Cell death can be executed by apoptosis or one of several nonapoptotic cell death mechanisms, including ferroptosis. Ferroptosis can be triggered by blocking the uptake of cystine by the system x<sup>−</sup>-cystine/glutamate antiporter<sup>1</sup>. Loss of cystine uptake leads to depletion of intracellular reduced glutathione (GSH), starving the phospholipid hydperoxidase glutathione peroxidase 4 (GPX4) of its essential cofactor. Ultimately, GPX4 inactivation allows for iron-dependent accumulation of lipid hydroperoxides that appear to act via cryptic off-target antioxidant or iron chelating activities. We show that the FDA-approved drug bazedoxifene acts as a potent radical trapping antioxidant inhibitor of ferroptosis both in vitro and in vivo. ATP-competitive mechanistic target of rapamycin (mTOR) inhibitors, by contrast, are on-target ferroptosis inhibitors. Further investigation revealed both mTOR-dependent and mTOR-independent mechanisms that link amino acid metabolism to ferroptosis sensitivity. These results highlight kinetic modulatory profiling as a useful tool to investigate cell death regulation.

**Results**

**Kinetic modulatory profiling of cell death.** We developed a kinetic modulatory profiling approach to identify new modulators of apoptosis and ferroptosis (Fig. 1a and Supplementary Fig. 1a,b). HT-1080<sup>7</sup> fibrosarcoma cells were treated with one of eight different proapoptotic or proferroptotic lethal ‘query’ compounds and simultaneously exposed to one of 1,833 different bioactive ‘modulator’ compounds or vehicle (dimethylsulfoxide, DMSO), for a total of roughly 16,000 different conditions. Cell death in each condition was measured over time using STACK<sup>12</sup>. We computed the expected cell death for each query-modulator combination using the Bliss independence model<sup>11</sup>, and then determined the deviation between the expected and the observed cell death for each compound combination (Supplementary Fig. 1c,d). To facilitate an initial exploration of this dataset, z-scored deviation values were hierarchically clustered in an unsupervised manner across both query and modulator compounds and plotted as a heat map (Fig. 1b).

Several features of this compendium were consistent with a high-quality dataset. First, the three ferroptosis-inducing query compounds and five apoptosis-inducing query compounds segregated into distinct clusters (Fig. 1c). Moreover, within the ferroptosis subcluster, the system x<sup>−</sup>-inhibitors erastin and sorafenib were more similar to each other than the GPX4 inhibitor ML162. This is consistent with ferroptosis induced by system x<sup>−</sup>-inhibition versus direct GPX4 inhibition having unique mechanisms of regulation<sup>11</sup>. Second, we observed functionally coherent clustering of modulator compounds. For example, eight known microtubule
disrupting agents clustered together as modulators, including three kinase inhibitors (rigosertib, buparlisib and KX2-391) that directly inhibit tubulin polymerization in an off-target manner15–17 (Fig. 1c). In another example, erastin (also present in the library as a modulator compound) clustered with the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) inhibitors fluvastatin, lovastatin and mevastatin, consistent with inhibition of HMGCR promoting ferroptosis11,18 (Fig. 1c). In a final example, eight known proteasome inhibitors formed a distinct cluster (Fig. 1c). This cluster also contained the opioid receptor antagonist JTC-801, which can induce an unusual form of nonapoptotic cancer cell death19. Based on the observed clustering pattern, we hypothesized

![Fig. 1](https://example.com/fig1.png)

**Fig. 1 | A kinetic modulatory profile.** a, Overview of the kinetic modulatory profiling analysis and compendium generation. b, Deviation $z$ scores between the expected and observed effect of each compound combination in the compendium are plotted as a heat map. Gray cells indicate no data available. Soraf., sorafenib; Tunic., tunicamycin; Vinbla., vinblastine; Campt., camptothecin; Etopo., etoposide; Bortez., bortezomib; Thaps., thapsigargin. c, Three heat map subclusters of interest. Orange arrows indicate kinase inhibitors that bind tubulin as off-targets. d, Cell death in HT-1080 cells as determined using STACK. Cpt, camptothecin; Era2, erastin2. e, Time of population cell death onset ($D_O$) for treatments shown in d. NL, not lethal. f, Cell death in HT-1080 cells as determined using STACK. Btz, bortezomib. g, $D_O$ values for treatments shown in f. Note that the DMSO and Cpt data shown in d–g are from the same experiment. Results in d,f are mean ± s.d. from three independent experiments. Results in e,g are mean ± 95% confidence interval, derived from the curves in d and f, respectively.
that JTC-801 inhibited proteasome function. Both JTC-801 and bortezomib induced cell death in HT-1080 cells with similar rapid kinetics that was only partially inhibited by a pan-caspase inhibitor (Extended Data Fig. 1a,b). However, only bortezomib stabilized the transcription factor NFE2L1/NRF1, whose levels are negatively regulated by the proteasome\(^2\) (Extended Data Fig. 1c). We infer that JTC-801 does not directly inhibit proteasome function, but possibly a related target that yields effects on cell death similar to proteasome inhibition.

Overall, most compound combinations profiled as part of the compendium yielded no evidence for interaction. However, 295 combinations yielded apparent enhancing interactions (deviation \(z\) scores \(\geq 2\)), and 795 combinations resulted in apparent suppressing interactions (deviation \(z\) \(\leq -2\)). Among the apparent suppressing interactions were several between lethal query and known lethal modulator compounds. These results may be explained in several ways. The Bliss model of drug interaction assumes distinct compound mechanisms of action\(^2\). Thus, trivially, combining a lethal compound with itself or a different compound with the same target results in less cell death than expected mathematically. This explains the apparent suppressive interactions between bortezomib (query) with itself and other proteosome inhibitors (modulators), and between vinblastine (query) with other microtubule disrupting agents (modulators) (Fig. 1b).

Other apparent suppressing interactions between lethal compounds may be explained by kinetic single agent dominance\(^2\), when one fast-acting lethal compound kills cells before a more slowly acting lethal compound has a chance to act. This phenomenon could explain the apparent suppressing interaction between erastin and the topoisomerase inhibitor camptothecin (Fig. 1c). In follow-up experiments, the erastin analog erastin2 consistently initiated cell death roughly 8h earlier than camptothecin, such that camptothecin may not have time to act before the induction of ferroptosis is already widespread (Fig. 1d,e). Kinetic single agent dominance may also be facilitated when different inducers of cell death activate distinct cell death mechanisms. Indeed, the combination of bortezomib and camptothecin, which both trigger apoptosis, yielded less evidence for single agent dominance (Fig. 1f,g). However, these compound interactions can be difficult to tease apart, especially when two lethal compounds both induce cell death so rapidly that little additional headroom is available to further accelerate cell death within the population, as observed for erastin2 and bortezomib (Extended Data Fig. 1d,e).

While many apparent suppressing interactions between lethal compounds are best explained by mathematical or kinetic phenomena, two lethal compounds could also exhibit apparent suppressing interactions in the compendium due to true mechanistic interference between cell death mechanisms. For example, we observed an apparent suppressing interaction in the compendium between the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (query) and erastin (modulator) (Fig. 1c). We confirmed in two additional cell lines that thapsigargin attenuated ferroptosis response to erastin2, but not in response to direct GPX4 inhibition, suggesting a specific mechanism of cell death interference (Extended Data Fig. 1f). Understanding the nature of this and other mechanisms of cell death interference will require detailed characterization. Further analysis of the compendium using alternative metrics (for example, Loewe additivity) to predict expected compound interactions may also yield a distinct set of predicted interactions. Nonetheless, the functionally coherent clustering observed here for both query and modulator compounds suggests the present approach is suitable for kinetic modulatory profiles.

**Exploration of ferroptosis suppressor clusters.** Within the compendium, our attention was attracted to a cluster of 48 modulator compounds that had apparent suppressing interactions with all three ferroptosis-inducing compounds (Fig. 2a). Compounds in this cluster included known radical trapping antioxidant (RTA) inhibitors of ferroptosis, including ferrostatin-1 (Fer-1), phenothiazine and carvedilol\(^{2,22,23}\). This cluster also contained the MEK1/2 inhibitor UO126, which also likely acts as a RTA ferroptosis inhibitor\(^2\) (Supplementary Figs. 2 and 3). These compounds were not lethal themselves, and so the apparent suppressive interactions observed here appeared more likely to reflect true mechanistic inhibition of cell death. Other compounds in this cluster had diverse structures and annotated targets, including the NF-kB pathway (JSH-23), sphingosine kinase (SKI II) and vascular endothelial growth factor receptor (linifanib). Structurally, many of these compounds contained conjugated primary or secondary amine reminiscent of Fer-1 (Fig. 2b). Several of these compounds were also outliers in their ability to suppress ferroptosis compared to other members of the same compound family, hinting at off-target activities (Extended Data Fig. 2a). Accordingly, we hypothesized that this cluster was enriched for RTA ferroptosis inhibitors.

To test this hypothesis, we examined the ability of eight structurally and functionally distinct compounds found within this cluster (BGJ398, linifanib, bazadexifene, WZ4003, laetrile, flurpilinate maleate, SKI II and JSH-23) to suppress ferroptosis-like cell death in an Saccharomyces cerevisiae-based assay\(^\text{-linolenic acid (Supplementary Fig. 4). We previously showed that death in this model is suppressed by Fer-1 and other ferroptosis-specific inhibitors}\(^\text{-hemoglobin} (Extended Data Fig. 2a). These results were consistent with these compounds inhibiting ferroptosis through off-target chemical reactivities.

In addition to putative RTAs, we observed a distinct cluster of structurally distinct pan-ferroptosis suppressors centered on the iron chelator ciclopirox\(^\text{-cyclic thiazine), substantially inhibited cell death in this model, while two randomly selected library compounds (VX-765, LGK-974) did not (Fig. 2c). These results are consistent with these compounds inhibiting ferroptosis through off-target chemical reactivities.

