MESH1 is a cytosolic NADPH phosphatase that regulates ferroptosis

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Critical to the bacterial stringent response is the rapid relocation of resources from proliferation toward stress survival through the respective accumulation and degradation of (p)ppGpp by RelA and SpoT homologues. While mammalian genomes encode MESH1, a homologue of the bacterial (p)ppGpp hydrolase SpoT, neither (p)ppGpp nor its synthetase has been identified in mammalian cells. Here, we show that human MESH1 is an efficient cytosolic NADPH phosphatase that facilitates ferroptosis. Visualization of the MESH1–NADPH crystal structure revealed a bona fide affinity for the NADPH substrate. Ferroptosis-inducing erastin or cystine deprivation elevates MESH1, whose overexpression depletes NADPH and sensitizes cells to ferroptosis, whereas MESH1 depletion promotes ferroptosis survival by sustaining the levels of NADPH and GSH and by reducing lipid peroxidation. The ferroptotic protection by MESH1 depletion is ablated by suppression of the cytosolic NAD(H) kinase, NADK, but not its mitochondrial counterpart NADK2. Collectively, these data shed light on the importance of cytosolic NADPH levels and their regulation under ferroptosis-inducing conditions in mammalian cells.

Stringent response is the main strategy used by bacteria to cope with fluctuating nutrient supplies and metabolic and oxidative stresses1-3. This process rapidly redirects energy from cell proliferation toward stress survival by reduction of biosynthesis, conservation of ATP and blockage of GTP production4. The stringent response is triggered by the accumulation of the bacterial ‘alarmone’ (p)ppGpp (guanosine tetra- or penta-phosphate, shortened as ppGpp below) through the regulation of ppGpp synthetases and hydrolases in the RelA and SpoT homologue family5. Recent studies suggest that the stringent response may also function in metazoans, as metazoan genomes encode a homologue of bacterial SpoT—MESH1 (Metazoan SpoT Homologue 1, encoded by HDDC3)—that can hydrolyse ppGpp in vitro and functionally complement SpoT in Escherichia coli6. Furthermore, Mesh1 deletion in Drosophila displays impaired starvation resistance and extensive transcriptional reprogramming7. Despite these supporting lines of evidence, neither ppGpp nor its synthetase has been discovered in mammalian cells and examined metabolites with a MESH1 may function through alternate metabolic substrate(s) of MESH1 in mammalian cells. Here, we show that human MESH1 is an efficient cytosolic NADPH phosphatase that facilitates ferroptosis.

Results

MESH1 is an efficient NADPH phosphatase. We reasoned that MESH1 may function through alternate metabolic substrate(s) from ppGpp in mammalian cells and examined metabolites with a similar molecular architecture to ppGpp. The metabolite NADPH shares many similarities with ppGpp, including a purine nucleoside, a 2′-phosphate group and a 5′-pyrophosphate group (Fig. 1a). Although NADPH differs from ppGpp in that it contains a 2′-phosphate instead of a 3′-pyrophosphate in ppGpp, the crystal structure of the bifunctional RelA/SpoT homologue from Streptococcus dysgalactiae subsp. equisimilis captured an unusual ppGpp derivative, GDP-2′,3′-cyclic monophosphate, in the active site of the hydrolase domain8, suggesting that the enzyme may accommodate a 2′-substituted phosphate group9. Based on the observation that SpoT catalyses the hydrolysis of the 3′-pyrophosphate group of ppGpp, we predicted that MESH1 would similarly hydrolyse the 2′-phosphate group of NADPH to yield NADH and an inorganic phosphate (Fig. 1a).

Indeed, treatment of NADPH with hMESH1 readily released inorganic phosphate, yielding a green solution in the malachite green assay (Fig. 1b). Importantly, the phosphate accumulation was linear over time (Fig. 1c), reflecting continuous enzymatic turnover of NADPH by hMESH1. Furthermore, mass spectrometry analysis revealed a peak at m/z 664.141 as [M-H]-, verifying the product as NADH (Fig. 1d). We then analysed the steady state kinetics parameters of hMESH1 toward NADPH (Fig. 1e). hMESH1 is an efficient NADPH phosphatase, with a catalytic efficiency (kcat/KM) of 14.4 ± 1.1 s−1M−1; its Michaelis constant (KM) value of 0.12 ± 0.01 mM is on par with that from other reported cellular enzymes utilizing NADPH (for example, Km value of 0.11 mM for the human phagocytic NADPH oxidase10), supporting its role as a physiologically relevant NADPH phosphatase in cells. While hMESH1 also displays measurable activity towards NADP+ in vitro, it is approximately tenfold less efficient (kcat/KM = 1.4 ± 0.1 s−1M−1), and its Km value of 0.43 ± 0.04 mM is higher than the estimated cytosolic concentration of NADP+, rendering hMESH1 an ineffective phosphatase for NADP+ in cells (Fig. 1e).
Previous biochemical and structural analysis has revealed MESH1 as a Mn$^{2+}$-dependent enzyme\textsuperscript{4}. Accordingly, we found that the NADPH phosphatase activity of hMESH1 was reduced by $>10$-fold when Mn$^{2+}$ was substituted with other metal ions, such as Zn$^{2+}$ (Fig. 1f). Likewise, mutations of hMESH1 residues near the Mn$^{2+}$ ion on the active site, including E65A and D66K, severely compromised the NADPH phosphatase activity by $>10$-fold in enzymatic assays (Fig. 1f).

To determine whether MESH1 is a significant contributor to the cellular NADPH phosphatase activity, we measured the enzymatic activity in cell lysates extracted from two human cell lines, RCC4 cells (a human clear cell renal carcinoma cell line) and HEK-293T cells (a human embryonic kidney cell line). We found that lysates from MESH1-silenced cells had a noticeable decrease of the NADPH phosphatase activity (Fig. 1g and Extended Data Fig. 1a). Conversely, overexpression of wild-type (WT) hMESH1, but not the catalytically deficient E65A hMESH1 mutant, markedly enhanced the cellular NADPH phosphatase activity (Extended Data Fig. 1b). We also investigated the primary subcellular compartment where MESH1 functions. Cellular fractionation experiments\textsuperscript{8} indicated that MESH1 was predominately enriched in the cytosolic, but not the mitochondrial or nuclear, pools (Fig. 1h). Taken together, these observations verify hMESH1 as a significant contributor to the cytosolic NADPH phosphatase activity in human cells. It is worth noting that the short interfering RNA (siRNA) of MESH1 did not suppress NADPH phosphatase activity completely, indicating the possible existence of another NADPH phosphatase or nonspecific phosphatase activity released during sample processing that also contributed to the NADPH phosphatase activity in mammalian cells. One possible candidate is the NADP(H) phosphatase activity previously observed in rat liver lysates\textsuperscript{30}, though the reported enzymatic activity favours NADP$^+$ and the identity of the enzyme has remained unknown in animal cells\textsuperscript{31}. Another contributor could be an unrelated mitochondrial NADPH phosphatase, Curl\textsuperscript{12}, reported after the release of our discovery at bioRxiv\textsuperscript{13}. Therefore, to the best of our knowledge, the cytosolic NADPH phosphatase activity of MESH1 is distinct from the previously observed activity and MESH1 represents a new instance of a cytosolic NADPH phosphatase in human cells.

**Molecular recognition of NADPH by hMESH1.** To visualize the molecular details of the NADPH recognition, we determined the
The cocrystal structure of the specific hMESH1–NADPH complex by comparing it with the catalytically compromised D66K mutant enzyme and by substituting the catalytic Mn2+ ion with Zn2+. The structure was refined to 2.1-Å resolution (Supplementary Table 1). The crystallographic asymmetric unit consists of two protomers of hMESH1 (Extended Data Fig. 2a), each adopting a compact fold of ten α-helices and a short β-hairpin (Fig. 2a) previously observed in the structure of apo hMESH1 (ref. 4). Although the active sites of both protomers were occupied by the adenosine portion of the substrate (Extended Data Fig. 2b), the molecular recognition of the entire NADPH molecule, including that of the nicotinamide moiety, was only visible in one of the two protomers (Extended Data Fig. 2b), which is described.

The catalytic site of hMESH1 is formed at the centre of the helical architecture, surrounded by the short β-hairpin (β1 and β2), α2, the α3–α4 loop, α4, α7, α8, the α8–α9 loop and α9 (Fig. 2a). Anomalous scattering revealed the presence of a single Zn2+ ion in the active site, which substitutes the catalytic Mn2+ ion reported in the apo hMESH1 structure (Extended Data Fig. 2c). The Zn2+ ion is hexa-coordinated in a distorted octahedral geometry by H35 of α2, H61 and D62—the signature HD motif of the α2–α3 loop that defines this family of enzymes, D122 of α8, the catalytic water molecule and the 2′-phosphate group of NADPH (Fig. 2b).

In addition to coordinating the active site metal ion, the 2′-phosphate group of NADPH forms additional polar interactions with Y32 of α2, K66 of α4 and N126 of α8 that are located one layer above the equatorial plane of the zinc ligands (Fig. 2c,d). K66, the mutated residue substituting D66 in the WT enzyme, not only significantly diminishes the catalytic activity, but also forms a direct salt bridge with the 2′-phosphate group of NADPH, a likely contributor to the successful capture of NADPH in the cocrystal structure. Another catalytically important residue, E65 of α4, the neighbouring residue of D66, is located over 4 Å away from the catalytic water molecule, and its indirect interactions with the catalytic water molecule and the 2′-phosphate of NADPH are bridged by a second water molecule in the active site (Fig. 2c,d).

The NADPH adenosine ribose group and its adjacent 5′-pyrophosphate group are extensively recognized (Fig. 2c,d). The 3′-hydroxyl group and the 4′-oxygen atom form hydrogen bonds with Y146 of α9 and K25 of β1, respectively. The 5′-diphosphate group adopts a tight turn, aided by salt bridges with K97 of α7, K25 of β1 and a sodium ion. While sodium is an unusual cation to coordinate the NADPH diphosphate, our purification procedure and crystallization condition lack divalent cations other than Zn2+ but contain over 600 mM sodium chloride. Given that this density lacks anomalous Zn2+ signals (Extended Data Fig. 2b), it is interpreted as a sodium ion, though Mg2+ or Ca2+ might be more suitable ions in vivo. The adenine moiety of NADPH is largely coordinated by π-stacking with R24 of β1 and a direct hydrogen bond between its N7 atom and the amide group of K25 of the α1 loop (Fig. 2c,d). As both of these interactions are also found in a guanine base, hMESH1 lacks the ability to differentiate the adenine nucleotide from the guanine nucleotide as was found in ppGpp.

