with a compound that exploits the metabolic stress associated with porphyrin accumulation.

To exploit the metabolic stress (*e.g.*, lipid peroxidation) associated with porphyrin accumulation, we treated the human erythroleukemia HEL cell line with a range of concentrations of RSL3, an inhibitor of the antioxidant enzyme glutathione peroxidase 4 (GPX4), a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation. As such, inhibition of GPX4 with RSL3 induces ferroptosis, an iron-dependent form of programmed cell death highly relevant to iron and heme metabolism [82]. Pretreatment of HEL cells with ALA increased RSL3 sensitivity at least 1000-fold (Fig. 4, C and D). In contrast, ALA pretreatment did not sensitize cells to ganetespib, a heat shock protein inhibitor with antineoplastic activity [83] suggesting that ALA is not a general inducer of cell death.

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Discussion

For over a century, the association between photosensitizing porphyrins and cancer has been recognized [84], and the preferential porphyrin accumulation in cancer cells has been used in the development of light-based therapies as early as 1911 [85]. Our results show that heme overdrive, characterized by an imbalanced and dynamic porphyrin production, is ubiquitous to cancer cells and provide the likely cellular basis for specific light-based therapies. With a new understanding of this aberrant metabolic pathway in cancer, heme overdrive may offer novel vulnerabilities and opportunities for cancer therapeutic intervention.

Cancer metabolic processes are often described in terms of aberrant gene regulation and epigenomic reconfiguration [86-88]. Heme overdrive likely operates through direct epigenetic regulation [89] with heme/porphyrin acting as a master regulator of chromatin, such as via binding to guanine quadruplex (G4) DNA structures [51]. Heme, a recognized molecular sensor [15], contributes - perhaps via a paracrine/autocrine mechanism, - to the regulation of genes associated with biological processes as diverse as angiogenesis, oxidative stress management, circadian rhythm adjustment [90], and anabolism [15]. Clearly, this recognition of heme as a signaling molecule has bolstered our understanding of the multifaceted roles of heme in cancer pathogenesis [91,92]. Our findings provide a new line of evidence for the signaling power of heme/porphyrin, particularly in relation to cellular redox stress and growth.

Cancer cells often employ bio-energetically favorable mechanisms to fuel anabolism and growth [78]. For rapidly dividing cancer cells, this means a heavy reliance on utilization of salvage and scavenging [93] pathways to provide structural and energetic metabolic intermediates to fuel unchecked proliferation [78]. With the analysis of our data on human AML single-cell transcriptomes, we found that cancer progenitor cells preferably express genes for enzymes involved in salvaging pathways for biomolecules such as NAD, purines, and pyrimidines. Interestingly, the genes *SLC28A3* and *SLC29A3*, encoding nucleotide importers, are under direct heme epigenetic control [89]. Thus, to support the heavy metabolite trafficking activities, cancer heme overdrive is also probably associated with a wide range of cancer microenvironments.

Cancer cells do not exist in isolation: aggressive cancers successfully remodel not just their metabolism but also their immediate cellular environment. In fact, recently, cancer-associated stromal cells, such as cancer-associated fibroblasts (CAFs), were shown to shape the cancer cell niche and promote cancer progression [94,95]. Preliminary evidence suggests that heme overdrive is present in cancer niche cells, leading us to raise the intriguing possibility that targeting heme overdrive can potentially kill both the cancer and the partner cells in the tumor microenvironment (based on our analysis of available single-cell solid tumor data (unpublished results)). Such dual lethality may be attributed to the fact that CAFs are highly anabolic and have increased collagen production and growth factor secretion [96,97], a set of features uniquely shared with cancer cells.

Cancer heme overdrive is similar, yet opposite in magnitude, to human porphyrias, a group of inborn genetic diseases mostly caused by reduced enzyme activities (Fig. S6A) [98]. It is of interest to note that, except the erythroid lineage ALAS2 gain-of-function

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porphyria, no gain-of-function of human porphyria's (similar to cancers) have ever been reported [98], suggesting possible developmental lethality. Thus, cancer's imbalanced heme biosynthesis likely causes elevated pools of heme metabolites, while most porphyria's likely cause reduced heme synthesis.