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**Cell-free profiling identifies ferroptosis inhibitors.** Given the above results, we sought to more systematically examine compound RTA and iron chelator chemical reactivities. Toward this end, we tested all 1,833 compounds in our library in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay\(^1\) and the ferrozone Fe\(^{2+}\) binding assay\(^1\) in a high-throughput format. Here, 95 and 117 compounds reduced signal by \(\geq 50\%\) in the DPPH and ferrozine assays, respectively (Extended Data Fig. 3a).
Thus, roughly 5% of all library compounds examined here showed evidence for off-target chemical reactivities relevant to ferroptosis.

We subsequently focused on the 95 compounds identified in the DPPH assay, plus five additional compounds just above the arbitrary 50% DPPH signal inhibition threshold. Overall, 43, 11 and six of these compounds suppressed erastin2-induced ferroptosis by at least 80% when tested in HT-1080\(\text{N}\) cells at 50, 5 and 0.5 \(\mu\text{M}\), respectively (Fig. 3a). The six most potent compounds (Fer-1, SKI II, phenothiazine and bazedoxifene) all suppressed erastin2 and ML162-induced ferroptosis with sub-150 nM potency in confirmatory dose-response experiments, with Fer-1, phenothiazine and bazedoxifene emerging as the most potent compounds in this analysis (Supplementary Table 1). Fer-1, phenothiazine and bazedoxifene were also found in the large cluster of pan-ferroptosis suppressors identified in the compendium, and inhibited cell death in the \(S.\) \(cerevisiae\) model of ferroptosis. VX-765 and LGK-974 were randomly selected negative control compounds.

Many compounds examined above scored highly in the cell-free DPPH assay yet did not inhibit ferroptosis in cells, even when tested at 50 \(\mu\text{M}\) (Extended Data Fig. 3b). We hypothesized that partitioning into lipid environments might be a crucial determinant of compound activity in cells. Consistent with this hypothesis, across all 100 compounds ferroptosis suppression correlated with higher predicted lipophilicity and lower predicted water solubility (Extended
Bazedoxifene is an RTA ferroptosis inhibitor. Bazedoxifene is a third-generation approved drug for the treatment of postmenopausal osteoporosis35 (Fig. 3b). We elected to characterize the ability of this compound to inhibit ferroptosis in greater detail given the possibility that this approved drug could be repurposed for ferroptosis inhibition in vivo. Much like the iron chelator deferoxamine (DFO), bazedoxifene suppressed lipid reactive oxygen species (ROS) accumulation in erastin2-treated HT-1080 cells, potentially accounting for how this compound inhibited ferroptosis (Fig. 3c). Several lines of evidence suggested that suppression of lipid ROS accumulation and ferroptosis were not explained by modulation of estrogen receptor function. First, out of 29 estrogen or progesterone receptor modulators profiled in the compendium, only bazedoxifene and the structurally related selective estrogen receptor modulator raloxifene appreciably suppressed ferroptosis (Extended Data Fig. 2a). Second, the ability of bazedoxifene to suppress erastin2-induced ferroptosis was not altered by cotreatment with the estrogen receptor antagonist 17β-estradiol, which has a roughly tenfold greater affinity for estrogen receptor than bazedoxifene39 (Extended Data Fig. 4a). Third, bazedoxifene potentely suppressed ferroptosis in MDA-MB-231, E0771 and 4T1 breast cancer cell lines that do not express the estrogen receptor31 (Extended Data Fig. 4b,c).

Given the totality of our previous results, we hypothesized that bazedoxifene inhibited ferroptosis by acting as an RTA. Consistent with this, bazedoxifene trapped phospholipid-derived peroxyl radicals in a liposome-based, hyponitrite-initiated cooxidation assay28 almost as effectively as the positive control Fer-1 (Fig. 3d). The compound also suppressed lipid ROS accumulation in erastin2-treated HT-1080 cells, potentially accounting for how this compound inhibited ferroptosis (Fig. 3c).

Fig. 3 | Widespread bioactive compound antioxidant and iron chelating activity. a, Cell death in cells cotreated with erastin2 and 100 different compounds that scored positively in the cell-free DPPH assay as determined using STACK. The 43 compounds that suppressed cell death by >80% are listed, color coded by potency. Ph., phenethyl ester; Diphos., diphosphate. b, Structures of bazedoxifene and raloxifene. c, Lipid ROS detected using C11 BODIPY 581/591 (C11) in HT-1080 cells following 11 h treatment. Scale bar, 20 µm. Representative images from two independent experiments are shown. d, Inhibited cooxidation of egg phosphatidylcholine (1 mM) and STY-BODIPY (10 µM) in phosphate buffered saline (pH 7.4) initiated with 0.2 mM DTPN. Inhibitors were used at 2 µM. Average inhibition rate constants (kinh) determined assuming a reaction stoichiometry (n) of unity. e, Outline of a C. elegans model of ferroptosis. f, Sterile animals ± DGLA (0.13 mM) ± inhibitors (150 µM). Each datapoint represents the mean for one independent experiment (n ≥ 50 worms per experiment).
In this connection, our attention was attracted to a cluster of eight specific inhibitors of erastin2-induced ferroptosis in response to system x c(-i). This clustering suggested that these modulators were unlikely to act as RTAs or iron chelators, which would also be expected to inhibit ferroptosis. Rather, we hypothesized that mTOR inhibition can suppress ferroptosis, seven of which appeared to suppress ferroptosis induced by system x c(-i) in HT-1080 cells and this correlated with weaker phospholipid-derived peroxyl radical trapping (k(float) = 1.3 × 10^4 M^(-1) s^(-1)) (Fig. 3b,d). These results are consistent with bazedoxifene inhibiting ferroptosis by acting as a RTA, and we propose that the greater potency of bazedoxifene relative to raloxifene may be due to the weaker O-H bond in the 5-hydroxyindole moiety in bazedoxifene compared to the 6-hydroxybenzothiophene moiety in raloxifene.

To examine whether bazedoxifene could suppress ferroptosis in vivo we used a new Caenorhabditis elegans-based animal model. Direct exposure to the polyunsaturated fatty acid dihomo-γ-linolenic acid (DGLA) induces ferroptotic germ cell death during C. elegans development and adult sterility (Fig. 3e). Like the positive control Fer-1, bazedoxifene inhibited DGLA-induced germ cell death and sterility (Fig. 3f and Extended Data Fig. 5a). We confirmed that bazedoxifene itself did not alter DGLA uptake (Extended Data Fig. 5b).

Together, these results suggest that bazedoxifene can act as a potent RTA inhibitor of ferroptosis.

**mTOR inhibition can suppress ferroptosis.** In the compendium, the system x_c(-i) inhibitors erastin and sorafenib clustered away from the GPX4 inhibitor ML162 (Fig. 1b). This suggested that certain compounds were likely to uniquely modulate ferroptosis by acting as a RTA, and we propose that the greater potency of bazedoxifene relative to raloxifene may be due to the weaker O-H bond in the 5-hydroxyindole moiety in bazedoxifene compared to the 6-hydroxybenzothiophene moiety in raloxifene.

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mTORC1 complex subunit RPTOR, but not the mTORC2-specific subunit RICTOR, inhibited erastin2-induced ferroptosis to the same extent as the ATP-competitive mTOR inhibitor INK128 (Extended Data Fig. 6a,b). Thus, mTORC1 inhibition appears sufficient to suppress ferroptosis in response to cystine deprivation.

INK128, along with another structurally distinct ATP-competitive mTOR inhibitor, AZD8055, both suppressed erastin2-induced ferroptosis and phosphorylation of the mTOR target 4E-binding protein 1 (4E-BP1) in HT-1080N, U-2 OSN and human embryonic kidney (HEK) 293T® cells (Extended Data Fig. 7a,b). Mechanistically, mTOR inhibitors reduced lipid ROS accumulation and enhanced residual intracellular GSH levels following erastin2 treatment (Fig. 4b,c and Extended Data Fig. 7c). The ability of INK128 to suppress ferroptosis was blunted when cells were cotreated with buthionine sulfoximine (BSO), indicating that de novo GSH synthesis was required for the protective effect (Fig. 4d). mTOR inhibition did not increase system x− activity; therefore, increased cystine import seemed unlikely to explain how mTOR inhibition helped maintain GSH levels and suppress ferroptosis (Extended Data Fig. 7d).

ATP-competitive mTOR inhibitors blocked ferroptosis better than rapamycin and related allosteric mTOR inhibitors (Fig. 4d, Extended Data Fig. 7e and Supplementary Fig. 5). Protein synthesis is one of several processes that are more sensitive to ATP-competitive mTOR inhibitors than rapalogues. We and others previously observed that direct protein synthesis inhibition using cycloheximide (CHX), or genetic silencing of the ribosomal large subunit gene RPL8, but not system x− inhibitors but not direct GPX4 inhibitors, reminiscent of the phenotype observed here with mTOR inhibition. Thus, we hypothesized that mTOR-dependent regulation of protein synthesis could modulate ferroptosis in response to cystine deprivation.

Supporting this model, ATP-competitive mTOR inhibitors blocked the phosphorylation of both 4E-BP1 and RPS6, two key regulators of mTOR-driven protein synthesis, while rapamycin inhibited RPS6 phosphorylation only, as expected (Extended Data Fig. 8a). Higher expression of ribosomal subunits (for example, RPS3, RPL3, RPLP2) was also more strongly correlated with sensitivity to erastin than the GPX4 inhibitors RSL3 and ML210, across hundreds of cancer cell lines profiled in the Cancer Therapeutics Response Portal (Extended Data Fig. 8b). Finally, we confirmed in five different cancer cell lines that direct inhibition of protein synthesis using CHX attenuated ferroptosis in response to erastin2 but not ML162, and that these protective effects were partially reverted by cotreatment with BSO (Extended Data Fig. 8c).