Fig. 2 | Structure of the hMESH1 (D66K)–NADPH complex. a, The architecture of the hMESH1 active site. MESH1 is shown in the ribbon diagram, NADPH in the stick model and the Zn2+ (grey) and Na+ (pink) ions are shown as spheres. Secondary structures are labelled, with MESH1–NADPH-interacting motifs annotated in magenta. b, Coordination of the active site Zn2+ ion in a distorted octahedral geometry. NADPH and side chains of the Zn2+-binding residues are shown in the stick model. The zinc ion (grey) and its coordinating water molecule (red) are shown as spheres. The signature HD motif is annotated in blue. c, The schematic illustration of the NADPH recognition by MESH1. Polar interactions are denoted with dashed lines, and van der Waals residues are shown in the stick model. The zinc ion (grey) and its coordinating water molecule (red) are shown as spheres. The signature hD motif is located in the equatorial plane of the zinc ligands (Fig. 2c,d). K66, the mutated residue substituting D66 in the WT enzyme, not only significantly diminishes the catalytic activity, but also forms a direct salt bridge with the 2′-phosphate group of NADPH, a likely contributor to the successful capture of NADPH in the cocrystal structure. Another catalytically important residue, E65 of α4, the neighbouring residue of D66, is located over 4 Å away from the catalytic water molecule, and its indirect interactions with the catalytic water molecule and the 2′-phosphate of NADPH are bridged by a second water molecule in the active site (Fig. 2c,d).
MESH1 regulates cellular NADPH levels and ferroptosis. After establishing hMESH1 as a significant contributor to the cellular NADPH phosphatase activity and elucidating its molecular recognition of NADPH, we investigated whether the NADPH phosphatase activity of MESH1 plays a role in ferroptosis, a regulated form of cell death driven by the lethal accumulation of lipid hydroperoxides. Various genetic determinants of ferroptosis have been identified. Importantly, ferroptosis can be triggered by erastin, an inhibitor of the cystine importer xCT, or by cystine deprivation, and is alleviated by high concentrations of NADPH. Accumulation of NADPH promotes the glutathione-reductase-catalysed regeneration of reduced glutathione (GSH), which serves as the reductive cofactor for glutathione peroxidase 4 (GPX4) to detoxify lipid hydroperoxides, thus protecting cells from ferroptotic cell death (Fig. 3a). We found that MESH1 was significantly upregulated in RCC4 cells at both messenger RNA and protein levels by erastin treatment or cystine deprivation (Fig. 3b). The percentage of cells showing increased fluorescence (indicated by the bar) for each treatment are labelled. The time course of cell death in RCC4 cells transfected with NT siRNA or two distinct MESH1-targeting siRNAs under erastin (0.625 M) treatment for up to 1 week. **P < 0.01; ***P < 0.005. Source data are provided in the Source Data File. RFU, relative fluorescence units.

The ribose ring of the nicotinamide riboside is indirectly recognized by a water-mediated hydrogen bond of its ring oxygen atom with R142 of o9, whereas the nicotinamide moiety is supported by a π-stacking network with W138 and R142 that emanate from the α8–α9 loop and from o9 (Fig. 2c,e). The reduced electron density of the nicotinamide ring in NADP⁺ would weaken such interaction and likely contribute to the reduced substrate affinity in comparison with NADPH. Finally, the terminal amide group is additionally but likely contribute to the reduced substrate affinity in comparison with NADPH. Finally, the terminal amide group is additionally but likely contribute to the reduced substrate affinity in comparison with NADPH. Finally, the terminal amide group is additionally but likely contribute to the reduced substrate affinity in comparison with NADPH.
deprivation (Fig. 3b and Extended Data Fig. 3a,b), implicating a functional role of MESH1 in these ferroptosis-inducing conditions. Overexpression of WT hMESH1 (MESH1-WT), but not the catalytically deficient mutant (MESH1-E65A), significantly lowered the intracellular NADP(H) level (Extended Data Fig. 3c) and sensitized cells to ferroptosis upon erasin treatment (Extended Data Fig. 3d). Taken together, these observations implicate a direct role of MESH1 in the execution of ferroptosis through degradation of NADPH.

To further assess the MESH1 function in ferroptosis, we investigated whether depletion of MESH1 and its associated NADPH phosphatase activity could mitigate lipid peroxidation and ferroptosis through the accumulation of NADPH and alteration of the glutathione redox potential. Ersarin treatment dramatically reduced the level of NADP(H) (Fig. 3c) and increased degree of lipid peroxidation (Fig. 3d) in control (nontargeting siRNA, siNT) cells. Importantly, MESH1 silencing sustained a significantly higher level of NADP(H) under ferroptosis-inducing conditions (Fig. 3c and Extended Data Fig. 3e), and markedly reduced the level of lipid peroxidation (Fig. 3d) under erasin. In addition, MESH1 silencing robustly protected cells from erasin-induced death over a broad range of erasin concentrations (Extended Data Fig. 3f) when monitored for over a 7-d period using CellTox-Green assay, which measures cell death by DNA staining (Fig. 3e). The ferroptosis protection of MESH1 silencing was further validated by multiple additional types of viability and death assays (Extended Data Fig. 4a–c). Such a ferroptosis-rescuing effect appeared to be general, as MESH1 depletion similarly promoted resistance to erasin-triggered ferroptosis in multiple cell lines, including HEK-293T, H1975, MDA-MB-231, PC3, HT1080, A673, PANCl and 786-O (Extended Data Fig. 5a–h).

After confirming the general role of MESH1 in ferroptosis regulation, we investigated at which signalling steps MESH1 functions during ferroptosis. We found that MESH1 depletion similarly conferred resistance to ferroptosis induced by cystine deprivation and sulfasalazine, another xCT inhibitor (Extended Data Fig. 3f,g), which trigger ferroptosis by depleting intracellular GSH. In comparison, inhibition of GPX4 downstream of NADPH and GSH also triggers ferroptosis (Fig. 3a), but MESH1 depletion could not rescue ferroptosis induced by different GPX4 inhibitors, such as ML-162 or RSL3 (Fig. 3h,i). These observations indicate that MESH1 depletion functions upstream of GPX4 and rescues ferroptosis by NADPH-driven repletion of GSH (Fig. 3a). Consistent with this analysis, although no statistically significant effects on the levels of intracellular cysteine or oxidized glutathione (GSSG) were observed in MESH1-depletion cells, (Extended Data Fig. 6a,b), we observed a significant increase of the GSH level and the GSH/GSSG ratio (Extended Data Fig. 6c,d).

NADPH phosphatase activity of MESH1 contributes to ferroptosis. Next, we investigated the specific roles of the enzymatic activities of MESH1 in the ferroptosis-related phenotypes. We found that the resistance to erasin-induced ferroptosis is directly correlated with the loss of the catalytic activity of MESH1, as the ferroptotic survival phenotype in MESH1-depletion cells was abolished when WT MESH1, but not the catalytically deficient E65A mutant of MESH1, was expressed (Fig. 4a).

Besides NADPH, the bacterial ppGpp is also a previously identified in vitro substrate of MESH1 (ref. 1), which has been suggested as a signalling molecule despite the lack of evidence of its presence in mammalian cells. To verify that the enhanced survival under
ferroptosis-inducing erasin treatment is dependent on a higher sustained level of NADPH, but not due to accumulation of any other in vitro substrate(s) of hMESH1, such as ppGpp, we tested whether the survival advantage of MESH1 depletion is reversed by simultaneous depletion of NAD(H) kinases that convert NAD(H) to NADPH (H). Human cells have two NAD kinases, NADK and NADK2, that are predominantly located in the cytosol and mitochondria\(^{23,24}\), respectively (Fig. 3a). As the cytosolic and mitochondrial pools of NADP(H) and NAD(H) are compartmentalized in mammalian cells due to the impermeability of these molecules across the mitochondrial membranes\(^{25}\), we reasoned that removal of the NAD kinase either from the cytosol (NADK) or mitochondria (NADK2) would reduce the distinct pools of NADP(H) and only the cytosolic NADK depletion would compromise the ferroptosis survival phenotypes of MESH1–silenced cells. Indeed, when the gene encoding the cytosolic enzyme NADK was silenced, the survival benefit of silencing MESH1 was largely eliminated (Fig. 4b and Extended Data Fig. 7). In contrast, silencing the gene encoding the mitochondrial enzyme NADK2 did not affect the MESH1–depletion-mediated ferroptosis survival (Fig. 4b and Extended Data Fig. 7). Consistent with this notion, we found that the cellular NADP(H) was significantly diminished when NADK was silenced, whereas silencing of NADK2 had little effect on cellular NADP(H) (Fig. 4c,d), suggesting a limited role of NADK2 in this process. Importantly, the mRNA expression level of MESH1, as measured by the normalized reads in the RNA sequencing, was comparable (within 0.5–4-fold) to that of other genes in the NADPH-glutathione pathway (Fig. 3a), including NADK, NADK2, NNT (NAD(P) transhydrogenase, mitochondrial), G6PD (glucose-6-phosphate dehydrogenase), PGD (phosphogluconate dehydrogenase), GSR (glutathione reductase) and NOX1 (NADPH oxidase 1) (Extended Data Fig. 8), supporting a functional role of MESH1 in mediating the essential cytosolic metabolite NADPH.

Taken together, our results establish MESH1 as a cytosolic NADPH phosphatase that is induced by ferroptosis-inducing conditions and is involved in the execution of ferroptosis by degrading NADPH. Conversely, depletion of MESH1 and its NADPH phosphatase activity under ferroptosis-inducing conditions promotes stress survival by preserving NADPH, elevating GSH and mitigating lipid peroxidation.

Discussion

MESH1, the metazoan homologue of the bacterial ppGpp hydrolase SpoT, has been implicated in the cellular response to metabolic starvation in Drosophila, though the functional role and pertinent mammalian cell substrate of MESH1 have not been elucidated\(^{14}\). The discovery of MESH1 as a cytosolic NADP phosphatase that contributes to the execution of ferroptosis through degradation of the central metabolite NADPH directly addresses this mystery and implicates a physiological role of MESH1 in disposing of cells damaged by metabolic and redox disorders.

Ferroptosis is a distinct form of iron-dependent cell death\(^{12}\) that is triggered by oxidative stresses and characterized by the accumulation of lipid peroxidation products\(^{14}\). Even with the recent discovery of many genetic determinants\(^{18-23}\) of ferroptosis by regulating lipid metabolism, iron availability, reactive oxygen species (ROS) and anti-oxidant capacity, much still remains unknown about the molecular mechanisms regulating ferroptosis. Our discovery of MESH1 as an NADP phosphatase reveals an important role of the MESH1–mediated NADPH depletion and impairment of the glutathione regeneration in the execution of ferroptosis. Ferroptosis also has emerging roles in other pathophysiological processes and human diseases, such as neurotoxicity\(^{25}\), acute renal failure\(^{26}\), hepatic\(^{27}\) and cardiac\(^{28}\) injuries, and ischaemia-reperfusion injury\(^{29}\). Therefore, modulating ferroptosis may have therapeutic potentials, and the newly discovered MESH1–NADPH phosphatase activity and our structural elucidation of the hMESH1–NADPH may be harnessed for developing potential therapeutics for the treatment of these devastating diseases involving dysregulated ferroptosis.