In conclusion, through whole genome CRISPR KO analysis, single-cell transcriptomics using patient-derived tumor cells, and characterization of the heme biosynthetic pathway in engineered mammalian cells, we found an unrecognized pivotal role of imbalanced and dynamic heme metabolism in oncogenesis. Heme overdrive is a process that is universal in cancer, cancer-specific and required for cancer cell survival, and may be exploitable with novel therapeutic approaches. Finally, our studies provide a basis for exploring new avenues of research on the metabolic features of cancer cells and tumor microenvironments.

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Author contributions:

Conceptualization: RHYJ, SRA, GCF, and GAH

Methodology: RHYJ, SRA, GCF, GAH, GWR, NEA, CM, AB, and EMS

Investigation: RHYJ, SRA, GCF, GAH, GWR, and SMS

Visualization: RHYJ

Funding acquisition: RHYJ, GCF, GWR, and SMS

Project administration: RHYJ, GCF, GWR, and SMS

Supervision: RHYJ, GCF, GWR, and SMS

Writing – original draft: RHYJ, GCF, GWR, and SMS

Writing – review & editing: RHYJ, GCF, GWR, SMS, SRA, and GAH

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Supplementary Materials

Materials and Methods

Figs. S1 to S8

Tables S1 to S8

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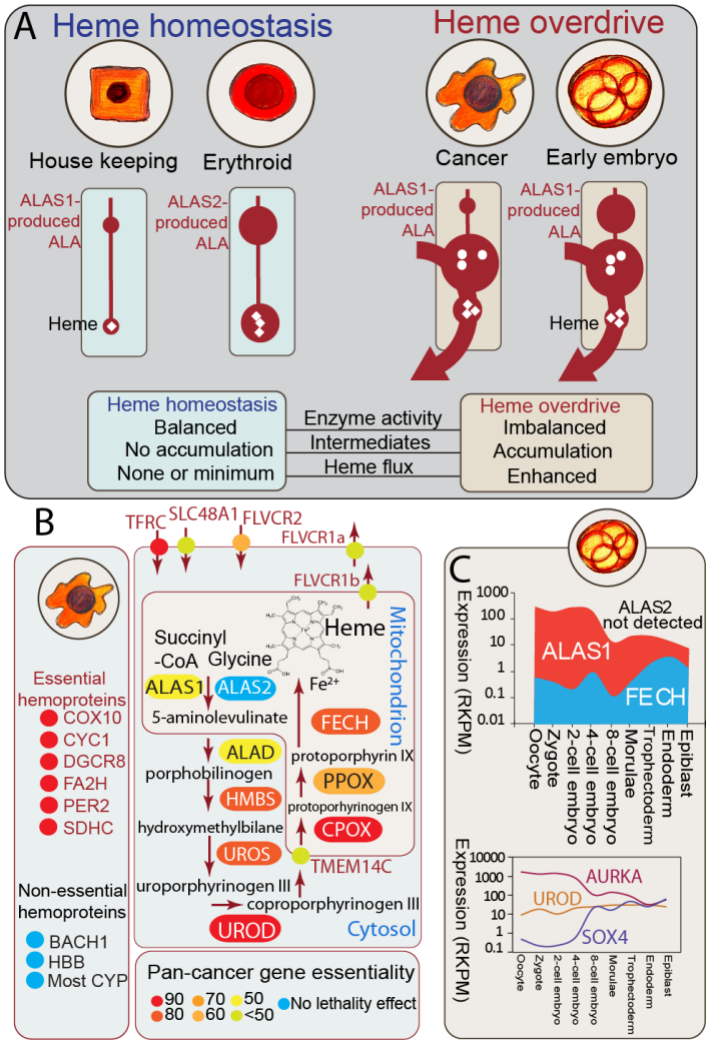