To more directly test the role of mTOR-regulated protein synthesis in ferroptosis regulation we over-expressed in U-2 OS cells a non-phosphorylatable 4E-BP1 mutant (that is, 4E-BP14A) . This mutant mimics the consequences of pharmacological mTOR inhibition on 4E-BP1 function, blocking protein synthesis by binding constitutively with eIF4E. Inducible 4E-BP1 4A expression was sufficient to lower the phosphorylation of both 4E-BP1 and RPS6 (Fig. 4e,f). Moreover, the protective effect of 4E-BP14A expression was reverted by cotreatment with BSO, and this corresponded to weaker retention of intracellular GSH following erastin2 treatment (Fig. 4f.h). Notably, 4E-BP1 and RPS6 phosphorylation were not altered by system x− inhibition itself, indicating that mTOR remained active in the context of cystine deprivation (Extended Data Fig. 9a). Indeed, ongoing mTOR activity could drive amino acid consumption in protein synthesis that otherwise could be used for GSH synthesis. Consistent with this possibility, INK128 treatment resulted in accumulation of most amino acids within the cell while rapamycin treatment had weaker effects (Extended Data Fig. 9b).
mTOR-independent ferroptosis regulation by amino acids. mTORC1 is sensitive to amino acid levels, especially leucine (Leu) and arginine (Arg)\(^40\). We therefore asked whether Leu or Arg withdrawal was sufficient to suppress erastin2-induced ferroptosis. Leu withdrawal did not inhibit erastin2-induced ferroptosis in U-2 OS\(^N\) cells (Fig. 5a). By contrast, Arg withdrawal potently suppressed erastin2-induced cell death and lipid ROS accumulation (Fig. 5a–c). The protective effect of Arg deprivation could be reverted by co-supplementation with l-Arg or the Arg metabolic precursor citrulline, but not d-Arg (Fig. 5d and Extended Data Fig. 10a). Arg deprivation protected against erastin2-induced ferroptosis in seven different cancer cell lines in addition to U-2 OS\(^N\), but did not inhibit ferroptosis in response to the GPX4 inhibitor ML162 (Fig. 5e and Extended Data Fig. 10b). This suggested that, like mTOR inhibition, Arg deprivation might protect from ferroptosis by modulating GSH metabolism. Consistent with this hypothesis, Arg deprivation was unable to inhibit erastin2-induced ferroptosis when BSO was included in the growth medium (Fig. 5f).

The above data were consistent with the expectation that Arg deprivation blocked ferroptosis by inhibiting mTORC1 activity. Arg withdrawal reduced the phosphorylation of RPS6 in 293T cells, as expected\(^39\). However, Arg withdrawal did not inhibit 4E-BP1 phosphorylation in 293T cells, or RPS6 and 4E-BP1 phosphorylation in U-2 OS cells (Fig. 6a). Consistent with the absence of effect on key regulators of protein synthesis, bulk protein synthesis was reduced in U-2 OS cells by INK128 treatment but not by Arg deprivation, as determined using a puromycylation assay (Extended Data Fig. 10c,d). These results indicated that Arg withdrawal may suppress ferroptosis without inhibiting mTOR function.

The GCN2/ATF4 pathway responds to amino acid deprivation and has been linked to the regulation of ferroptosis in some contexts\(^40,41\). The transcription factor ATF4 controls the expression of transsulfuration pathway enzymes (that is, CBS, CTH) that could convert methionine to cysteine under conditions of cysteine limitation, and thereby potentially suppress ferroptosis. In U-2 OS cells, Arg deprivation increased ATF4 protein levels and the expression of CBS and CTH in a manner sensitive to the specific GCN2 inhibitor GCN2iB\(^42\) (Fig. 6a–c). However, despite blocking ATF4 expression and function, GCN2iB did not prevent Arg deprivation from inhibiting ferroptosis (Fig. 6d). Likewise, Arg deprivation suppressed erastin2-induced ferroptosis in HT-1080 cells stably expressing an shRNA targeting ATF4 (ref. \(^43\)) (Extended Data Fig. 10e,f). Thus, increased GCN2/ATF4 pathway activity appeared unable to explain the ability of Arg deprivation to suppress ferroptosis.

Given these unexpected results, we investigated more broadly how depriving cells of different individual amino acids impacted ferroptosis. Using STACK technology, we quantified the effects of 14 single amino acid withdrawal conditions on ferroptosis induced by erastin2 or cystine co-withdrawal. Deprivation of glutamine, lysine, valine, methionine and arginine all suppressed ferroptosis in both U-2 OS\(^N\) and HT-1080\(^N\) cells in response to erastin2 or cystine co-withdrawal (Fig. 6e). By contrast, deprivation of glycine, tryptophan, phenylalanine and serine had little or no ability to inhibit ferroptosis. These effects were not obviously related to differences in amino acid physical properties or shared metabolic roles. However, the ability of single amino acid withdrawal to prevent ferroptosis did track with the ability of each condition to inhibit proliferation in the absence of erastin2 or cystine co-withdrawal (Fig. 6e). Thus, acute...
amino acid deprivation-induced proliferative arrest correlates with protection from ferroptosis in a manner that can be independent of mTOR inhibition and GCN2/ATF4 pathway activation.

Discussion

We generated a large compendium of kinetic cell death modulatory profiles and used this compendium to identify new chemical modulators of ferroptosis. Many compounds, including numerous drugs, appear to suppress ferroptosis in an off-target manner as cryptic RTAs or iron chelators. These results reinforce the concept that such activities are relatively common and. This may confound mechanistic studies, when one compound is assumed to be acting to inhibit ferroptosis through a particular protein target. However, cryptic drug RTA or iron chelator activities could be of interest for drug repurposing. We find that bazedoxifene is a potent RTA inhibitor of ferroptosis. This is notable given that bazedoxifene has been shown to inhibit pathological cell death in several contexts, including the brain and liver. We speculate that these positive effects could be due in part to off-target ferroptosis suppression.

We find that off-target mTORC1 inhibition can delay ferroptosis triggered by system x− inhibition or direct cystine deprivation. Protein synthesis normally consumes most intracellular cysteine. A model consistent with our data and earlier results is that reduced protein synthesis allows for intracellular cysteine to be shunted toward GSH synthesis. We speculate that direct inhibition of ribosome activity blocks ferroptosis selectively in response to system x− inhibitors for similar reasons. Our results support the concept that ongoing mTOR activity can have a paradoxical pro-death effect in cancer cells when it drives the consumption of a metabolic resource that is limiting for survival. However, given the pleiotropic effects of mTORC1 signaling on metabolism, it may not be unexpected that this pathway has different effects on ferroptosis sensitivity in other contexts.

We find that cystine deprivation is largely unable to induce ferroptosis when other amino acids (Gln, Lys, Arg, Met and Val) are absent from the environment. Inhibition of mTORC1 function or activation of the GCN2/ATF4 pathway is not sufficient to explain how Arg withdrawal suppresses ferroptosis. It will be interesting to investigate whether this also holds true for Gln, Lys, Met and Val, and whether the protective effect of withdrawing these amino acids is explained by a shared mechanism or linked to distinct roles for each individual amino acid in ferroptosis. At present, our results suggest that slower proliferation following the withdrawal of these amino acids could be one shared mechanism that contributes to ferroptosis suppression, possibly via cystine or GSH sparing. These results indicate that therapeutic anticancer cystine deprivation strategies may be most effective at inducing ferroptosis when other amino acids are present in the environment at levels sufficient to stimulate normal proliferation.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41589-021-00751-4.

Received: 28 October 2019; Accepted: 27 January 2021; Published online: 08 March 2021