Methods

Purification of recombinant hMESH1. The gene encoding hMESH1 was codon optimized for E. coli expression, synthesized and cloned into a modified pET26 vector as a C-terminal fusion to the His\(_6\)-tagged StrepII. Cultures of transformed E. coli strain BL21 (DE3) were grown to an optical density at 600 nm between 0.4 and 0.5 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 ºC for 2 h. Following cell lysis, the target protein was purified using Ni\(^{2+}\)-nitrilotriacetic acid affinity chromatography following standard protocols (Qiagen). The SUMO tag was cleaved using the SENP1 protease, and potential impurities were removed by a second round of Ni\(^{2+}\)-nitrilotriacetic acid chromatography. The target protein was further purified using size-exclusion chromatography (Superdex 75, GE Life Sciences) in a buffer containing 50 mM Tris, pH 8.0, 200 mM NaCl and 0.1% 2-mercaptoethanol. Mutants of hMESH1 were generated using the QuikChange site-directed mutagenesis kit (Agilent) and prepared using the same procedure.

Mass spectrometry. Reversed-phase liquid chromatography-electrospray ionization tandem mass spectrometry for NADPH and NADP was performed using a Shimadzu liquid chromatography system (comprising a solvent degasser, two LC-10A pumps and an SCL-10A system controller) coupled to a high-resolution TripleTOF5600 mass spectrometer (Sciex). Liquid chromatography was operated at a flow rate of 200 μl/min\(^{-1}\) with a linear gradient as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 5 min, and held at 100% B for 2 min. Mobile phase A was a mixture of water/acetonitrile (98:2, v/v) containing 0.1% acetic acid. Mobile phase B was a mixture of water/acetonitrile (10:90, v/v) containing 0.1% acetic acid. The column (150 mm × 2.1 mm) was a Kinetex ECLIPSE C18 reversed-phase column (5 μm; 2.1 mm × 50 mm) obtained from Agilent. The liquid chromatography eluent was introduced into the electrospray ionization (ESI) source of the mass spectrometer. Instrument settings for negative ion electrospray ionization/mass spectrometry and tandem mass spectrometry analysis of lipid species were as follows: ion spray voltage = −4,500 V; curtain gas = 20 pounds per square inch (psi); ion source gas 1 = 20 psi; de-clustering potential = −53 V; focusing potential = −150 V. Data acquisition and analysis were performed using the Analyst TF1.5 software (Sciex).

The sample preparation process for quantification of cysteine, GSH and GSSG was carried out using an automated MicroLab STAR system (Hamilton). After removal of the protein fraction, the extract was divided into two fractions: one for analysis by liquid chromatography (GSSH, GSSG) and one for analysis by gas chromatography (cysteine). The liquid chromatography/mass spectrometry portion of the platform is based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consists of an ESI source and linear ion-trap mass analyser. The sample extract was analysed using acidic positive ion optimized conditions. Extracts reconstituted in acidic conditions were analysed by electrospraying using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The mass spectrometry analysis alternated between mass spectrometry and data-dependent tandem mass spectrometry scans using dynamic exclusion. The samples destined for gas chromatography/mass spectrometry analysis were re-dried under nitrogen at 24 h before being reconstituted using bistrimethyl-silyl-triflouroacetamide. The gas chromatography column was 5% phenyl and the temperature ramp was from 40 °C to 300 °C in a 16-min period. Samples were analysed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The information output from the raw data files was automatically extracted. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 p.p.m. Identification of known chemical entities was based on comparison with metabolomic library entries of purified standards. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity.

Enzymology. Enzymatic assays for hMESH1 were performed in a buffer containing 50 mM Tris, pH 8, 200 mM NaCl and 1 mM MnCl\(_2\) or ZnCl\(_2\). Michaelis–Menten kinetics assays for NADPH and NADP were carried out using 50 nM or 500 nM enzyme, respectively, and serial dilutions of substrate concentration starting at 1 mM. Reactions were stopped by the addition of formic acid (3 M final concentration) at 2 min, 4 min, 6 min, 8 min, 10 min and 15 min time points. Specific activities of purified hMESH1 mutants were determined using 93 μM Zn-replaced, 33 μM E65K, 100 μM D66K or 50 nM WT enzyme with 1 mM NADP, with the reaction stopped at 30 min for each mutant and 10 min for the WT. Specific activities of the knockdown cell lysate were performed using diluted cell lysate (1:100 dilution in Tris–NaCl with 1 mM MnCl\(_2\)) and reactions were performed at 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. The amount of released phosphate was assessed using the malachite green reagent\(^\text{a}\) by mixing equal parts of reaction
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Transfection Reagent (Mirus, MIR2305). pMD2.G and psPAX2 were gifts from HEK-293T cells with a 0.1:1:1 ratio of pMD2.G/psPAX2/pLX302 with TransIT-LT1 without expression of the V5 tag. Briefly, virus was generated by transfecting

of virus to a 60-mm dish of RCC4 cells with 8

cultured in DMEM with 4.5 g l

The RCC4 cell line was provided by Denise Chan (University of

vector or with MESH1-WT or MESH1-E65A) was transfected with TransIT-LT1

Crystals of hMESH1 bound to NADPH were formed using

X-ray crystallography. Crystals of hMESH1 bound to NADPH were formed using the hanging drop vapour diffusion method. Immediately before crystallization, hMESH1 was treated with 10 mM EDTA for 30 min and exchanged into a buffer containing no metal before addition of 1 mM ZnCl2. Diffraction-quality crystals were obtained by mixing equal volumes of the protein solution (9 mg ml

2-mercaptoethanol) with the mother liquor solution (200 mM ammonium acetate, 100 mM sodium citrate and 25% PEG 4000) by streak-seeding with apo hMESH1 crystals. To improve NADPH occupancy, crystals were additionally soaked in the mother liquor solution containing 750 mM NADPH for 1 h before cryoprotection in a solution containing 525 mM NADPH, 10 mM ZnCl2, 20% ethylene glycol and 70% precipitant solution and flash freezing in liquid nitrogen.

X-ray data were collected at the Southeast Regional Collaborative Access Team 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory and processed using the XDS package

The structure was determined by molecular replacement with the program Phaser

and by using the previously reported structure of apo hMESH1 (SNR1) as the search model. Iterative model building and refinement were carried out using COOT

and PHENIX

MESH1 silencing using RNA interference. siNT was purchased from Qiagen (ABStar Negative control siRNA, S036583-01). Three individual siRNAs targeting the hMESH1 CDS (target sequence GGAAUACAGCUACUGUUG, D-031786-01, Dharmacon). siSNR1 (target sequence CGUGCACAAU, CCAAUCAGAUAGACUUCAU, M-006318-01, Dharmacon) siMESH1-CDS (target sequence GGGAAUCACUGACAUUGUG, D-031786-01, Dharmacon). If not specified, siMESH1 indicates siMESH1-CDS. The efficiencies of these siRNAs were assessed by rt-qPCR, western blots or both. For enzymatic analysis, 8 x 104 RCC4 cells were seeded in a 100- mm plate, and transfected after 1 d of growth with 600 pmol of siRNA and 40 μl of Lipofectamine RNAiMAX (ThermoFisher Scientific, no. 13778150) for 72 h before the collection of cell lysates. For NADP(H) measurement, 8 x 104 cells were seeded per well on six-well plates with 40 μl of DNA and transfected with 3 μl of Lipofectamine RNAiMAX for 48 h before drug treatment for 4 d. For viability assays, 2,800 RCC4 cells were seeded per well on 96-well plates with 5 pmol of siRNA and 0.4 μl of Lipofectamine RNAiMAX for at least 48 h before drug treatment for 1 d. To collect RNA or protein, 10% RCC4 cells were seeded in a well of a six-well plate with 40 pmol of siRNA and 3 μl of Lipofectamine RNAiMAX for 72 h before collection.

MESH1 overexpression. Stable RCC4 cell lines with MESH1-WT (ReSeq-NM_001286415.1) or MESH1-E65A expression were generated using lentiviral vector pLX302 (ref. 32). A gift from David Root, Addgene plasmid no. 25896

without expression of the V5 tag. Briefly, virus was generated by transfecting HEK-293T cells with a 0.1:1:1 ratio of pMD2.G/pSPAX2/pLX302 with TransIT-LT1 Transfection Reagent (Mirus, MR2305). pMD2.G and psPAX2 were gifts from HEK-293T cells with a 0.1:1:1 ratio of pMD2.G/psPAX2/pLX302 with TransIT-LT1 without expression of the V5 tag. The NADP(H) content was measured and normalized by protein content of the lysate, and quantified by the bicinchoninic acid assay. In cells treated with erastin or cystine deprivation, the NADP(H) change is normalized with NADP(H) measured in dimethylsulfoxide-treated cells or cells in regular full media.

NADPH sensor experiments. For the NADPH biosensor assay, HEK-293T cells (6,000 cells per well in a 96-well plate) were counted and reverse transected with control or indicated MESH1 siRNA using Lipofectamine RNAiMAX (Invitrogen). After 24 h, the cells were transfected with niAP1 biosensor

Transfection Reagent (Mirus, MIR2305). pMD2.G and psPAX2 were gifts from HEK-293T cells with a 0.1:1:1 ratio of pMD2.G/psPAX2/pLX302 with TransIT-LT1 without expression of the V5 tag. Briefly, virus was generated by transfecting

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30 mg l

NADPH was measured using the CellTiter-Glo assay (Promega, no. G9260), which quantified protein release from broken cells in the cell culture media. The fluorescence intensity, absorbance and luminescence intensity were measured by using a plate reader (FLUOstar Optima, BMG Labtech).

Lipid peroxidation measurement. The C11-BODIPY dye (D3861, Thermofisher Scientific) was used to determine lipid ROS levels according to the manufacturer's instructions. Cells were reverse transected with siRNAs as indicated in six-well plates for 2 d, and then treated with 1 μM erastin overnight. The next day, the cells were incubated with 10 μM C11-BODIPY for 2 h. After 1 h, the cells were collected by 0.25% trypsin-Epox-7- EDTA treatment and washed three times with ice-cold PBS followed by re-suspension in PBS containing 1% BSA. The amount of ROS within cells was determined by flow cytometry analysis (FACScanJo II, BD Biosciences).