Figure 1. Defining heme overdrive with CRISPR data and early human embryonic stem cell single cell RNAseq. (**A**) **Schematic illustration of heme homeostasis and heme overdrive.** Heme homeostasis refers to the heme biosynthetic pathway in normal cells, with the housekeeping enzyme ALAS1 catalyzing the first and rate-limiting step in all cells, with the exception of precursor erythroid cells where the erythroid-specific ALAS isozyme ALAS2 catalyzes the rate-determining step. Heme overdrive refers to the imbalanced (i.e. with aberrantly increased and suppressed enzyme activities) pathway in cancer cells and early human embryonic stem cells, with the ALAS1 isozyme controlling ALA production regardless of the cell type. White circles and white diamonds represent porphyrin intermediates and heme, respectively; Erythroid refers to erythroid precursor cell. ALA, 5-aminolevulinate; ALAS, ALA synthase. (**B**) **Defining heme overdrive with CRISPR KO-derived essentiality data in over 300 cancer cell lines.** Analysis of CRISPR essentiality data enables mapping of heme overdrive in 27 major types of cancer. Color mapping indicates average cancer cell growth dependence revealed by CRISPR KO of a given gene. UROD has the highest pan-cancer essentiality. (**C**) Early human embryonic stem cells show features of heme overdrive. The expression levels for *ALAS1* and *FECH* differ over 100-fold during the first rounds of embryonic stem cell divisions, but they normalize with the early embryonic development progression. AURKA and SOX4 show expected patterns of expression dynamics during embryogenesis. [Abbreviations: ALA, 5-aminolevulinate; ALAS1, housekeeping ALA synthase; ALAS2, erythroid-specific ALA synthase; ALAD, ALA dehydratase (aka porphobilinogen synthase); BACH, transcription regulator BACH (BTB and CNC homology); CPOX, coproporphyrinogen oxidase; COX10, cytochrome C oxidase assembly homolog

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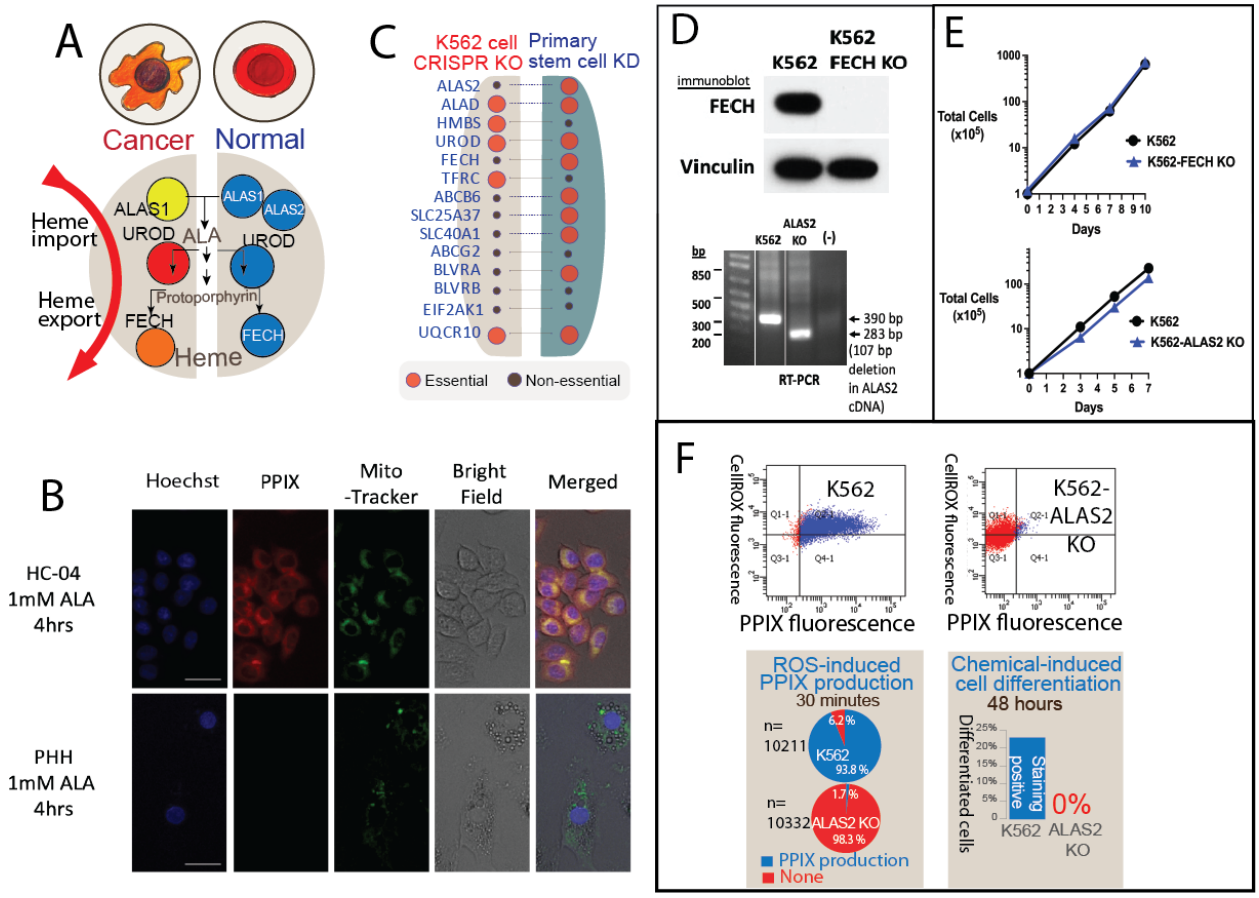