References

1. Dixon, S. J. et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 149, 1060–1072 (2012).
2. Yang, W. S. et al. Regulation of ferroptotic cancer cell death by GPX4. Cell 156, 317–331 (2014).
3. Stockwell, B. R., Jiang, X. & Gu, W. Emerging mechanisms and disease relevance of ferroptosis. Trends Cell Biol. 30, 478–490 (2020).
4. Conrad, M. & Pratt, D. A. The chemical basis of ferroptosis. Nat. Chem. Biol. 15, 1137–1147 (2019).
5. Li, Y., Qian, L. & Yuan, J. Small molecule probes for cellular death machines. Curr. Opin. Chem. Biol. 39, 74–82 (2017).
6. Smith, C. E. et al. Non-steroidal anti-inflammatory drugs are caspase inhibitors. Cell Chem. Biol. 24, 281–292 (2017).
7. Corsello, S. M. et al. The Drug Repurposing Hub: a next-generation drug library and information resource. Nat. Med. 23, 405–408 (2017).
8. Mishima, E. et al. Drugs repurposed as antioxidant agents suppress organ damage, including AKI, by functioning as lipid peroxyl radical scavengers. J. Am. Soc. Nephrol. 31, 280–296 (2020).
9. Shah, R., Shchebinov, M. S. & Pratt, D. A. Resolving the role of lipoxynigenases in the initiation and execution of ferroptosis. ACS Cent. Sci. 4, 387–396 (2018).
10. Wolpaw, A. J. et al. Modulatory profiling identifies mechanisms of small molecule-induced cell death. Proc. Natl Acad. Sci. USA 108, E771–E780 (2011).
11. Shimada, K. et al. Global survey of cell death mechanisms reveals metabolic regulation of ferroptosis. Nat. Chem. Biol. 12, 497–503 (2016).
12. Forcina, G. C., Conlon, M., Wells, A., Cao, J. Y. & Dixon, S. J. Systematic quantification of population cell death kinetics in mammalian cells. Cell Syst. 4, 600–610.e6 (2017).
13. Fitzgerald, J. B., Schoeberl, B., Nielsen, U. B. & Sorger, P. K. Systems biology and combination therapy in the quest for clinical efficacy. Nat. Chem. Biol. 2, 458–466 (2006).
14. Soula, M. et al. Metabolic determinants of cancer cell sensitivity to canonical ferroptosis inducers. Nat. Chem. Biol. 16, 1351–1360 (2020).
15. Jost, M. et al. Combined CRISPRa/a-based chemical genetic screens reveal that gosirgosib is a microtubule-destabilizing agent. Mol. Cell 68, 210–223.e6 (2017).
16. Bohnacker, T. et al. Deconvolution of Buparlisib's mechanism of action defines specific PI3K and tubulin inhibitors for therapeutic intervention. Nat. Commun. 8, 14683 (2017).
17. Smolinski, M. P. et al. Discovery of novel dual mechanism of action SRC signaling and tubulin polymerization inhibitors (KX2-391 and KX2-361). J. Med. Chem. 61, 4704–4719 (2018).
18. Viswanathan, V. S. et al. Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. Nature 547, 453–457 (2017).
19. Song, X. et al. ITC801 induces pH-dependent death specifically in cancer cells and slows growth of tumors in mice. Gastroenterology 154, 1480–1493 (2018).
20. Donlin, F. M. et al. Inhibition of NGLY1 inactivates the transcription factor Nrf1 and potentiates proteasome inhibitor cytotoxicity. ACS Cent. Sci. 3, 1143–1155 (2017).
21. Richards, R. et al. Drug antagonism and single-agent dominance result from differences in death kinetics. Nat. Chem. Biol. 16, 791–800 (2020).
22. Zilka, O. et al. On the mechanism of cytoprotection by ferrostatin-1 and lipostatin-1 and the role of lipid peroxidation in ferroptotic cell death. ACS Cent. Sci. 3, 232–243 (2017).
23. Shah, R., Margison, K. & Pratt, D. A. The potency of diarylamine radical-trapping antioxidants as inhibitors of ferroptosis underscores the role of autodestruction in the mechanism of cell death. ACS Chem. Biol. 12, 2538–2545 (2017).
24. Gao, M., Monian, P., Quadri, N., Ramasamy, R. & Jiang, X. Glutaminolysis and transferrin regulate ferroptosis. Mol. Cell 59, 298–308 (2015).
25. Skouta, R. et al. Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. J. Am. Chem. Soc. 136, 4551–4556 (2014).
26. Lu, T. et al. Up-regulation of hypoxia-inducible factor antisense as a novel approach to treat ovarian cancer. Thrombos. 10, 6959–6976 (2020).
27. Jeitner, T. M. Optimized ferrozone-based assay for dissolved iron. Anal. Biochem. 454, 36–37 (2014).
28. Shah, R., Farmer, I. A., Zilka, O., Van Kessel, A. T. M. & Pratt, D. A. Beyond DPPH: use of fluorescence-enabled inhibited autodestruction to predict oxidative cell death. Cell Chem. Biol. 26, 1594–1607.e7 (2019).
29. Silverman, S. L. et al. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. J. Bone Miner. Res. 23, 1923–1934 (2008).
30. Komin, B. S. et al. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology 146, 3999–4008 (2005).
31. Le Naour, A. et al. EO717, the first luminal B mammary cancer cell line from C57BL/6 mice. Cancer Cell Int 20, 328 (2020).
32. Perez, M. A., Magtanong, L., Dixon, S. J. & Watts, J. L. Dietary lipids induce ferroptosis in caenorhabditis elegans and human cancer cells. Dev. Cell 54, 447–454.e4 (2020).
33. Valvezan, A. J. & Manning, B. D. Molecular logic of mTORC1 signalling as a metabolic rheostat. Nat. Med. 1, 321–333 (2019).
34. Yang, W. S. & Stockwell, B. R. Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. Chem. Biol. 15, 234–245 (2008).
35. Kang, S. A. et al. mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin. Science 341, 1236566 (2013).
36. Rees, M. G. et al. Correlating chemical sensitivity and basal gene expression reveals mechanism of action. Nat. Chem. Biol. 12, 109–116 (2016).
37. Thoreen, C. C. et al. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109–113 (2012).
38. Wolfson, R. L. & Sabatini, D. M. The dawn of the age of amino acid sensors for the mTORC1 pathway. Cell Metab. 26, 301–309 (2017).
39. Chantranupong, L. et al. The CASTOR proteins are arginine sensors for the mTORC1 pathway. Cell 165, 153–164 (2016).
40. Harding, H. P. et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11, 619–633 (2003).
41. Dai, C. et al. Transcription factors in ferroptotic cell death. Cancer Gene Ther. 27, 645–656 (2020).
42. Nakamura, A. et al. Inhibition of GCN2 sensitizes ASNS-low cancer cells to asparaginase by disrupting the amino acid response. Proc. Natl Acad. Sci. USA 115, E7776–E7785 (2018).
43. Ye, J. et al. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. EMBO J. 29, 2082–2096 (2010).
44. Jover-Mengual, T. et al. Molecular mechanisms mediating the neuroprotective role of the selective estrogen receptor modulator, bazedoxifene, in acute ischemic stroke: a comparative study with 17beta-estradiol. J. Steroid Biochem. Mol. Biol. 171, 296–304 (2017).
45. Lan, Y. L. et al. Bazedoxifene protects cerebral autoregulation after traumatic brain injury and attenuates impairments in blood-brain barrier damage: involvement of anti-inflammatory pathways by blocking MAPK signaling. Inflamm. Res. 68, 311–323 (2019).
46. Yu, X. & Long, Y. C. Crosstalk between cystine and glutathione is critical for the regulation of amino acid signaling pathways and ferroptosis. Sci. Rep. 6, 30033 (2016).
47. Ratan, R. R., Murphy, T. H. & Baraban, J. M. Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. J. Neurosci. 14, 4385–4392 (1994).
48. Nofal, M., Zhang, K., Han, S. & Rabinowitz, J. D. mTOR inhibition restores amino acid balance in cells dependent on catabolism of extracellular protein. Mol. Cell 67, 936–946.e5 (2017).
49. Baba, Y. et al. Protective effects of the mechanistic target of rapamycin against excess iron and ferroptosis in cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 314, H659–H668 (2018).
50. Cramer, S. L. et al. Systemic depletion of l-cyst(e)ine with cyst(e)inase increases reactive oxygen species and suppresses tumor growth. Nat. Med. 23, 120–127 (2017).

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Methods

Chemicals and reagents. An 1,833-member bioactive compound library, and an independent 86-member PI3K signaling inhibitor library comprising mTOR, PI3K and AKT pathway inhibitors, were obtained from Selleck Chemicals and stored at −80 °C. Fractions were annotated as compound SMEM28 (Ref. 35) and ML162 were synthesized by Acme Bioscience. Erastin was the kind gift of B. Stockwell (Columbia). Chemicals used were DMSO, ferrostatin-1, thapsigargin, tunicamycin, CHX, t-arginine, d-arginine and l-citrulline (Sigma-Aldrich Corporation); bortezomib, rapamycin, etoposide and BSO (Thermo Fisher Scientific); INK-128, AZD8055, vinblastine, camptothecin, sorafenib, bezafibrate, rolaufen and JTC-81 (Selleck Chemicals) and GCN2iB (MedChemExpress). BSO was dissolved directly into cell media. All other drugs were prepared as stock solutions in DMSO. Stock solutions were stored at −20 °C.

Cell culture. HT-1080 (CCL-121), U-2 OS (HTB-96), MBA-M231 (HTB-26), HEK293T (CRL-3216, hereafter 293T), NCI-H1129 (CRL-5803, hereafter H129), A549 (CCL-185), T98G (CRL-1697), Caki-1 (HTB-46) and A375 (CRL-1619) were obtained from ATCC. Mouse 4T1 and E0771 triple negative breast cancer cells were originally obtained from ATCC and CHB Biosystems, and obtained via Lingying Li (Stanford, Department of Biochemistry). HT-1080 cells stably expressing a nontargeting shRNA (sh-NLNT) and an shRNA targeting ATF4 were the kind gift of I. Ye (Stanford, Department of Radiation Oncology). H23 cells stably expressing Cas9 (H23Cas9) were the kind gift of M. Bassik (Stanford, Department of Genetics). The polyclonal nuclear mKate2-expressing (denoted by superscript ‘N’) cell lines HT-1080-N, U-2 OS-N, 293T-N and H129-N were described previously (Ref. 36). Polyclonal mKate2-expressing cells were generated from the respective parental cell lines via transduction with the NucLight Red lentivirus, which directs the expression of nuclear-localized mKate2 (Essen Biosciences/Sartorius). Polyclonal mKate2-expressing populations were selected using puromycin (Life Technologies, catalog no. A11318-03, 1.5 µg ml−1, for 48–72 h).

All HT-1080 cells were cultured normally in DMEM Hi-glucose media (Corning Life Sciences) catalog no. 444001 used with 1% nonessential amino acids (NEAAs, Thermo Fisher Scientific). A549, 293 T, MBA-M231 and T98G cells were cultured in DMEM Hi-glucose medium without supplemental NEAAs. U-2 OS, Caki-1 and A375 cells were cultured in McCoy’s 5A medium (Gibco) with 10% fetal bovine serum. U-2 OS, Caki-1 and A375 cells were originally obtained from ATCC and CH3 Biosystems, and obtained via Lingying Li (Stanford, Department of Biochemistry). T98G cells were cultured in DMEM High glucose medium without supplemental NEAAs. U-2 OS, Caki-1 and A375 cells were cultured in McCoy’s 5A medium (Gibco) with 10% fetal bovine serum. U-2 OS, Caki-1 and A375 cells were originally obtained from ATCC and CH3 Biosystems, and obtained via Lingying Li (Stanford, Department of Biochemistry). T98G cells were cultured in DMEM High glucose medium without supplemental NEAAs. U-2 OS, Caki-1 and A375 cells were cultured in McCoy’s 5A medium (Gibco) with 10% fetal bovine serum. U-2 OS, Caki-1 and A375 cells were originally obtained from ATCC and CH3 Biosystems, and obtained via Lingying Li (Stanford, Department of Biochemistry).

Bioactive compound library profiling in cells. Large-scale bioactive compound profiling was performed as described (Ref. 40). HT-1080 cells were grown in T-175 flasks (Corning Life Sciences) and trypsinized and counted using a Cellometer Auto T4 cell counter (Nexcelom). Then, 40 µl of cell solution was added manually to each well of a 384-well clear-bottom tissue culture plate (Corning) at a final density of 1,500 ± 150 cells per well. The plate was spun briefly (500 rpm, 2 s) to settle the cells evenly at the bottom of the wells. The next day, the medium was removed and replaced with 360 µl of media containing the dead cell probe SYTOX Green (20 nM, final concentration; Life Technologies) and DMSO or one of ten lethal compounds (final concentration), thapsigargin (12.5 µM), tunicamycin (10 µg ml−1), camptothecin (100 µg ml−1), etoposide (100 µg ml−1), vinblastine (0.1 µg ml−1), erastin (10 µg ml−1), sorafenib (10 µM) and ML162 (5 µM). Next, 4 µl of medium containing one of 1,833 different bioactive library compounds was added to the mixture (final concentration 50 nM) and 10 µl of SYTOX Green positive counts were subtracted from live cell counts. Some experiments used unmarked cells and SG+ cells only were counted as a metric of cell death. These scores were hierarchical clustered in an unsupervised manner using default settings available in the Morpheus suite (https://software.broadinstitute.org/morpheus/). These settings were: metric, one minus Pearson correlation; linkage, average; cluster, rows and columns.