Protein lystate collection and western blots. Cell lysates were collected in a buffer containing 50 mM Tris, pH 8.0, and 200 mM NaCl (for enzymology), in the NADP(H) lysis buffer (for NADP(H) measurement) or in the radioimmunoprecipitation assay buffer (Sigma, R0278) with protease inhibitors (Roche, 1183617001). For western blots, 15–50 μg of lysates were loaded on SDS–PAGE gels, semi-dry transferred to the polyvinylidene fluoride membrane, blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20, then incubated with primary antibodies overnight at 4°C. Anti-GAPDH antibody (Santa Cruz, sc-25778, lot number [I416], anti-MESH1 antibody (Abcam, ab18325, lot numbers GR926347-1 and GR75995-5), anti-β-tubulin antibody (Cell Signaling Technology, no. 2128S, lot number 2, Synthesis antibody, antibodies for proteins), ML-162 (Cayman no. 20455) or RSL3 (Cayman no. 19288) at 10 μM for 2 h, then washed with PBS. After 1 h incubation with primary antibodies, the blots were washed with 1:5000 secondary antibody, then probed with horseradish peroxidase conjugated secondary antibody and chemiluminescent substrate (GE Healthcare Life Sciences, no. 1705064). Total RNA (500 ng) with or without reverse transcriptase was prepared using a SensiFast cDNA Synthesis Kit (Qiagen, 74104) following the manufacturer's instructions. Total RNA (500 ng) with or without reverse transcriptase was prepared using a SensiFast cDNA Synthesis Kit (Bioline, BIO-60504) for real-time PCR comparison with the Power SYBRGreen Mix (ThermoFisher Scientific, 4367669). Primers were designed across exons

mixture and malachite green reagent and measuring the absorbance at 630 nm. Values for Km and maximum enzyme rate, Vmax, were calculated from the Michaelis– Menten equation.

X-ray crystallography. Crystals of hMESH1 bound to NADPH were formed using the hanging drop vapour diffusion method. Immediately before crystallization, hMESH1 was treated with 10 mM EDTA for 30 min and exchanged into a buffer containing no metal before addition of 1 mM ZnCl2. Diffraction-quality crystals were obtained by mixing equal volumes of the protein solution (9 mg ml

100 mM NADPH, 50 mM Tris, pH 8.0, 200 mM NaCl and 0.1% 2-mercaptoethanol) with the mother liquor solution (200 mM ammonium acetate, 100 mM sodium citrate and 25% PEG 4000) by streak-seeding with apo hMESH1 crystals. To improve NADPH occupancy, crystals were additionally soaked in the mother liquor solution containing 750 mM NADPH for 1 h before cryoprotection in a solution containing 525 mM NADPH, 10 mM ZnCl2, 20% ethylene glycol and 70% precipitant solution and flash freezing in liquid nitrogen.

X-ray data were collected at the Southeast Regional Collaborative Access Team 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory and processed using the XDS package. The structure was determined by molecular replacement with the program Phaser and by using the previously reported structure of apo hMESH1 (SNR1) as the search model. Iterative model building and refinement were carried out using COOT and PHENIX.

Articles

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whenever possible using PrimerBot developed by Jeff Jasper at Duke University. Primer sequences used in this study are listed here: MESH1-rt-F: GAGGCGGG
AATCACTGACAT; MESH1-rt-R: TGGTGCCCACAAAGTGGACCT; NADK-rt-F: CACAATGGCTGCTGAGGAA; NADK-rt-R: TGAGAAGTGGAGGAGGAGG; NADK-rt-F: GCCCTACAGTCCGGAAGAACAC; NADK-rt-R: GCATCCCAACAAGGAGAACG; beta-actin-rt-F: GGGGTGTTGAGGGTCTCACA; beta-actin-
rt-R: GGGACTCTCACCCTGAGGTA. The product of PCR was validated for specificity by DNA electrophoresis.

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

**Data availability**

The data that support the findings of this study are available from the corresponding authors upon request. The coordinates of the hMesh1 D66K–NADPH complex have been deposited in the Protein Data Bank under accession code 5JXA.

The RNA-seq data have been deposited into NCBI GEO with accession number GSE114282. Source data for Figs. 1, 3 and 4 are presented with the paper.

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**Author contributions**

The experimental strategy was conceived by J.-T.C. and P.Z. and further developed by C.-K.C.D. and J.R. X-ray crystallography was conducted by J.R. and analysed by J.R. and P.Z. Enzymatic assays were performed and analysed by J.R., J.Z. and P.Z. Mass spectrometry measurements were carried out by Z.G. Measurements of the enzymatic activity and NADPH concentration in the cell lysates were carried out by C.-K.C.D., J.W. and J.R. Cell culture and transcriptome profiling experiments were carried out by C.-K.C.D. and K.-Y.C. with assistance from J.W., T.S., P.-H.C., H. M. and J.L. and K.-C.L. contributed to the NADPH sensor and phenotype experiments. Lipid peroxidation measurements were carried out by W. H. Y. C. K. C. D., J.-T.C. and P.Z. wrote the manuscript with input from all co-authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Author contributions**

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The Hippo Pathway Effector TAZ Regulates Ferroptosis in Renal Cell Carcinoma

Graphical Abstract

Highlights
- Ferroptosis susceptibility can be affected by cell density
- TAZ is activated in renal cell carcinoma cell lines and early-passage tumor cells
- The activation status of TAZ regulates the susceptibility of RCC to ferroptosis
- TAZ regulates ferroptosis through affecting EMP1-NOX4 expression

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In Brief
Yang et al. show that ferroptosis sensitivity in renal cell carcinoma (RCC) is regulated by cell density through the TAZ-EMP1-NOX4 pathway. These findings reveal TAZ as a genetic determinant of ferroptosis in RCC. In addition, ferroptosis may hold therapeutic potential for RCC and other TAZ-activated tumors.
The Hippo Pathway Effector TAZ Regulates Ferroptosis in Renal Cell Carcinoma

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SUMMARY

Despite recent advances, the poor outcomes in renal cell carcinoma (RCC) suggest novel therapeutics are needed. Ferroptosis is a form of regulated cell death, which may have therapeutic potential toward RCC; however, much remains unknown about the determinants of ferroptosis susceptibility. We found that ferroptosis susceptibility is highly influenced by cell density and confluence. Because cell density regulates the Hippo-YAP/TAZ pathway, we investigated the roles of the Hippo pathway effectors in ferroptosis. TAZ is abundantly expressed in RCC and undergoes density-dependent nuclear or cytosolic translocation. TAZ removal confers ferroptosis resistance, whereas overexpression of TAZS89A sensitizes cells to ferroptosis. Furthermore, TAZ regulates the expression of Epithelial Membrane Protein 1 (EMP1), which, in turn, induces the expression of nicotinamide adenine dinucleotide phosphate (NADPH) Oxidase 4 (NOX4), a renal-enriched reactive oxygen species (ROS)-generating enzyme essential for ferroptosis. These findings reveal that cell density-regulated ferroptosis is mediated by TAZ through the regulation of EMP1-NOX4, suggesting its therapeutic potential for RCC and other TAZ-activated tumors.

INTRODUCTION

Renal cell carcinoma (RCC) annually affects ~338,000 new cases worldwide (Medina-Rico et al., 2018). Despite recent inclusion of anti-angiogenic agents or immune checkpoint inhibition for treating RCC, the median overall survival rate for patients remains unsatisfactory (Hsieh et al., 2017). Thus, identifying new therapeutics is urgent. RCCs are particularly susceptible to ferroptosis, a form of iron-dependent programmed death that is morphologically, genetically, and biochemically distinct from other cell deaths (Dixon et al., 2012; Yang et al., 2014a), suggesting its therapeutic potential for RCC. Ferroptosis can be induced by erastin (Dolma et al., 2003), which inhibits the glutamate-cystine antiporter system, xCT, resulting in the redox imbalance by decreasing intracellular glutathione (GSH) levels and accumulation of lipid reactive oxygen species (ROS). Lipid ROS can be accumulated by either impaired detoxification of lipid peroxidation via reducing the expression of glutathione peroxidase 4 (GPX4) (Yang et al., 2014a) or by the generation of superoxide and hydrogen peroxide involving upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) (Dixon et al., 2012). In kidney, NOX4 is highly expressed as an important source of renal ROS (Gorin et al., 2005; Sedeek et al., 2013). In addition, inhibition of NOX4 reduces the cystine deprivation-induced cell death and lipid ROS, suggesting its essential role in ferroptosis (Poursaitidis et al., 2017). However, much remains unknown about the genetic determinants of ferroptosis in RCC.

Here, we demonstrate how cell density regulates the ferroptosis sensitivity of RCC. We found that the Hippo pathway regulator WW Domain Containing Transcription Regulator 1 (TAZ) regulates ferroptosis through Epithelial Membrane Protein 1 (EMP1)-NOX4, implying that ferroptosis can be a therapeutic approach for RCC and other TAZ-activated tumors.

RESULTS

Cell Density Affects the Ferroptosis Sensitivity of RCC Cell Lines

RCCs are susceptible to ferroptosis induced by erastin and cystine deprivation (Tang et al., 2016; Yang et al., 2014a). We found that erastin sensitivity is determined by cell density. When RCC4, a renal cancer cell line, was grown at low cell density (<50% confluency), it was highly sensitive to erastin-induced ferroptosis as the morphological change of cell rounding shown under light microscopy. In contrast, when cells were grown at high cell density (>80% confluency), they became less sensitive to erastin (Figure 1A). Next, we used a cell viability assay to define the erastin 50% effective concentration (EC50) of high or low densities were due to the availability of erastin to its target, xCT; we conducted two experiments. First, we seeded high/low densities were due to the availability of erastin to its target, xCT; we conducted two experiments. First, we seeded...
Figure 1. Cell Density Regulates the Ferroptosis Sensitivity of RCC

(A) Bright-field images of RCC4 cells cultured in low/high densities with 0 to 1 μM erastin; n = 3; scale bar, 200 μm.

(B) CelltiterGlo assay and EC50 after RCC4 cells of low/high densities treated with erastin for 24 h; n = 3; mean ± SEM; two-way ANOVA.

(C) Cell death assay of RCC4 grown at low/high densities treated with DMSO or 4 μM erastin for 16 h; n = 2.

(D) Crystal violet staining of RCC4 cultured in low/high densities treated with DMSO or 2 μM erastin; n = 3.

(E) Protein expression of YAP and TAZ; n = 3.

(F) YAP/TAZ and phospho-TAZ (Ser89) expressions in cytosol and/or nucleus of RCC PDX 13-789 cells. H3, nuclear marker; β-actin, cytosolic marker.

(legend continued on next page)
the same number of cells in a larger or smaller area to represent low or high cell densities. Crystal violet staining and quantification (Figures 1D and S1C) revealed that the RCC4 cells seeded at high cell density are more resistant to erasin. Second, the upregulation of CHAC1 gene has been reported as a biomarker for inhibition of xCT by erasin (Dixon et al., 2014). We found comparable CHAC1 upregulation under high/low densities (Figure S1D). Cell density-dependent erasin sensitivities were also found in human embryonic kidney cells, 293T (Figure S1E), and an early passaged, patient-derived xenograft (PDX) RCC cell line, 13–789 (Figure S1F). To exclude the possibility that cell density alters the mode of cell death induced by erasin, we found that the erasin-induced cell death in low cell density can be rescued by ferrostatin-1, but not by Z-VAD-FMK (Figure S1G). In addition, the cell density also affects the ferroptosis triggered by RSL3, a GPX4 inhibitor (Figure S1H). Together, these data indicate that cell density regulates ferroptosis sensitivity.