Figure 2. Heme overdrive is unique to cancer cells and absent in normal cells. (**A).** Schematic illustrations of the key differences of heme metabolism in cancer *vs.* normal cells. Red, orange and yellow shadings represent cancer gene essentiality in Fig 1B. Blue colors show the normal heme biosynthesis process **(B).** PPIX autofluorescence (red) is detected in HC-04 liver cancer cells but not in primary human hepatocytes (PHH) following ALA addition, indicating PPIX accumulation in cancer cells but not normal cells. Mitotracker green staining indicates viable cells. Over 1000 cells were assessed for each sample (representative cells shown). PPIX red was not observe in any normal cells. (**C**). Distinct heme metabolic gene essentiality in stem cell and cancer cells (lethality after loss/deletion of a specific gene). Human primary bone marrow stem cell gene knock down essentiality data from Egan et al (63). Cancer CRISPR KO data are from pan-cancer essentiality analysis (Depmap) (**D).** Validation of K562-FECH KO cell line generated by CRISPR/Cas9 gene editing. Immunoblots of wild-type K562 and K562-FECH KO whole cell lysates show a complete loss of FECH protein in K562-FECH KO cells, with vinculin used as a loading control (top). A 107 bp out of frame CRISPR-induced deletion was identified in the *ALAS2* gene (not shown) and due to in inability to identify a definitely specific ALAS2 antibody for immunoblotting, presence of this deletion was confirmed in RT-PCR analysis of K562-ALAS2 KO cells (bottom). (**E**) Total viable K562 and K562-FECH KO cells (top) and K562 and K562-ALAS2 KO cells (bottom) were determined over time using trypan blue exclusion, and no growth differences were detected. **(F)**. ALAS2 KO K562 cells are arrested in an undifferentiable state. K562 cells readily differentiated upon ROS induction (tert-butyl-