Identification of known antioxidants and iron chelators. To identify known antioxidants, literature searches were performed using PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and the search terms (‘compound’) AND (‘antioxidant’ OR ‘ferroptosis’) on 18 February 2018.

Analysis of NRF1/NFE2L1 stabilization. Here, 200,000 HT-1080 cells were seeded into six-well plates (Corning). The next day, media on these cells was aspirated and replaced with media containing DMSO (vehicle), bortezomib (200 nM) or JTC-801 (5 µM). DMSO and Bortezomib treated cells were collected after 4 h while JTC-801 treated cells were collected both at 4 h and after 8 h. Both media were washed once with PBS (50 nM), followed by a wash step with 1 ml of HBSS (Life Technologies) and trypsinized to collect. Trp53 was quantified with medium growth. Cells were subsequently spun at 350 g for 5 min. Media was aspirated from the pellets followed by a wash step with 1 ml of HBSS. Pellets were then resuspended in 100 µl of 9 M urea, sonicated, and spun for 15 min in a centrifuge at maximum speed. The resulting lysate was transferred to a new Eppendorf tube and protein abundance was quantified using the BCA assay. Then, 30 µg of protein from each lysate was loaded onto a Bolt 4–12% Bis-Tris Plus SDS gel (Life Technologies) for separation for 75 min at 100 V. The gel was transferred to a nitrocellulose membrane using the iBlot2 system (Life Technologies). Membranes were probed with a rabbit monoclonal antibody directed against the TRAF2/NFE2L1 (Cell Signaling Technology, dilution 1:1,000) and a mouse monoclonal antibody directed against tubulin (Fisher Scientific, dilution 1:2,000). Donkey anti-rabbit secondary antibody (IRDye 800LT, LI-COR Biosciences) and donkey antimouse secondary antibody (IRDye 680LT, LI-COR Biosciences) were used at 1:15,000 dilution to visualize bands. Membranes were imaged using the LI-COR CLx Imaging System.

Analysis of ERK phosphorylation. In this stage, 200,000 HT-1080 cells per well were grown overnight in a six-well tissue culture plate. Cells were washed with 1 ml of HBSS and the media was replaced with serum-free DMEM high glucose media containing 1% penicillin-streptomycin. After 16 h of serum starvation, HT-1080 cells were stimulated with 1 µM TNFα, 50 nM TRAIL, 100 nM TNFα or 100 nM etretinate (250 nM) for 30 min, washed with 1 ml of HBSS and lysed in 100 µl of 9 M urea containing protease and phosphatase inhibitor cocktail (Cell Signaling Technology). Samples were analyzed by western blotting as described above. Rabbit monoclonal antibodies directed against the diphosphorylated (Thr202/Tyr204) form

Compendium data analysis and visualization. Cell death within each population was analyzed using the STACK approach using counts of live (mKate2+ and dead (SYTOX Green, SG+) cells to compute lethal fraction scores at each timepoint, as described (Ref. 40). At this stage, several data quality filters were applied. First, results obtained for ten autofluorescent compounds were removed from all subsequent analyses: nintedanib (BBF 1120), sunitinib malate, enzastaurin (LY317615), PHA-665752, SB216763, SU11274, darubicin HCl, TSU-68 (SU6688, orantinib), quinacrine ZH13 and Ro 31-8220 mesylate. Second, we removed from the analysis of bioactive compound modulatory effects between the lethal queries that were each observed for different lengths of time. AUC values for each population were normalized to the maximum possible cell death within the observation period. Thus, the normalized AUC (nAUC) was used to compute the expected nAUC (nAUCexp) using the Bliss independence model (Q + M − (Q × M)). The difference between nAUCexp and the experimentally observed nAUC (nAUCobs) were calculated for each compound combination (nAUC = nAUCobs - nAUCexp). To account for differences in the overall ‘modulatability’ of each lethal query by the bioactive compound libraries, all difference values were z scored separately for each lethal query across all tested bioactive modulator compounds. Z scores were hierarchically clustered in an unsupervised manner using default settings available in the Morpheus suite (https://software.broadinstitute.org/morpheus/). These settings were: metric, one minus Pearson correlation; linkage, average; cluster, rows and columns.
of ERK1/2 (p42/44 MAPK, Cell Signaling Technology, dilution: 1:1,000) and total ERK1/2 (Cell Signaling Technology, dilution: 1:1,000) were the primary antibodies.

**Yeast experiments.** The *S. cerevisiae* strain used in this study was coqΔΔ (BY4741 MALA his3Δ1 leu2Δ1 met15Δ1 ura3Δ1 coqΔΔ:AdaMX4). coqΔΔ was grown in YPD (1% yeast extract peptone dextrose, BD Biosciences), 2% peptone (BD Biosciences) and 2% dextrose (Fisher BioReagents). The day before the experiment, a single yeast coqΔΔ colony was used to inoculate 5 ml of YPD. The culture was grown overnight (30°C, 160 r.p.m.). The next morning, the culture was diluted to an optical density (OD600) of 0.1 in fresh YPD and incubated at 30°C, 160 r.p.m. for roughly 4 h to mid-log phase (OD600 = 0.2–0.5). At mid-log phase, cells were collected and washed twice with 2 volumes of sterile water, resuspended to a final OD600 = 0.8 in 0.1 M sodium phosphate buffer pH 6.2/25 mM SYTOX Green and 50 μl of cell suspension was added to the appropriate wells of a 96-well flat-bottom plate (Costar). The final SYTOX Green concentration was 250 nM. α-linolenic acid (Cayman Chemical) and vehicle (ethanol) were diluted to 1 mM in 0.1 M sodium phosphate buffer pH 6.2/2% glucose and 100 μl of the appropriate mixture was added to the appropriate wells. The final fatty acid concentration was 500 μM. Candidate antioxidants, iron chelators and DMSO were diluted to 40 μM in 0.1 M sodium phosphate buffer pH 6.2/2% glucose and 100 μl of the appropriate mixture was added to the appropriate wells (final antioxidant concentration, 10 μM). Assay plates were incubated for 24 h (30°C, 160 r.p.m.) in a CytoTect cell imaging multimode reader (BioTek Instruments). At 24 h, the SYTOX Green fluorescence was measured on the CytoTect using the following settings of 488/633 nm background signal. 100 μl of sodium phosphate buffer pH 6.2/25 mM SYTOX Green (only) was subtracted from all samples, and the final percentage of cell death was determined using the 500 μM α-LA + DMSO condition set to 100% cell death.

**DPPH assay.** For the DPPH profiling experiment of the 1,833-compound library, the stable radical DPPH was dissolved in methanol (MeOH) to a concentration of 53.3 μM. Here, 60 μl of DPPH solution was added to 20 μl of diluted library compound (160 μM). The final concentrations of DPPH and test compounds were 40 μM. Samples were incubated in the dark at room temperature for 30 min. After incubation, absorbance was measured at 517 nm using a CytoTion3 multimode reader. Each plate had eight wells with DPPH and vehicle (DMSO) only and eight wells with MeOH only for background subtraction. Each plate was blank subtracted using the average MeOH signal from eight wells, and comparison to DMSO only were used to compute a percentage inhibition value for each compound. The entire experiment was performed twice on separate days. All test compounds had average normalized DPPH signals ≤150% of the negative controls and were excluded from further analysis (obatoclax mesylate, crystal violet, dolafazinamide, indirubin, vitamin B12, daunorubicin HCl, epirubicin HCl, enzastaurin, doxorubicin, iraducibin HCl, GW441756, WO and pirarubicin). Of the remaining 1,820 compounds, 5.2% (95/1818) exhibited normalized DPPH signals between 0 and 50% of the DMSO negative controls, with <20% standard deviation between the two replicates being used as a consistency filter to help select compounds for subsequent analysis.

When the DPPH assay was performed for single compound follow-up experiments, DPPH was dissolved in methanol to a final concentration of 40.2 μM. Here, 498 μl of DPPH solution was added to 2 μl of 10 mM compound dissolved in DMSO. Fifty micrograms of DPPH and DMSO were used. Samples were briefly vortexed and allowed to incubate in the dark at room temperature for 60 min. Then, 150 μl aliquots of each DPPH-test compound solution were added to three wells of 96-well clear-bottom tissue culture plates (Corning) and absorbance at 517 nm was recorded using a CytoTion3 multimode reader (BioTek). Absorbance at 517 nm was averaged across the three technical replicates, blank (methanol only) subtracted and normalized to average DPPH absorbance. The entire experiment was performed three times on separate days.

**Ferrozine–iron chelation assay.** The 1,833-member bioactive compound library was examined using the ferrozine assay as follows. First, 4 μl of each library compound (2 μM) was spotted to a final density of 1 μl of each compound and dried. Then, 2 μl of a 5% solution of ferrozine (2-methoxyestradiol, TCD, epinephrine HCl, milciclib and PYR-41) were prepared. The sublibrary of 100 compounds was tested in HT-1080 cells against three fixed concentrations of erastin: 50, 5 and 0.5 μM. Cell death was analyzed over 48 h, scanning in 2 h intervals. Lethal fraction and nAUC scores were computed for each treatment. Data represent the mean of two independent experiments.

**Analysis of cell death with 17β-estradiol competition assay.** HT-1080 cells were seeded in a clear-bottom 384-well plate at a final density of 1,500 cells per well. Cells were treated with 17β-estradiol (1 μM) + β-oxadiazene or raloxofene (both 1 μM) + 17β-estradiol (100 nM). Counts of live and dead cells were acquired using the STACK method every 2 h for 36 h.

**Lipophilicity prediction.** Compound lipophilicity prediction was predicted using ALOGPS v2.1 to predict log, and log, values (http://www.vcllab.org/lab/alogp/).