The Hippo pathway regulators, YAP and TAZ, are molecular sensors that regulate the density-dependent proliferation of cancer cells (Mori et al., 2014; Zhao et al., 2007). Thus, we investigated the role of YAP and TAZ in ferroptosis. First, we determined the expression of YAP and TAZ proteins in RCC4, 786O, and PDX 13–789 cell lines. With MDA-MB-231 as a control, we found that TAZ protein, but not YAP, was the predominant coactivator in RCC (Figures 1E and 1F). In addition, knockdown of TAZ in RCC4 and 293T cells increased YAP expression, suggesting a compensatory mechanism between TAZ and YAP (Figure S1J).

YAP/TAZ activities are regulated by their phosphorylation and intracellular localization. In high density, YAP and TAZ are phosphorylated, cytosolically retained, and subjected to proteasomal degradation; in low density, YAP and TAZ become dephosphorylated and translocate into the nuclei to associate with TEAD protein to drive gene expression regulating cell proliferation, differentiation, and migration (Hsiao et al., 2016; Zhao et al., 2007). When the expression levels of TAZ in RCC4 were compared between different cell densities, higher cell density led to a lower level of TAZ protein (Figure 1G). In addition, when RCC4 and PDX 13–789 cells were shifted from high density to low density in vitro, TAZ translocated from cytosol to the nuclei (Figures 1H and 1I). To verify that the activated TAZ expression also occurred in vivo, we performed cytosolic and nuclear fractionations of RCC PDX tissues. As shown in Figures 1F and S1I, the combination of nuclear TAZ expression with a low level of phosphorylation indicated that TAZ was activated in the PDX 13-789 model. Thus, these results suggest that TAZ is the predominant Hippo effector in RCC, and its subcellular localization is regulated by cell density. Consistent with the ferroptosis sensitivity of RCC, RCC cell lines and renal tumors express the highest level of WWTR1 mRNA (encoding TAZ) from the analysis of the Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) (Figures S1K and S1L). Therefore, we focused on TAZ as the main Hippo effector that regulates ferroptosis in RCC cells.

**TAZ Regulates Sensitivity to Erastin-Induced Ferroptosis**

To investigate whether TAZ regulates ferroptosis, we found that TAZ knockdown (Figure 2A) reduces erastin-induced death (Figures 2B and 2C). TAZ knockdown in the low-density RCC4 cells also conferred ferroptosis resistance as in high cell density (Figure 2D), suggesting that TAZ activation contributes to the density-dependent ferroptosis sensitivity. Moreover, knockdowns of TAZ in RCC4 by multiple independent small interfering RNAs (siRNAs) all reduce sensitivity to erasin (Figures 2E and S2A). Similar reduced erastin sensitivities by TAZ knockdown were also observed in other RCC cells (786O and 13–789) (Figures 2F, 2G, S2B, and S2C) and breast cancer cells MDA-MB-231 (Figures S2D–S2H), suggesting the general relevance of TAZ for ferroptosis. Conversely, the expression of a constitutively active form of TAZ, TAZS89A (Lei et al., 2008), increased sensitivities to erasin (Figures 2H and 2I). Together, these data indicate that the activation status of TAZ regulates the ferroptosis sensitivities under different cell density.

To characterize the in vivo relevance of TAZ-regulated ferroptosis, we determined how the TAZ knockdown (Figure S2I) affects the erastin response of 786O xenografts. Erasin administration reduced the tumor growth in the control 786O xenografts, but not in the TAZ knockdown xenografts (Figures S2J and S2K). The effects of TAZ knockdown on erasin sensitivity were further tested ex vivo by 3D Matrigel culture. We found that erasin decreases the sphere size of the control 786O, but not TAZ-knockdown cells (Figures S2J and S2K). These data support the in vivo relevance of ferroptosis regulation by TAZ.

**EMP1 Is a Direct Target Gene of TAZ That Regulates Ferroptosis Sensitivity**

Next, we sought to identify how TAZ regulates erasin sensitivity in RCC4. TAZ is a transcriptional coactivator that affects phenotypes through gene expression. Based on the assumption that TAZ silencing may repress genes essential for ferroptosis, we focused on genes that were both repressed during TAZ knockdown (Figure S2I) and essential for the cystine-deprived death of RCC4 (RNAi screen) (Figures 3A, S3A, and S3B). From these comparisons, we identified 11 candidate genes, including TAZ. After excluding TAZ and two other genes encoding only one subunit of multi-component complexes, we prioritized the eight remaining candidate genes. First, we used qRT-PCR to validate TAZ-knockdown cells (Figures S2J and S2K). These data support the in vivo relevance of ferroptosis regulation by TAZ.

(G) Protein expression of YAP and TAZ of RCC4 at low/high densities; n = 3. (H and I) Confocal immunofluorescence images of TAZ in RCC4 cells (H) or RCC PDX 13-789 cells (I), when grown at low/high densities. DAPI, nucleus; F, actin, cell boundaries. Scale bars, 50 μm. See also Figure S1.
CTGF and CYR61 mRNAs, two well-known YAP/TAZ target genes in TCGA (Figures S3E and S3F) and CCLE (Figures S3G and S3H). EMP1 knockdown by multiple siRNAs also conferred ferroptosis resistance in RCC4 cells (Figures S3I, S3J, and S3K), and MDA-MB-231 cells (Figures S3L and S3M). Thus, the downregulation of EMP1 may contribute to the ferroptosis resistance conferred by the TAZ knockdown. Conversely, overexpression of EMP1 sensitizes RCC4 cells to erastin-induced ferroptosis (Figures 3E and S3M). Furthermore, EMP1 overexpression reduces the ferroptosis protection conferred by TAZ knockdown (Figure 3F), indicating that EMP1 genetically works downstream of TAZ to regulate ferroptosis.

Previous chromatin immunoprecipitation sequencing (ChIP-seq) studies have suggested that the regulatory regions of EMP1 were physically associated with YAP/TAZ/TEAD complexes (Stein et al., 2015; Walko et al., 2017; Zanconato et al., 2015). To validate that EMP1 is a direct target gene of TAZ, we performed ChIP-qPCR using an antibody specific for endogenous TAZ protein. As shown in Figure 3G, EMP1 was enriched in the TAZ pull-down as the positive control connective tissue growth factor (CTGF), indicating that EMP1 promoter is directly associated with TAZ. Together, these data support EMP1 as a direct target gene of TAZ that regulates ferroptosis sensitivity.

**EMP1 Regulates Ferroptosis through NOX4**

To investigate the mechanistic link between EMP1 and ferroptosis, we sought to determine whether EMP1 would affect the levels of GPX4 or NOX4, two key regulators of lipid peroxidation for ferroptosis (Dixon et al., 2012; Poursaitidis et al., 2017; Yang et al., 2014a). We found that EMP1 knockdown decreased the mRNA expression of NOX4, but not GPX4 (Figures 4A, S4A, and S4B). Conversely, EMP1 overexpression increases the mRNA level of NOX4, but not GPX4 (Figures 4B, S4C, and S4D). Overexpression of EMP1 also increases NOX4 protein level (Figure 4C), but not GPX4 protein level (Figure S4E). Thus, we reasoned that EMP1 may regulate ferroptosis by affecting the levels and activities of NOX4. Consistent with its ability to...
induce EMP1, TAZS89A also increased the level of NOX4 protein (Figure S4F), but not GPX4 (Figure S4E). To rule out that elevated NOX4 may affect ferroptosis through GSH, we measured the GSH levels when the control and TAZ-knockdown cells were exposed to erastin. We found that erastin treatments significantly reduced the GSH levels (Figure S4G), as previously reported (Yang et al., 2014a). However, the TAZ knockdown did not affect the GSH levels before or after erastin treatments (Figure S4G). We further found that the NOX1/NOX4 inhibitor, GKT136901 (Laleu et al., 2010), protected RCC4 from ferroptosis (Figure 4D). Because NOX1 was not detectable in RCC cell lines (Gregg et al., 2014), GKT136901 probably mediated the ferroptosis protection through NOX4. Consistently, knockdowns of NOX4 by siRNAs also conferred ferroptosis resistance (Figures S4H). Conversely, overexpression of NOX4 increased the sensitivity of RCC4 to erastin treatment (Figures S4I–S4K).

To investigate the genetic interaction between EMP1 and NOX4, we treated the NOX4 inhibitor, GKT136901, in EMP1-overexpressing RCC4. We found that the EMP1 overexpression increased erastin sensitivity, but that increased sensitivity was abolished by GKT136901 (Figure 4F).

As p38 mitogen-activated protein kinase (MAPK) has been reported to regulate NOX4 reciprocally (Dougherty et al., 2017; Huang et al., 2018; Park et al., 2010; Peng et al., 2013), we examined the role of p38 activity in the NOX4 induction by EMP1. We found that EMP1 expression increased the phosphorylation and activation of p38 (Figure 4G). Furthermore, EMP1 silencing reduced the p38 phosphorylation (Figure 4H). In addition, a p38 inhibitor, SB203580, abrogated the increase of NOX4 associated with EMP1 expression (Figure 4I).

Finally, we measured the lipid ROS, which is crucial for ferroptosis (Dixon et al., 2012), by C11-BODIPY staining. We found that the erastin-induced accumulation of lipid ROS is abolished by knockdown of TAZ, knockdown of EMP1, or NOX4 inhibitor, GKT136901 (Figures 4J and S4L). Taken together, we propose a molecular model (Figure 4K) by which TAZ regulates cell...
density-dependent ferroptosis by affecting the levels of EMP1, which, in turn, regulates NOX4, lipid peroxidation, and ferroptotic death of RCC.

**DISCUSSION**

Previous studies of ferroptosis have identified certain genetic determinants (Chen et al., 2019; Ding et al., 2018; Dixon et al., 2012), but the role of non-genetic factors are unknown. Here, we identify that a non-genetic factor, cell density, regulates ferroptosis sensitivity. A similar observation is also reported on BioRxiv (Panzilius et al., 2018). Importantly, we have elucidated the molecular mechanism by which cell density regulates ferroptosis in RCC; TAZ affects the levels of EMP1, NOX4, and resulting lipid peroxidation and ferroptosis. Therefore, TAZ activation may promote ferroptosis and predict ferroptosis sensitivity.

Although our current study focuses on the regulation of ferroptosis by TAZ, the relevant roles of other components of the Hippo pathway remain unknown. For example, YAP may also regulate ferroptosis in cells with a high level of YAP protein. Furthermore, the Hippo pathway integrates a wide variety of non-genetic factors, such as mechanical properties and metabolic status (Zanconato et al., 2016). Therefore, our findings may suggest that these Hippo-sensitive, non-genetic factors may also regulate ferroptosis sensitivities. For example, epithelial-mesenchymal transition and fibrosis are prominent features of RCC, which...
may lead to the “stiff” environment known to activate the YAP/TAZ and promote ferroptosis. In addition, YAP/TAZ is also regulated by metabolic pathways, which may help to explain the essential role of glutamine metabolism in ferroptosis (Gao et al., 2015).