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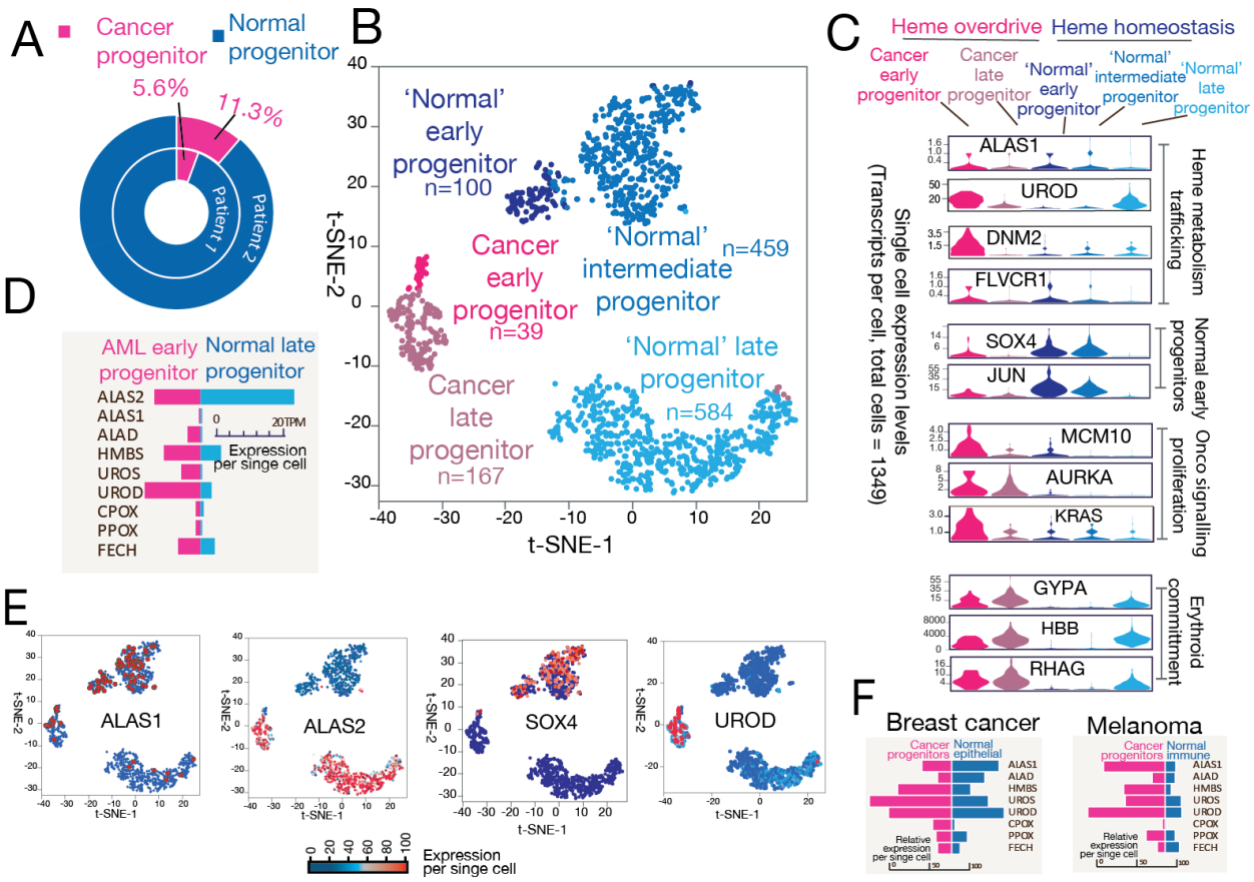

Figure. 3. Single-cell RNA sequencing of human AML bone marrow samples supports heme overdrive as a hallmark of cancer cells. Single-cell transcriptomes were obtained from bone marrow biopsies of AML donors with over 60% blasts. (**A**) Single-cell population composition shows only a minor fraction of the cells are cancer progenitors, which are defined by transcriptome embedding and clustering analysis. (**B**) Identification of distinct single cell populations in the AML patient bone marrow samples by t-SNE (t-distributed **S**tochastic **N**eighbor **E**mbedding) analysis. A total of 1349 high-quality transcriptomes from patient biopsies were used. (**C**) Representative genes for heme metabolism, normal early erythroid development, cell proliferation and erythroid commitment process are plotted. (**D**) *UROD*, the gene encoding the fifth enzyme of the heme biosynthetic pathway, is highly expressed in cancer early progenitors. *HMS* and *UROS*, which encode other intermediate step-catalyzing enzymes, are also over expressed in cancer progenitors. (**E**) Marker genes delineate cell populations from the patient samples. Note the complementary expression patterns of *ALAS1 vs*. *ALAS2*. (**F**) Solid tumor single cell RNAseq shows overexpression of the genes for the enzymes responsible for the intermediate heme biosynthetic pathway steps in breast cancer and melanoma.