**Kinetic analysis of RTA activity.** All chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. STY-BODIPY and di-tert-undeyl hyponitrite (DTUN) were prepared as reported. Egg-phosphatidylcholine liposomes were prepared as previously reported. Ultraviolet-visible (UV-vis) light spectra and kinetics were measured on a 9400 UV-vis spectrophotometer equipped with a 2-M unit cell (Corning) and absorbance at 517 nm was recorded using a Cytation3 multimode reader. A cuvette of 2.34 ml of 10 mM phosphate buffered saline (150 mM) at pH 7.4 was added 125 μl of 20 mM stock of 100 mM unilamellar egg-phosphatidylcholine liposomes in the same buffer, and the cuvette was placed into the thermostatted sample holder of a UV-vis spectrophotometer and equilibrated to 37°C. An aliquot (12.5 μl) of a 2.0 mM solution of STY-BODIPY in DMSO was added, followed by 10 μl of a 50 mM solution of DTUN in EOH, and the solution was thoroughly mixed. The absorbance of the sample at 571 nm was monitored for around 20 min to ensure that STY-BODIPY consumption was proceeding at a constant rate, after which 10 μl of 500 mM solution of the test antioxidant was added. The solution was then thoroughly mixed and the absorbance readings resumed. The initial rate and inhibited period were then used to calculate υ and n as described.

**C. elegans whole animal cell death assay and lipid analysis.** Germ cell death in *C. elegans* was analyzed as reported. Briefly, N2 Bristol (wild-type) nematodes were maintained on nematode growth media plates seeded with bacteria (E. coli OP50) at 20°C. Experiments with DGLA were performed using nematode growth media plates formulated with 0.1% Tergitol NP40 (Sigma Chemicals) and 0.125 mM DGLA sodium salt (NuChek Prep, Inc.) or Tergitol alone (vehicle). Dry plates were seeded with OP50 and then three days later ferrostatin-1 or bazedoxifene were dissolved into the plates at 300 μM and allowed to dry for 30 min, before roughly 50 synchronized L1 larvae were transferred to each plate. Sterility was scored 72–96 h later, as determined by light microscopic examination of uterine embryos. In total, six separate populations of nematodes were scored for each condition. Fatty acids were analyzed from two independently treated worm populations using gas chromatography/mass spectrometry following direct transesterification using 2.5% H2SO4 in methanol (1 h, 70°C) to generate fatty acid methyl esters, as described.

**Analysis of cell death with mTOR inhibitors.** HT-1080, U-2 OS® and 293TN® cells were seeded in clear-bottom 12-well plates (Corning) at densities of 75,000, 50,000 and 500,000 cells per well, respectively, in 40 μl of medium. The next day, the medium was replaced with fresh medium containing SYTOX Green (final concentration, 20 nM) along with either DMSO or erastin (1 μM), INK 128 (1 μM) and AZD8055 (1 μM). Cells were then transferred into the IncuCyte Zoom enclosed within a tissue culture incubator and images were acquired using the x10 objective every 4 h for 48 h. Lethal fraction scores were computed over time as described above. In some experiments, U-2 OS® cells were cotreated with BSO (1 μM) or rapamycin (100 μM). In one experiment, ferroptosis was induced in U-2 OS® cells by placing the cells medium lacking cystine, as described.

**PI3K library mini-screen.** The effects of 86 different small-molecule inhibitors of PI3K and related pathways were examined in U-2 OS® cells. Cells were seeded in 384-well plates at a density of 1,500 cells per well in 40 μl of medium. The next day, the medium was removed and 36 μl of fresh medium containing SYTOX Green (20 nM final concentration) and the PI3K library compounds were added. The PI3K library compounds were added to final concentrations of 100, 250 and 10 mM in separate plates. Then, either immediately or following a 6 h preincubation, 4 μl of
10X erasin solution (10μM final concentration) was added to cells, and cell were imaged using an IncuCyte microscope. Images were acquired using a x10 objective lens in phase contrast and green fluorescence (excitation 460 ± 20, emission 510 ± 30 nm). Counts of response to erasin treatment alone were used to compute a normalized cell death for each condition, set equal to 1. The effect of each individual PI3K pathway inhibitor was assessed relative to this baseline, with a value of 0 being equal to no dead cells observed at 24h. The entire experiment was repeated three times on separate days and the results shown are the average of these three experiments, where each dot represents an individual compound.

shRNA analysis. To generate lentiviruses bearing shRNAs, plasmids encoding shRNA were cotransfected with the packaging plasmids pMDG/ePRES, pRSV-Rev, which was gifted from D. Trono, Addgene plasmids nos. 12251 and 12253, respectively, and pCMV-VSV-G, which was a gift from R. Weinberg, Addgene plasmid no. 8454) into HEK293T cells using PolyJet (SignaGen Laboratories, catalog no. SL100688) as per the manufacturer’s instructions. Viral supernatants were harvested and 24 and 72 h later combined, filtered through a 0.45 μm polycarbonate filter (EMD Millipore) and stored in single-use aliquots at −80 °C until use.

For functional studies, HT-1080 (immunoblot) or HT-1080IC50 (STACK viability cells) were seeded in either six-well plates (immunoblot, at 30,000 cells per well) or 12-well plates (viability, at 10,000 cells per well). The next day, medium was removed and replaced with HT-1080 medium containing 2 mM l-glutamine for 15–20 min, and the supernatant was isolated to exclude debris. Protein concentration was quantified using the Pierce Microplate BCA Assay Kit (Thermo Fisher). Lysates were centrifuged at 12,700 r.p.m. speed (15,000 g) at 4 °C for 15–20 min, the C11 BODIPY 581/591/Hoechst mixture was removed and fresh HBSS was added to cells, and incubated for 60 min at 37 °C. Cell media was then collected and added to a 96-well assay plate (Corning). For normalization purposes, cells were trypsinized and cell number was quantified using a Cellometer Auto T4 Bright Field Cell Counter. Glutamate release was detected using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Thermo Fisher) per the manufacturer’s instructions. 10 mM H2O2 and 25 μM l-glutamate were included as positive controls. Fluorescence readings were recorded at excitation/emission 530/590 on a BioTek Synergy Neo2 multimode reader. Background fluorescence from blank uptake media was subtracted and samples were normalized to cell number.

Determination of metabolite abundance by mass spectrometry. HT-1080 cells were seeded in 15 cm2 dishes and treated with 1μM erasin2 (1 μM ± INK 128 (5 μM) or rapamycin (100 μM) for 10h. Cells were trypsinized, collected and cell number was quantified using a Cellometer Auto T4 Bright Field Cell Counter. Cells pelleted at 1,500g for 5 min and pellets were frozen at −80 °C. Four biological replicates were collected and analyzed by Metabolon as described previously.

Cancer therapeutics response portal analysis. We obtained data from the Cancer Therapeutics Response Portal v.2.1 at https://cancerrxgene.org/cancertherapeuticsresponseportal/ and statistically compared conditions by performing pairwise two-sample t-tests. We used false discovery rate correction for multiple comparisons between basal gene expression and sensitivity to erasin, RSL3 (denoted 1S,3R-BSL-3 in the dataset) and MLI210 from all cancer cell lines available for analysis were extracted from the v21.data.gex_global_analysis.txt table and plotted using Prism v.7.0.

Amino acid deprivation and resupplementation. All amino acid deprivation experiments were conducted by treating cells in starvation media, resupplemented with stock solutions of the missing amino acid. DMEM minus arginine was constituted by supplementing DMEM for SILAC (Thermo Fisher), which lacks l-lysine and l-arginine, with a 1000X stock solution of L-lysine·HCl at 130 g l−1, l-lysine in stock DMEM at 114.6 g l−1, but once fully diluted with FBS and P/S, DMEM constitutes only 89% of the final volume. Thus, a 1000X stock was 130 g l−1. Similarly, DMEM minus leucine was constituted by supplementing DMEM-LM (Thermo Fisher), which lacks l-leucine and l-methionine, with 1000X stock solution of L-methionine at 26.7 g l−1, 89% of the 30 mg l−1 contained in DMEM. To minimize the contribution of monomeric amino acids contained in normal FBS, all media were supplemented with 10% dialyzed FBS (IRFBS, Thermofisher) and 1% P/S.

Small-scale cell death analysis for amino acid manipulations and small-molecule treatments. The effects of amino acid deprivation and inhibitors on cell death were investigated in several ways. For amino acid deprivation experiments, most experiments used U-2 OS cells. These cells were seeded overnight at 20,000 cells per well in 24 well plates (BD Falcon), 40,000 cells per well in 12-well plates (Corning) or 100,000 cells per well in six-well plates (Corning), in McCoy’s 5A medium. The next day, cells were washed once in prewarmed HBSS, the amino acid deprivation medium was then replaced with DMEM-base resupplementation medium, as described above, containing different treatment conditions, and incubated in a Thermofisher tissue culture incubator (37 °C, 5% CO2). For 48h. Treatment conditions were medium lacking Arg only, medium lacking leucine only, medium lacking Arg and re-supplemented with l-Arg, d-Arg or L-citrulline (all at 356 μM), medium lacking Arg re-supplemented with increasing concentrations of l-Arg to enable the calculation of the l-Arg EC50 using four-point logistic regression in Prism 7, and medium lacking Arg and supplemented with (BSO (1 mM). BSO was dissolved directly into the medium at the final concentration.

Cell death ± erasin ± Arg was examined in HT-1080, T98G, A549, Caki-1, H1929, H2951, U-2 OS and A549 cells, all seeded in 24-well plates at 20,000 cells per well, with treatments starting the next day. The experimental conditions used are given in the legend for Fig. 5c. Lethal fraction scores at 48h were computed using STACK as described above. In one experiment, A549, Caki-1, H1929, H2951 and U-2 OS cells were seeded at a density of 3,400 cells per well in 96-well plates then, the next day, cotreated with erasin2 or ML162±CHX (2.5 μM) and BSO (1 mM) and cell death determined over 72h using STACK.