Although inducing ferroptosis may have anti-tumor potential, it is important to identify genetic influences on the response to those ferroptosis-inducing agents. Our results suggest that TAZ-activated tumors may be particularly responsive. Previous studies have revealed that chemo-resistant persister cells are highly sensitive to ferroptosis, based on a high level of GPX4 (Hangauer et al., 2017; Viswanathan et al., 2017). The drug resistance and persister cells are also known to be associated with YAP/TAZ activation (Lin et al., 2015; Zanconato et al., 2016) and EMP1 expression (Jain et al., 2005). Our results suggest that TAZ-EMP1-NOX4 and ZEB1-GPX4 may represent two distinct pathways linking the chemo-resistance with ferroptosis, which may operate in different resistant settings.

Besides cancer, ferroptosis also has emerging roles in other pathophysiological processes and diseases, such as neurotoxicity (Skouta et al., 2014), acute renal failure (Friedmann Angeli et al., 2014), cardiac injury (Gao et al., 2015), and ischemia-reperfusion injury (Linkermann et al., 2014). Therefore, modulating ferroptosis may have therapeutic potentials. In addition, the ability of NOX4 inhibitors to abolish ferroptosis suggests the potential for developing combinational therapies. Currently, several oral NOX4 inhibitors are under preclinical studies (Borbély et al., 2010) and may be tested for their efficacy in mitigating these ferroptosis-associated diseases.

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**AUTHOR CONTRIBUTIONS**

W.-H.Y. and J.-T.C. conceived the project, designed the experiments, and wrote the manuscript. All other authors edited and commented on the manuscript. J.-T.C. secured funding and supervised the research, D.H. designed the mice study and provided the PDX cell line. W.-H.Y. and G.R. performed the mice study. W.-H.Y. performed cell experiments and analyzed the data with assistance from C.-K.C.D., C.-C.L., and T.S.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| YAP/TAZ antibody     | Cell Signaling Technology | Cat# 8418, RRID:AB_10950494 |
| Phospho-YAP (Ser127) antibody | Cell Signaling Technology | Cat# 4911, RRID:AB_2218913 |
| TAZ antibody         | Cell Signaling Technology | Cat#70148, RRID:AB_2799776 |
| β-tubulin antibody   | Cell Signaling Technology | Cat# 86298, RRID:AB_2715541 |
| β-actin antibody     | Santa Cruz Biotechnology | Cat# sc-130301, RRID:AB_2223360 |
| V5 tag antibody      | Thermo Fisher Scientific | Cat# MA5-15253, RRID:AB_10977225 |
| NOX4 antibody        | Abcam  | Cat# ab133303, RRID:AB_11155321 |
| GPX4 antibody        | Abcam  | Cat# ab125066, RRID:AB_10973901 |
| Anti-rabbit IgG, HRP-linked antibody | Cell Signaling Technology | Cat# 7074, RRID:AB_2099233 |
| Anti-mouse IgG, HRP-linked antibody | Cell Signaling Technology | Cat# 7076, RRID:AB_330924 |
| **Biological Samples** |        |            |
| Patient-derived xenografts (PDX) | Duke University Medical Center | IRB protocol (Pro000022289) |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Erastin              | Duke University Small Molecular Synthesis Facility | Batch DG11_65 |
| Ferrostatin-1        | Sigma  | SML0583    |
| Z-VAD-FMK            | MilliporeSigma | Cat# 627610 |
| Growth factor reduced Matrigel | Corning | Cat#356231 |
| TransIT®-LT1 Transfection Reagent | Mirus Bio | MIR2305 |
| Lipofectamine RNAiMAX Transfection Reagent | Thermo Fisher Scientific | Cat# 13778150 |
| **Critical Commercial Assays** |        |            |
| CellTiterGlo® Luminescent Cell Viability Assay Kit | Promega | Cat# G7571 |
| CytoTox-Fluor Cytotoxicity Assay | Promega | Cat# G9260 |
| GSH/GSSG-Glo Assay   | Promega | Cat# V6611 |
| RNAeasy Mini Kit     | QIAGEN | Cat# 74104 |
| RNase-Free DNase Set | QIAGEN | Cat# 79254 |
| SuperScript II Reverse Transcriptase | Thermo Fisher Scientific | Cat# 18064014 |
| Power SYBR Green Master Mix | Thermo Fisher Scientific | Cat# 4368577 |
| SYTOX Green Dead Cell Stain | Thermo Fisher Scientific | Cat# S34860 |
| **Deposited Data**   |        |            |
| siTAZ microarray     | This paper | GSE: 121689 |
| **Experimental Models: Cell Lines** |        |            |
| RCC4                 | Denise Chan | Authenticated by DNA Diagnostics Center at Nov 2015 |
| 786O                 | Denise Chan | Authenticated by DNA Diagnostics Center at Nov 2015 |
| HEK293T              | Duke Cell Culture Facility | CRL-11268 RRID: CVCL_1926 |
| MDA-MB-231           | Duke Cell Culture Facility | HTB-26 RRID:CVCL_0062 |
| PDX 13-789           | This paper | Duke University DNA Analysis Facility Human cell line authentication |
| **Experimental Models: Organisms/Strains** |        |            |
| JAX NOD.CB17-PrkdcSCID-J mice | Duke University Rodent Genetic and Breeding Core | Stock No: 001303 RRID:IMSR_JAX:001303 |
| Oligonucleotides     |        |            |
| siRNA/shRNA/RT-qPCR sequences see Table S1 | This paper | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jen-Tsan Chi (jentsan.chi@duke.edu).

MATERIALS AVAILABILITY STATEMENT

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture Studies

RCC cell lines (RCC4 and 786O) were kind gifts from Dr. Denise Chan (Department of Radiation Oncology, University of California, San Francisco), which were further authenticated by DDC (DNA Diagnostics Center) Medical using the short tandem repeat method in November 2015. HEK293T and MDA-MB-231 were acquired from the Duke Cell Culture Facility. All cells are cultured in Dulbecco’s Modified Eagle Medium (11995-DMEM, ThermoFisher Scientific) with 10% heat-inactivated Fetal Bovine Serum (HyClone™ FBS, GE Healthcare Life Sciences #SH30070.03HI) in a humidified incubator at 37°C and 5% CO₂. Transfections were performed according to the manufacturer’s instructions with TransIT-LT1 transfection reagent (Mirus Bio) or RNAiMax transfection reagent (ThermoFisher Scientific).

Stable cell lines were generated with the pLKO.1 puro control vector or shTAZ constructs purchased from the Duke Functional Genomics Shared Resource. The virus was generated by transfecting HEK293T cells with a 1: 0.1: 1 ratio of psPAX2: pMD2.G: pLKO.1 with Mirus transit reagent. Media was changed after 16 h and the virus was collected 40 h and 64 h after transfection. The virus soup was further centrifuge and went through the filter to remove cell debris. Stable cell lines were generated by adding 1ml virus soup to a 10 cm dish of parental cells with a final concentration of 8 µg/ml polybrene and selected by 0.5 µg/ml puromycin.

250 786O stable control (pLKO.1) or TAZ knockdown (shTAZ) cells were resuspended in 5ml medium and further mixed with 55 ml growth factor reduced Matrigel (Corning #356231) and then seeded in the center of 24 well plates. After the mixture became solid (37°C for 30 min), the 3D culture was kept in the puromycin-containing medium for 1 week. Later, the culture medium with or without 0.5µM erastin was changed every other day for 7-10 days; representative views from three independent experiments were photographed, and the colony sizes were measured.

Animal Models

All experiments were performed following Duke Institutional Animal Care and Use Committee approval in accordance with the institutional and national guidelines. Mice were housed with no more than 5 animals per cage and given free access to food and water. One million 786O cells with or without shTAZ were implanted subcutaneously into the healthy 8-week-old JAX NOD.CB17-PrkdcSCID-J mice; both male and female mice were used. Later, tumors were measured by caliper and the volumes were determined as (width² x length)/2. Once tumor volume reached 120 mm³, mice were randomized into control or erastin treatment group. The vehicle (ORA-plus) or erastin (0.1ml of 4mg/ml erastin) was administrated by oral gavage twice daily for 20 days.

Patient-Derived Xenograft

Renal cell carcinoma (RCC) tissue sample (13-789) was collected under a Duke IRB-approved protocol (Pro000022289) and all participants provided written informed consent to participate in the study. The patient-derived xenograft (PDX) model of 13-789 was then generated as described previously (Kim et al., 2012; Uronis et al., 2012), and the in vivo PDX generation was performed in accordance
with the animal guidelines and with the approval of the Institutional Animal Care and Use Committee (IACUC) at the Duke University Medical Center. Briefly, to generate PDXs, the tissue sample was washed in phosphate-buffered saline (PBS), dissected into small pieces (< 2 mm), and injected into the flanks of 8-10-week-old JAX NOD.CB17-PrkdcSCID-J mice obtained from the Duke University Rodent Genetic and Breeding Core. The matched PDX cell line (13-789) was then generated from the PDX as follows. Once the PDX tumors reached a size of >1000 mm³, tumors were harvested, homogenized, and grown in 10 cm² tissue culture-treated dishes in cell culture media (DMEM media, 10% fetal bovine serum (FBS), 10 U/ml penicillin and streptomycin) at 37°C and 5% CO₂. Clonal populations of each cell line were then obtained by isolating a single clone using trypsinization of the clone sealed off from the dish by an O ring. Finally, the 13-789 cell line was authenticated using the Duke University DNA Analysis Facility Human cell line authentication (CLA) service by analyzing DNA samples from each individual cell line for polymorphic short tandem repeat (STR) markers using the GenePrint 10 kit from Promega (Madison, WI, USA).

**METHOD DETAILS**

**Determine of cell viability, cell death, and glutathione level**

Unless otherwise stated in the figure legend, the genetic experiments were conducted when the cell density is around 50%–80% confluency after the cells were seeding and transfected with siRNAs for 2 days. The cells are further treated with erastin for additional 24 h-72 h. Cell viability was evaluated using crystal violet staining or the CellTiterGlo luminescent cell viability assay kit (Promega) which determined cellular viability using ATP levels; cytotoxicity was determined by SYTOX Green staining (Thermo) or CytoTox-Fluor assay (Promega); and the level of reduced glutathione (GSH) was detected by GSH/GSSG-Glo assay (Promega) by subtracting the oxidized glutathione (GSSG) level from the total (GSH plus GSSG) glutathione according to the manufacturer’s instructions.

**Western blot analysis**

For immunoblotting, cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in RIPA buffer (Sigma), supplemented with protease inhibitor (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche). Proteins were quantified by BCA protein assay (ThermoFisher Scientific). Equal amounts of proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked with 5% non-fat milk or BSA and then probed with indicated antibodies following by HRP-conjugated secondary antibodies. The immune-signals were achieved by the Amersham ECL prime western blotting detection reagent (GE Healthcare Life Sciences RPN2232) and detected by a Bio-Rad ChemiDoc™ Imaging System.