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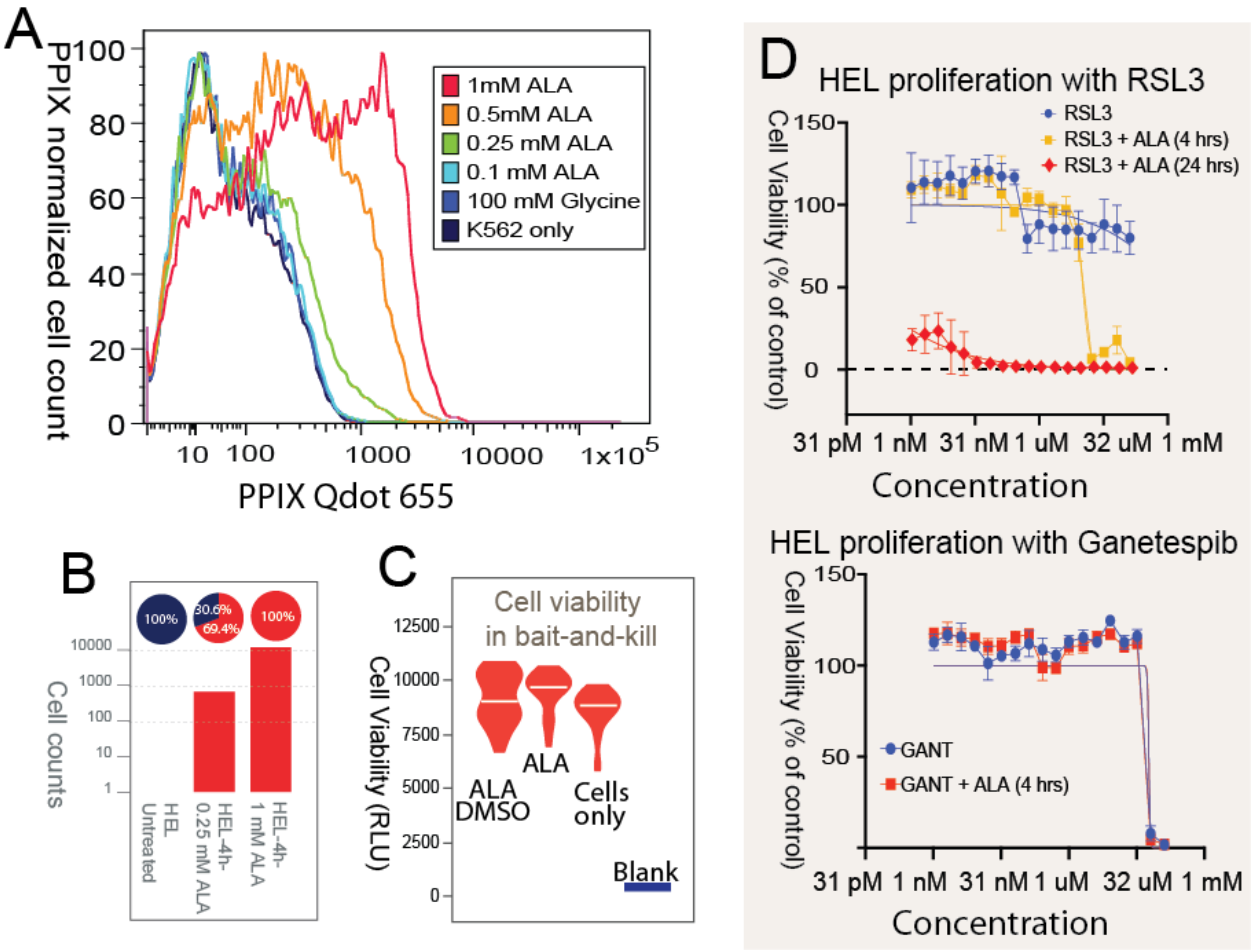

Figure 4: Targeting heme overdrive with a bait-and-kill strategy. (**A**) The concentration of PPIX accumulated in K562 cells depends on the ALA concentration supplemented to the culture medium. K562 cells were cultured in the absence or presence of 100 mM glycine or ALA (0.1 mM, 0.25 mM, 0.5 m and 1.0 mM) at 37 °C for 24 h, and PPIX fluorescence was measured by flow cytometry (**B**) ALA-induced PPIX accumulation in HEL cells. PPIX accumulated in 100% of the HEL cells (n >10,000 cells) as detected by PPIX fluorescence 4 hours after treatment with 1.0 mM ALA. Blue shows cells with negative PPIX in flowcytometry; red shows cells with positive PPIX. (**C**) Cell viability is unaffected by using ALA ("bait") as inducer of PPIX accumulation. HEL cells were treated with ALA (1 mM) at 37 °C for 24 h, and their viability was calculated from the generated luminescence upon reaction with CellTiter-Glo. The assays were conducted in triplicate. [RLU, relative luminescence units]. (**D**)

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