Determination of GSH levels using Ellman’s reagent. Total GSH levels were analyzed using the Glutathione Assay Kit (Cayman Chemical, catalog no. 703002). U-2 OS cells were seeded at a density of 100,000 cells per well in McCoy’s 5A medium in six-well plates. Following treatment, cells were washed once in ice cold PBS. DMSO-treated cells were mixed and resuspended into 500 μl of DEAS collection buffer while Er2a-treated cells were scraped into 50 μl to concentrate depleted GSH due to low assay sensitivity. GSH measurements were normalized to protein concentration that was equivalently concentrated with this method. Cells were sonicated with ten 1-s pulses at maximum amplitude with a Fisher Scientific Model 120 Sonic Dismembrator (Thermo Fisher) to lysis. To exclude debris, lysate
was centrifuged at 12,700 r.p.m. for 15 min at 4°C and supernatant was isolated. Then, 25μl of lysate was aliquoted and stored frozen at −20°C for under 1 week before a Bradford Assay was performed to determine the protein concentration for normalization. The rest of the lysate was deproteinized by adding one volume of 0.1 g·mL⁻¹ metaphosphoric acid (Acros Organics), vortexing thoroughly, then centrifuging at 12,700 r.p.m. for 3 min at room temperature. Metabolite extract was stored at −20°C for under 1 week before total GSH and glutathione disulfide (GSSG) levels were determined using Ellman's reagent (DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid) according to the manufacturer's instructions. Data are presented as DNTB fluorescence normalized to total protein or to a known GSH standard curve.

Puromycylation assay. U-2 OS cells were seeded at a density of 100,000 cells per well in McCoy’s 5A medium in six-well plates. The next day, cells were washed once in warm HBSS, and the medium was replaced with DMEM-base resupplementation medium, as described above, with or without Arg, leucine, or INK 128 (1μM). After 16h, the treatment medium was removed and cells were pulse labeled for 15 min in the same medium containing puromycin (10μg·mL⁻¹). After 15 min, cells were collected and processed via western blot as described above. The primary antibodies were mouse anti-puromycin (EMD Millipore, dilution 1:5,000) and rabbit anti-GAPDH (dilution 1:1,000). Results were quantified and normalized to one by computing the ratio of anti-puromycin signal to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal within each treatment condition.

Real-time quantitative PCR (RT-qPCR). U-2 OS cells were seeded at a density of 100,000 cells per well in McCoy’s 5A medium in six-well plates. The next day, cells were washed once in HBSS then the medium was replaced with DMEM-base resupplementation medium ± Arg for 16h. After 16h, cells were carefully washed twice with ice cold HBSS. Cells were then gently scraped into 1 ml HBSS and centrifuged at 3,000 r.p.m. for 2 min. The supernatant was removed and RNA extracted using the QuaShredder (Qiagen) and RNeasy (Qiagen) kits according to the manufacturer’s instructions. RNA was eluted in 65μl DNase/RNase free water and stored at −80°C. Complementary DNA synthesis reaction was performed using the Taqman Reverse Transciptase Kit (Life Technologies), with 5.5 mM MgCl₂, 500μM dNTPs, 2.5μM DT oligos, 2.5μM hexamers, 0.4 units per μl RNase inhibitor and 3.125 units per μl reverse transcriptase. Then, 1 μg of RNA was used per reaction. cDNA was synthesized using a ProFlex PCR System (Applied Biosystems) thermocycler with the following program: 25°C for 10 min, 48°C for 40 min and 95°C for 5 min. cDNAs were stored at −20°C. qPCR reactions were run using SYBR Green Master Mix (Life Technologies) and run on the Applied Biosystems QuantStudio 3 real-time PCR machine (Thermo Fisher). Data were analyzed using the ΔΔCT method using ACTB as a control. Primers for qPCR were as follows: ACTB (F: CCAGCACTCGGGTTTTGAATA R: TGCCACTTGCCTGAAGTACC), TGAGATCCTGCAGCAGTGTG R: CTCCTTCAGCTTCCTGGCAA) and CTH (F: CCAGCACCCTGGAGCTTTGTA R: TGCCACCTGCCCTGAAGTACC).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Uncropped western blots are available in the Supplementary Information. Source data are provided with this paper.

Code availability
The manuscript does not report any custom code.

References
51. Dixon, S. J. et al. Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. Cell 3, e02523 (2014).
52. Tarangelo, A. et al. p53 suppresses metabolic stress-induced ferroptosis in cancer cells. Cell Rep. 22, 569–575 (2018).
53. Haidasz, E. A., Van Kessel, A. T. & Pratt, D. A. A continuous visible light spectrophotometric approach to accurately determine the reactivity of radical-trapping antioxidants. J. Org. Chem. 81, 737–744 (2016).
54. Li, B. et al. Besting vitamin E: sidechain substitution is key to the reactivity of naphthyridinol antioxidants in lipid bilayers. J. Am. Chem. Soc. 135, 1394–1405 (2013).
55. Watta, J. L. & Browse, J. Genetic dissection of polyunsaturated fatty acid synthesis in Caenorhabditis elegans. Proc. Natl Acad. Sci. USA 99, 5854–5859 (2002).
56. Cao, J. Y. et al. A genome-wide haploid genetic screen identifies regulators of glutathione abundance and ferroptosis sensitivity. Cell Rep. 26, 1544–1556.e8 (2019).

Acknowledgements
We thank J. Cao, A. Tarangelo, Z. Inde and E. Kolebrander Ho for experimental assistance, and B. Stockwell, L. Li, M. Bassik, J. Ye and M. Cyert for reagents. Certain constructs were obtained from Addgene. This work was supported by awards from the NSERC (grant no. RGPIN-06741-2016) to D.A.P., and the National Institutes of Health (grant no. ST25GM07276) to D.A.A. (grant no. 1R01GM133883) to J.L.W. and (grant nos. 8R00CA166517-03 and 1R01GM122923) to S.J.D.

Author contributions
M.C., G.C.F. and A.W. performed kinetic modulatory profiling and follow-up experiments. A.K. and L.M. performed S. cerevisiae experiments. M.A.P. performed kinetic experiments. J.L.W. performed C. elegans experiments. A.K. and L.M. performed S. cerevisiae experiments. M.A.P. performed M.P. experiments. M.M. performed liposomal experiments. C.D.P. and D.A.A. performed mTOR and amino acid deprivation experiments. J.L.W., D.A.P. and S.J.D. supervised experiments. All authors analyzed the data. M.C., C.D.P. and S.J.D. wrote the manuscript.

Competing interests
S.J.D. is a member of the scientific advisory board for Ferro Therapeutics, has consulted for Toray Industries and AbbVie Inc., and is an inventor on patents related to ferroptosis.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41589-021-00751-4.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41589-021-00751-4.
Correspondence and requests for materials should be addressed to S.J.D.

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Extended Data Fig. 1 | Examination of compound interactions on cell death. a, Chemical structures. b, Cell death determined using STACK. c, NRF1 (NFE2L1) protein levels. Blot is representative of three independent experiments. d, Cell death determined using STACK. e, Quantification of the timing of population cell death onset (DO) from the lethal fraction curves in d. f, Cell death in two different cell lines determined using STACK. Results in b, d and e are mean ± SD from three independent experiments, while in f results are from two or three independent experiments.
Extended Data Fig. 2 | Investigating ferroptosis inhibitors. a, Cell death data extracted from the compendium for erastin treatment. Each dot represents a single modulator compound, tested once at 5 µM, organized together by major target class. Lower nAUC values indicate greater death suppression. MEKi: mitogen activated protein kinase kinase 1/2 inhibitors (n = 14); AdRm: adrenergic receptor modulators (n = 55); ER/PRm: estrogen/progesterone receptor modulators (n = 29); VEGFRI: vascular endothelial growth factor receptor inhibitors (n = 20); K⁺ Cm: potassium channel modulators (n = 19). The vertical dotted line indicates the mean lethality of the control erastin + DMSO conditions. b, Cell death determined by counting SYTOX Green positive (SG⁺) objects. The experiment was performed twice on different days and data represents mean ± SD.
Extended Data Fig. 3 | Cell free RTA and iron chelator profiling.  

**a.** Overview of cell free compound profiling for radical trapping and Fe$^{3+}$-binding activity.  

**b.** Cell death at 48 h in HT-1080$^*$ cells treated with erastin2 (1µM) and candidate radical trapping compounds (50µM, n = 100) plotted against predicted hydrophilicity (LogS), predicted lipophilicity (LogP) and the %DPPH inhibition values from the cell-free assay. Dotted lines indicate a lethal fraction of 0.2. Spearman correlation values are reported with the 95% confidence interval (C.I.). Exact P values (two-tailed) are reported where computable.
Extended Data Fig. 4 | Bazedoxifene suppresses ferroptosis in mammalian cells.  

**a**, Cell death quantified as the number of SYTOX Green positive (SG+) objects (that is dead cells) over time. Data are from two independent experiments. **b,c**, Cell death quantified by SG+ object counting. Data are from three or four independent experiments. **d**, Outline of the STY-BODIPY kinetic competition assay. Egg-phosphatidylcholine (1 mM) and STY-BODIPY (10 µM) are incubated with 0.2 mM di-tert-undecyl hyponitrite (DTUN), in addition to a radical trapping antioxidant (RTA-H).
Extended Data Fig. 5 | Bazedoxifene prevents ferroptosis in *C. elegans*. **a**, Representative images of DAPI-stained adult *C. elegans* under the different treatment conditions indicated below each image. The gonads of fertile worms are indicated (arrows). DGLA: dihomo-γ-linolenic acid (125 µM); Baz: bazedoxifene (150 µM). Scale bar = 100 µm. Imaging was repeated twice and representative animals from one experiment are shown. **b**, Polyunsaturated fatty acid levels as a function of total lipids determined in worms using gas chromatography/mass spectrometry. Results are from two independent experiments on separate populations of worms.
Extended Data Fig. 6 | mTOR regulates ferroptosis sensitivity. a, Cell death over time determined using STACK. Cells were infected with control (scrambled) shRNA or shRNAs targeting RPTOR or RICTOR for 72 h prior to compound treatment. INK128 was used as a positive control. Results are mean ± SD from three independent experiments. b, Expression and phosphorylation of proteins in the mTOR pathway following infection of HT-1080 cells as in a. Blot is representative of three independent experiments.
Extended Data Fig. 7 | mTOR inhibitors suppress ferroptosis. a, Cell death determined using STACK in three different cell lines. Results are mean ± SD from three independent experiments. b, 4E-BP1 protein phosphorylation and total levels. Blot is representative of three independent experiments. c, Total glutathione (GSH + GSSG) levels measured using Ellman’s reagent (DNTB). d, System x vít activity inferred from glutamate release over 2 h from HT-1080 and U-2 OS cells treated as indicated. e, Cell death determined using STACK. Results in c–e are from three independent experiments.
Extended Data Fig. 8 | mTOR and protein synthesis regulate ferroptosis. a, Phosphorylation and levels of mTOR pathway effectors in U-2 OS cells. Blot is representative of three independent experiments.