**RNA isolation and quantitative real-time PCR**

Total RNAs of culture cells were extracted by using the RNeasy Mini Kit (QIAGEN #74104) with DNase I treatment (QIAGEN #79254) and the cDNAs were synthesized from 1 μg of the RNA template using SuperScript™ II Reverse Transcriptase (ThermoFisher Scientific #18064) with random hexamers following protocols from the manufactures. The levels of gene expression were measured by quantitative PCR (qPCR) with Power SYBR Green PCR Mix (Applied Biosystems, ThermoFisher Scientific).

**Microarray**

RCC4 cells exposed to knockdown control, siNT, or knockdown TAZ, siTAZ for two days and then treated with or without 1 μM erastin for 7 h in biological triplicates. RNAs were extracted by RNeasy Kit (QIAGEN), labeled, and hybridized to Affymetrix U133A 2.0 arrays. The intensities of Affymetrix probes were normalized by Robust Multi-array Average (RMA) method and zero transformation (Δlog₂) against the control group, siNT (DMSO) as previously performed (Keenan et al., 2015; Tang et al., 2017). Then, the probe sets that varied by 2³.⁸-fold in at least two samples were selected for hierarchical clustering. The microarray data have been deposited into NCBI GEO with accession number: GSE121689.

**Immunofluorescence staining**

Cells were cultured on chamber slide (ThermoFisher Scientific #177437) to appropriate density. Cells were fixed with 4% formaldehyde for 10 min and then washed three times with PBS. After blocking in 5% BSA and permeabilized with 0.1% Saponin in 1 h, slides were incubated with the TAZ antibody (BD Biosciences #560235) 1:100 diluted in 1% BSA with 0.1% Saponin overnight. After washing with PBS, slides were incubated with Alexa Fluor 488-conjugated secondary antibodies (1:200 dilution, ThermoFisher Scientific #A12380) for 1 h. The slides were then washed and mounted with SlowFade Glod antifade mountant with DAPI ((ThermoFisher Scientific #S36938). Images were acquired using a Leica TCS SP8 confocal microscope equipped with a 40X objective.

**ChIP analysis**

ChIP-qPCR experiment was carried out according to the Myers Lab ChIP-seq protocol (Johnson et al., 2007). Briefly, RCC4 cells were incubated in cross-linking solution (1% formaldehyde) at room temperature for 10 min and then added 0.125M final concentration of glycine to stop cross-linking. The cells were then washed with cold PBS and suspended in Farnham lysis buffer (5mM PIPES pH8.0, 86mM KCl and 0.5% NP-40) with freshly added protease inhibitor. The lysate was subsequently passed through a 20-gauge
needle 20 times to break cells while keeping intact nuclei. After centrifugation, the pellet was resuspended with RIPA buffer with freshly added protease inhibitor. Chromatin fragmentation was performed by sonication using the Bioruptor (Diagenode) high speed for 30 min (30 s ON, 30 s OFF). Proteins were immunoprecipitated in PBS/BSA buffer using TAZ antibody (Cell signaling #70148) or control antibody, rabbit IgG (Cell signaling #2729) which have been conjugated to Dynabeads™ protein G magnetic beads (Thermo Fisher Scientific #10004D) at 4°C for 2 hr. The antibody-chromatin complexes were washed with LiCl wash buffer for 5 times and then washed with TE buffer. The crosslinking was reversed by incubation with elution buffer (1% SDS, 0.1M NaHCO₃) at 65°C overnight followed by incubation with RNase A and proteinase K. DNA was recovered by using QIAquick PCR purification kit (QIAGEN #28104). Precipitated DNA was analyzed by qPCR using primers targeting TEAD-binding sites found in CTGF (Stein et al., 2015) and EMP1 promoter regions as well as negative control chromatin 14 (Stein et al., 2015). Primer sequences are listed below.

**Lipid ROS assay using flow cytometry**

Lipid ROS levels were determined using 10 μM of C11-BODIPY dye (D3861, ThermoFisher Scientific) with the positive control, cumene hydroperoxide, according to the manufacturer’s instructions. Cells were seeded and treated with siRNAs in six-well plates for two days, then the culture medium was replaced with 1 μM erastin treatment for overnight. The next day, the medium was replaced with 10 μM C11-BODIPY-containing medium for 1 h. Later, the cells were harvested by trypsin and washed three times with ice-cold PBS followed by re-suspending in PBS plus 1% BSA. The amount of ROS within cells was examined by flow cytometry analysis (FACSCanto™ II, BD Biosciences).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Graphs were drawn and statistics were analyzed by using GraphPad Prism 8 Software (GraphPad La Jolla, CA). Data were analyzed using two-tailed unpaired Student’s t test or ANOVA (one-way or two-way) and expressed as mean ± SEM p values less than 0.05 were considered significant (* < 0.05; ** < 0.01; *** < 0.001). The exact values of “n” used are described in the corresponding figure legends. Unless otherwise stated in the figure legend, n refers to the number of biological replicates and includes either number of mice or replicates of cell studies.

**DATA AND CODE AVAILABILITY**

The microarray data of TAZ knockdown have been deposited into NCBI GEO with accession number: GSE121689. The siRNA screen of cystine deprivation has not been deposited in a public repository because the manuscript in preparation, but are available from the corresponding author on request.
Zinc transporter ZIP7 is a novel determinant of ferroptosis

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Abstract
Ferroptosis is a newly described form of regulated cell death triggered by oxidative stresses and characterized by extensive lipid peroxidation and membrane damages. The name of ferroptosis indicates that the ferroptotic death process depends on iron, but not other metals, as one of its canonical features. Here, we reported that zinc is also essential for ferroptosis in breast and renal cancer cells. Zinc chelator suppressed ferroptosis, and zinc addition promoted ferroptosis, even during iron chelation. By interrogating zinc-related genes in a genome-wide RNAi screen of ferroptosis, we identified SLC39A7, encoding ZIP7 that controls zinc transport from endoplasmic reticulum (ER) to cytosol, as a novel genetic determinant of ferroptosis. Genetic and chemical inhibition of the ZIP7 protected cells against ferroptosis, and the ferroptosis protection upon ZIP7 knockdown can be abolished by zinc supplementation. We found that the genetic and chemical inhibition of ZIP7 triggered ER stresses, including the induction of the expression of HERPUD1 and ATF3. Importantly, the knockdown of HERPUD1 abolished the ferroptosis protection phenotypes of ZIP7 inhibition. Together, we have uncovered an unexpected role of ZIP7 in ferroptosis by maintaining ER homeostasis. These findings may have therapeutic implications for human diseases involving ferroptosis and zinc dysregulations.

Introduction
Ferroptosis is a novel form of regulated cell death1,2 with distinct morphological, genetic, and biochemical features. Ferroptosis was first described as a death mechanism by which erastin-induced cell death3. Erastin was found to be an inhibitor of xCT (encoded by SLC7A11), a cystine importer in exchange for glutamate export. Therefore, erastin blocked cystine import, deplete intracellular glutathione, resulting in the accumulation of lipid-based reactive oxygen species (ROS), membrane damage, and ferroptotic death. The lipid peroxidation can be neutralized by two different mechanisms of ferroptosis protection, either GPX4 (glutathione peroxidase 4) or ferroptosis suppressor protein 1 (FSP1). GPX4 is a phospholipid hydroperoxidase that utilizes GSH as a cofactor to neutralize ROS2. Therefore, ferroptosis can also be triggered by either the depletion of GSH or direct inhibition of GPX4. Reciprocally, the activation of NRF2 and GSH generation can robustly protect cells from ferroptosis4,5. Recently, FSP1 is discovered as a new ferroptosis protection mechanism via CoQ10 as a lipophilic radical-trapping antioxidant6,7.

As the name implies, one canonical feature of “ferroptosis” is the iron-dependency. The iron chelator blocked ferroptosis and the addition of iron sensitized cells to ferroptosis. The hemochromatosis hepatocytes8 and erythrocyte-ingested macrophages9, with elevated iron levels, are highly susceptible to ferroptosis. Furthermore, many genetic determinants of ferroptosis regulate ferroptosis by affecting iron metabolism10–12. Indeed, a recent report has identified the transferrin receptor as a
biomarker of ferroptosis. While the role of iron in ferroptosis is well recognized, much remains unknown regarding the underlying mechanisms. Iron is postulated to promote lipid peroxidation and ferroptosis via the non-enzymatic Fenton reaction that amplifies ROS.

Other than iron, it is not clear whether any other transition metals also regulate ferroptosis. In the original ferroptosis study that revealed the essential role of iron, several different metals, including manganese, nickel, cobalt, or copper, were also tested together with iron, but their effects on ferroptosis were limited. Here, we show that ferroptosis sensitivity is also significantly affected by zinc, a divalent metal ion crucial for many biological processes. We found that zinc chelator protected ferroptosis, and zinc addition promoted ferroptosis. Since zinc itself cannot move across the membrane, its movement among different cell compartments is controlled by two classes of transporters: the SLC39 family (ZIP, Zrt-like, and Irt-like protein family members) and SLC30 (ZNT, zinc transporter) family. SLC39 family members transport zinc into the cytosol from either the extracellular space or intracellular stores such as the endoplasmic reticulum (ER). In contrast, the SLC30 family members mediate zinc efflux from the cytosol to other cellular compartments. Among these zinc transporters, we found ZIP7, a member of the SLC39 transporter that promotes cytosolic zinc levels, was essential for ferroptosis. The genetic knockdown and chemical inhibition of ZIP7 conferred robust ferroptosis protection. Mechanically, ZIP7 inhibition triggers the ER stress response, especially the induction of HERPUD1, which contributes to ferroptosis protection. Together, these data revealed the unexpected role of zinc and ZIP7 in regulating ferroptosis via organellar communication between ER and nuclei.

Results
Manipulations of zinc affected ferroptosis sensitivity
Ferroptosis is a newly recognized form of regulated cell death. As the name of ferroptosis indicates, this form of regulated cell death is an iron-dependent process. Therefore, ferroptosis can be blocked by iron chelators, such as deferoxamine (DFO). Also, excessive iron in cells promotes ferroptosis. In the original ferroptosis study, iron was tested together with manganese, nickel, cobalt, or copper. Only iron was shown to affected erastin sensitivity. However, the role of zinc was not examined. To determine the potential of zinc in regulating ferroptosis, we used erastin to induce ferroptosis in two ferroptosis-sensitive cell lines MDA-MB-231 and HT-1080. We then treated them with the chelators of either iron (DFO) or zinc (N,N′,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine, TPEN). As expected, DFO significantly rescued erastin-induced ferroptosis (Fig. 1A). Surprisingly, zinc chelator TPEN also protected cells from erastin-induced death in MDA-MB-231 cells (Fig. 1A). Although HT-1080 was sensitive to TPEN under normal conditions, TPEN also significantly rescued erastin-mediated cell death (Fig. 1B). Conversely, the addition of zinc chloride (ZnCl2), similar to ferric citrate (FC), significantly sensitized cells to ferroptosis (Fig. 1C). This observation is consistent with a recent paper showing zinc toxicity killed A549 lung cancer cells via ferroptosis.