b, Analysis of Cancer Therapeutics Response Portal (CTRP) dataset for ferroptosis-inducing compounds. CTRP.

c, Cell death determined using STACK. Erastin2 was used at 2 µM in all cell lines except Caki-1 (1 µM), ML162 was used at 4 µM in all cell lines except Caki-1 (2 µM). CHX: cycloheximide, BSO: buthionine sulfoximine. Results are mean ± SD from three independent experiments.
Extended Data Fig. 9 | a, Phosphorylation and levels of mTOR pathway effectors. Blot is representative of two independent experiments. b, Fold-change in amino acids levels in HT-1080 cells determined using liquid chromatography coupled to mass spectrometry.
Extended Data Fig. 10 | Arginine uptake regulates ferroptosis. **a**, Dose-dependent effect of arginine (Arg) resupplementation on erastin2-induced cell death determined using STACK. Erastin2 was used at 2 µM (U-2 OS)* or 4 µM (A549*). **b**, Cell death as determined using STACK in cells grown in complete medium (CM), switched to -Arg medium at the time of compound addition, or 24 h before compound addition. **c**, Detection of puromycylated peptides. Results are representative of three independent experiments. **d**, Quantification of results from three puromycylation experiments, as in **c**. **e**, SYTOX Green positive (SG+) dead cell counts normalized to initial cell confluence. **f**, Confirmation of ATF4 knockdown at the protein level. Blot is representative of two independent experiments. Results in **a**, **b** and **e** represent mean ± SD from three independent experiments.
Reporting Summary

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Give P values as exact values whenever suitable.

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☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

For cell death imaging analysis, automated object detection was performed in parallel to data acquisition using the Zoom software package (V2016A/B) using a routine with parameter values as described in the methods. No custom code was used.

Data analysis

Cell death data was analyzed using Microsoft Excel v16.4 and GraphPad Prism 8.4 using default functions, with analyses performed as described in the Online Methods. Data was clustered using Morpheus (no version number available; https://software.broadinstitute.org/morpheus/) with default settings as described in the Online Methods. Chemical properties were evaluated using ALGOPS (v2.1, http://www.vcclab.org/lab/algos/) No custom data analysis software was used.

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Z-scores for the kinetic modulatory profile reported in Fig. 1b, and % signal suppression for the cell free DPPH and ferrozine analyses reported in Extended Data Fig. 4a are available as Source Data.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Large-scale chemical screening was performed once, as in Shimada et al., (2016) Nat Chem Biol; all follow up biological studies were performed two to four times on different days as in Dixon et al., (2012) Cell. |
|-------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | A small number of datapoints in the modulatory profile were excluded, as described fully in the Methods, due to compound autofluorescence or due to cell seeding anomalies, which interfere with the analysis of cell death using the STACK method. |
| Replication | Given the nature of resources required to construct the compendium (e.g. limited supplies of the 1,833 member library), large-scale chemical screening was performed once, as in Shimada et al., (2016) Nat Chem Biol, and other similar large-scale screening studies. However, our chemical library typically contained >1 structurally-related compound for a given target (e.g. proteosone inhibitors) allowing us to compare the effects of independent compounds in the same experiment. As shown, these independent compounds with shared targets typically yielded similar results. Key compounds from the primary chemical screen were subsequently functionally validated in different cell-based and cell-free assays, providing independent validation. All follow up chemical biological studies were performed two to four times on different days, as in Dixon et al., (2012) Cell and the vast majority of current studies. |
| Randomization | For all cell-based studies, cells were randomly assigned to different treatment conditions. For animal studies, animals were randomly assigned to treatment and control groups. |
| Blinding | For the modulatory profiling, experimenters were blinded to the contents of the 1,833 library during data collection. Data collection was fully automated. The individuals responsible for data collection were not responsible for the subsequent data analysis. For follow-up chemical and cell-based studies investigators were not blinded to the experimental designs as this is impossible. However, subsequent data analysis was typically conducted from a different investigator than who collected that data. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
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| • Antibodies | • ChiP-seq |
| • Eukaryotic cell lines | • Flow cytometry |
| • Palaeontology and archaeology | • MRI-based neuroimaging |
| • Animals and other organisms | • Dual use research of concern |
| • Human research participants | • Clinical data |
| • Clinical data | • Dual use research of concern |

Antibodies

All antibodies were obtained from reputable commercial suppliers, including: diphosphorylated (Thr202/Tyr204) ERK1/2 (p42/44 MAPK, Cat. No. 4372), total ERK1/2 (Cat. No. 4695), rabbit anti-RPS6 (56, Cat. No. 22175), rabbit anti-phospho-RPS6 Ser235/236 (Cat. No. 4858), rabbit anti-4E-BP1 (Cat. No. 9644S), rabbit anti-phospho-4E-BP1 Thr37/46 (Cat. No. 9459S), rabbit anti-ATF4 (Cat. No. 11815S), rabbit anti-phospho-Akt Ser473 (Cat. No. 9271), rabbit anti-Akt (Cat. No. 9272), rabbit anti-Nrf1/NFE2L1 (Clone D5810, Cat. No. 8052) and rabbit anti-GAPDH (Cat. No. 21185) were from Cell Signaling Technologies, IRDye 680RD Donkey anti-Mouse IgG (Cat. No. 926-68072), IRDye 680RD Donkey anti-Goat (Cat. No. 926-68074), and IRDye 800 Donkey anti-Rabbit (Cat. No. 926-32213) were from LI-COR Biosciences (Lincoln, NE, USA), anti-pirolympcin (Clone 12010, Cat No. MA8F343) was from EMD Millipore, goat anti-actin (I-19, sc-1616) was from Santa Cruz, and mouse anti-tubulin (M5S81P, 1:10,000) from Fisher Scientific.

Validation

All antibodies used in this study were validated previously by the commercial supplier and/or in our own hands. For example, we show that the mTOR-specific inhibitor INK128 reduces the phosphorylation of the mTOR substrate 4E-BP1 in cancer cells, as reported by Hayman et al. (2014) Clinical Cancer Research. We show that phosphorylation of RPS6 is reduced in 293T cells by arginine deprivation as reported by Chantarupong et al. (2016) Cell. We confirm the specificity of the ATF4 antibody by showing that
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  
HT-1080 [CCL-121], U-2 OS [HTB-96], MDA-MB-231 [HTB-26], HEK293T [CRL-3216, hereafter 293T], NCI-H1299 [CRL-S803, hereafter 1299], A549 [CCL-185], 7986 [CRL-1697], Caki-1 [HTB-46], and A375 [CRL-1619] were obtained from ATCC (Manassas, VA, USA). Mouse 4T1 and E0771 triple negative breast cancer cells were the kind gift of Lingyin Li (Stanford, Dept. of Biochemistry). 4T1 and E0771 cells were from ATCC and CH3 Biosystems, respectively, obtained via the laboratory of Dr. Lingyin Li (Stanford, Dept. of Biochemistry). HT-1080 cells stably expressing a non-targeting shRNA (sh-NT) and an shRNA targeting ATF4 were the kind gift of Jiabin Ye (Stanford, Dept. of Radiation Oncology). H23 cells stably expressing Cas9 (H23Cas9) were the kind gift of Michael Bassik (Stanford, Dept. of Genetics). The polyclonal nuclear mKate2-expressing (denoted by superscript ‘N’) cell lines HT-1080N, U-2 OSN, 293TN and H1299N were described previously. Polyclonal populations of Caki-1N, A375N and H23Cas9N cells were generated from the respective parental cells via transduction with the NucLight Red lentivirus, which directs the expression of nuclear-localized mKate2.

Authentication  
All human cell lines used for this research were acquired from a trusted source, American Type Culture Collection (ATCC), and were used in culture for one passage and then frozen down immediately in small aliquots. These cell lines display morphological features, growth characteristics and phenotypic responses in culture that are consistent with the original description of these lines, which were validated to be correct when shipped from the original supplier. These cell lines were not further validated before use. Low passage mouse 4T1 and E0771 were originally obtained directly from reputable commercial sources (ATCC and CH3 Biosystems) and not further validated. The phenotype of HT-1080 cells stably expressing a non-targeting shRNA (sh-NT) and an shRNA targeting ATF4 was was originally reported in Ye et al., (2010) EMBO and validated in our experiments. H23 cells stably expressing Cas9 (H23Cas9) were generated in and the kind gift of Michael Bassik (Stanford, Dept. of Genetics; Reference: Kelly et al., (2020) Cancer Discovery). Experiments reported here use freshly-thawed aliquots of cells, passed in culture less than thirty times.

Mycoplasma contamination  
Mycoplasma-contaminated cell lines exhibit a diffuse intracellular green fluorescent staining when exposed to SYTOX Green. As the majority of our experiments employ SYTOX Green to detect cell death, we have included these experiments in our validation for mycoplasma contamination. Indeed, our cell death counting method would not be able to distinguish mycoplasma-infected cells due to this background. Thus, in each experiment, we confirm that all tested cultures are mycoplasma-free. In some cases, we have also independently confirmed the absence of contamination with mycoplasma in our cultures using a PCR-based assay.

Commonly misidentified lines  
No commonly mis-identified cell lines were used in the study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  
C. elegans nematode worms, strain N2.

Wild animals  
N/A

Field-collected samples  
N/A

Ethics oversight  
No ethical approval was required for C. elegans studies.

Note that full information on the approval of the study protocol must also be provided in the manuscript.