Next, we explored the relationship between iron and zinc metabolisms. We first blocked the erastin-induced ferroptosis by DFO, followed by the addition of either FC or ZnCl2. As expected, FC overcame the ferroptosis protection of DFO (Fig. 1D, E). Unexpectedly, ZnCl2 overcame the ferroptosis protection of DFO and re-sensitized cells to ferroptosis in both MDA-MB-231 (Fig. 1D) and HT-1080 (Fig. 1E). Therefore, the zinc levels significantly affect ferroptosis’s sensitivity, a cell death mechanism intimately associated with iron-dependency.

Genome-wide RNAi screen reveals novel genetic determinants of ferroptosis
To identify the genetic elements and biological processes involved in the ferroptosis triggered by cystine deprivation, we performed a genome-wide siRNA screen using Qiagen Human whole-genome siRNA library v1.0 covered more than 22,000 genes in the human genome (Fig. 2A). There are at least four siRNAs for each target gene, of which two siRNAs are combined into two independent pools. The siRNAs were transfected to RCC4 for 72 h, and ferroptosis was triggered by cystine deprivation. Then, each treatment’s relative cell viability was measured by ATP content (CellTiterGlo) and normalized to cells grown under full media. The gene was identified as a putative hit when both RNAi pools mitigated the cell death to at least 40% viability. These criteria identified 388 genes as essential for ferroptosis (supplemental information, Table S1).

The completed siRNA identified several genes previously known to be essential for ferroptosis. One top hit is CSAD (Cysteine Sulfinic Acid Decarboxylase), which mediates the limiting steps of taurine synthesis from cysteine. As previously shown, the knockdown of CSAD could block the taurine synthesis to preserve the cysteine for GSH synthesis to neutralize the ROS and rescue ferroptosis. In addition, another top hit is CARS, which encodes the cysteinyl-tRNA synthetase. CARS was reported to be essential for ferroptosis since its depletion enhanced the transsulfuration pathway. The genomic screen also identified several genes necessary for ferroptosis, including MESH (the first cystolic NADPH phosphatase) and relevant TAZ-regulated target genes. From the top identified hits, several processes were enriched based on Gene Ontology (GO) analysis, including amino acid metabolism processes, ubiquitin-
dependent proteasome activity, mitochondrial complex I, and vacuole ATPase activity (Fig. 2B). String analysis of protein-protein interactions identified the pathways of GPCR (G-protein coupled receptor) signaling, spliceosome, proteolysis, and tRNA synthetases (Fig. 2C). Some of these biological processes uncovered in our screens were also found to be essential for ferroptosis by other genome-wide screens.
Fig. 2 Genome-wide RNAi screens of cystine-deprivation mediated ferroptosis. A RCC4 cells were transfected with individual siRNA for 72 h. Cystine deprived medium (2 µM, 1% of regular media) was then applied for an additional 24 h before cell viability analysis. Cell viability is normalized to the viability in a regular medium with non-targeting siRNA on each microplate, then plotted against the rank of the viability of each siRNAs. CSAD is a positive control as shown in red squares. SLC39A7 (ZIP7) is shown in green triangles. B, C Gene Ontology (B), and String (C) analysis of 388 candidates were found to essential for ferroptosis on the screen. (FDR: false discovery rate, \( P < 0.05 \); \( p \)-value test type: Fisher’s exact).
The identification of zinc-related genes essential for ferroptosis

From the completed screens, we wish to identify zinc-related genes essential for ferroptosis. As mentioned above, zinc movement between different cell compartments is controlled by transporters in either the SLC39 family (ZIP) or SLC30 (ZNT) family. When all members of the ZnT/SLC30 and ZIP/SLC39A family were examined in the RNAi screening data, we found siRNA pools targeting ZIP7 (SLC39A7) and ZNT8 (SLC30A8) conferred robust ferroptosis protection (Fig. 2C). However, ZNT8 is known to be expressed mainly in the insulin-secreting β cells27 and not robustly expressed in most carcinoma cancer cells. Therefore, we focus on the ZIP7 as a potential zinc-related regulator of ferroptosis for further investigation.

The genetic and chemical inhibition of ZIP7 protect cells against ferroptosis

Next, we wished to validate that ZIP7 was essential for ferroptosis. First, we found that ZIP7 knockdown by additional independent sets of siRNAs significantly rescued the ferroptosis of MDA-MB-231 cells induced by cystine deprivation (Fig. 3A, B) or erastin (Fig. 3C). Furthermore, the ferroptosis protection effects of the ZIP7 knockdown can be abolished by zinc supplementation in RCC4 (Fig 3D) and MDA-MB231 (Fig. 3E, F). This result was also validated by the CellTox-Green assay, which measured cell death based on the released cellular DNA (Fig S1A). Besides RCC4 and MDA-MB-231, we also observed ZIP7/zinc-dependency in other ferroptosis sensitive cells, such as HT-1080 (Fig S1B). Together, ZIP7 knockdown rescued ferroptosis of MDA-MB-231, RCC4, and HT-1080. Furthermore, ZnCl2 treatment abolished ferroptosis protection by ZIP7 knockdown.

A recent study identified a potent and specific ZIP7 inhibitor NVS-ZP7-418. To determine whether the chemical inhibition of ZIP7 also protected ferroptosis, we treated the MDA-MB-231 with NVS-ZP7-4 together with erastin. We found that NVS-ZP7-4, similar to ZIP7 siRNAs, significantly reduced the erastin-induced death of MDA-MB-231 (Fig. 3G). Similar ferroptosis rescuing effects of NVS-ZP7-4 were also seen for RCC4 (Fig. 3H). Therefore, the inhibition of ZIP7 by both genetic and chemical means provided significant protection against ferroptosis. Collectively, these data indicate the critical role of ZIP7 and zinc transport for ferroptotic cell death.

Transcriptome response to the ZIP7 knockdown

We hypothesized that the depletion of ZIP7 might protect ferroptosis by affecting gene expression. Therefore, we used RNA-Seq to profile the transcriptional response to ZIP7 knockdown when MDA-MB-231 was transfected with control or two ZIP7-targeting siRNAs in triplicates (Fig. 4A). We performed zero transformation against the average of the three control (siNC) samples, filtered the genes based on the change of at least 1.8-fold in four samples, and arranged by hierarchical clustering (Fig. 4A). We found that both ZIP7 siRNA consistently repressed 1012 genes and induced 1649 genes (Fig. 4A).

The repressed genes include ZIP7 (SLC39A7) and many Major Histocompatibility Complex (MHC) genes, including HLA-F, HLA-DQB2, HLA-QB1, and HLA-DMB. The induced genes included genes involved in the endoplasmic reticulum (ER) stress (ATF3-Activating transcription factor 3) and HERPUD1-(Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1), nucleosome assembly (HIST1H1C and HIST1H2AC), and cellular communications (GDF9, GDF15, FLT1, JAG2, and CCR10). The top ten enriched biological pathways identified by Gene Set Enrichment Analysis (GSEA) include the enrichment of genes involved in autophagosome, N-glycan, or protein trafficking (Fig. 4B, C), consistent with previous reports18,26. Besides, ZIP7 knockdown also leads to the depletion of rRNA processing, ncRNA processing, and ribosome biogenesis (Fig. 4B, D).

ZIP7 inhibition induced ER stresses

As noted, ZIP7 knockdown induced ATF3 and HERPUD1 in the ER stress or unfolded protein response (UPR) (Fig. 4A)29,30. Therefore, we examined the effects of ZIP7 knockdown on the genes in the ER/UPR pathways. From our transcriptome analysis, ZIP7 knockdown in MDA-MB-231 significantly induced the expression of HSPA5 (heat shock protein family A member 5, or BIP), HERPUD1 (homocysteine induced ER protein with ubiquitin-like domain 1), DDIT3 (DNA-damage-inducible transcript 3, or CHOP), ATFD3 (activating transcription factor 3), and SLC7A11 (cystine/glutamate-inducible transporter) (Fig. 4E). Consistently, one independent profiling of the transcriptional response of four different cell lines to ZIP7 inhibitors also revealed the induction of HSPA5, HERPUD1, DDIT3, ATFD3, and SLC7A11 mRNA (Fig. 4F)18.

Therefore, ZIP7 inhibition by genetic and chemical means both robustly activated ER stress expression program. This finding is consistent with previous reports of the induction of ER stresses upon ZIP7 removal in multiple biological contexts and model organisms18,31,32.

Since ER stress was induced by erastin33 and implicated in ferroptosis34,35, we tested if induction of ER stress is associated with ZIP7-mediated ferroptosis protection in our systems. ER stress can be triggered by brefeldin A (BFA) or tunicamycin, both of which protected cells against ferroptosis (Fig. 5A, B). Therefore, we speculated that the induction of ER stress genes might contribute to the ferroptosis protection upon ZIP7 inhibition. Among
Fig. 3 (See legend on next page.)
these ER stress genes induced by ZIP7 inhibition, HSPA5 has been previously reported to protect against ferroptosis. However, the knockdown of HSPA5, together with ZIP7, did not mitigate the ferroptosis protection (data not shown). Therefore, we tested the role of other ER stress genes induced by ZIP7 knockdown.

HERPUD1 is a well-established ER stress-induced gene and a potential candidate in our study since its expression is strongly induced by both siZIP7 (Fig. 4D) and ZIP7 inhibitors (Fig. 4E). First, qRT-PCR revealed that HERPUD1 mRNA was significantly upregulated upon the ZIP7 knockdown (Fig. 5C). HERPUD1 protein was also elevated by tunicamycin and by both siZIP7s siRNA (Fig. 5D). Meanwhile, we also observed induction of xCT protein, but not HSPA5, by two ZIP7 siRNAs (Fig. 5D). The lack of increased HSPA5 protein was consistent with the inability of HSPA5 to mitigate the ferroptosis protection upon the ZIP7 knockdown. Most importantly, the knockdown of HERPUD1 significantly mitigated the ferroptosis protection by ZIP7 inhibition in both MDA-MB-231 and HT-1080 (Fig. 5E, F). Therefore, the induction of HERPUD1 contributed to the ferroptosis protection of ZIP7 inhibition. In sum, our study supports a model in which ZIP7 depletion and inhibition protect ferroptosis. The ZIP7 inhibition triggered the ER stresses, which triggered the induction of the HERPUD1 mRNA. The translated HERPUD1 protein will then migrate back to the ER and mediate an unknown process to protect ferroptosis (Fig. 5G).

Discussion

This study has provided compelling evidence that zinc and ZIP7 regulate ferroptosis, a regulated cell death process previously only known to be iron-dependent. Through the careful analysis of all zinc transporters in our RNAi screens, we identified ZIP7 as a novel determinant of ferroptosis. The genetic and chemical inhibition of ZIP7 robustly protects cancer cells from ferroptosis. We also found that ZIP7 knockdown may protect ferroptosis by upregulating HERPUD1, a well-known gene induced during ER stresses. Together, these data strongly indicate the unexpected role of ZIP7 in regulating ferroptosis through maintaining ER homeostasis and organellar communication.

Zinc is an essential metal required for the regulation of proliferation, metabolism, and cell signaling. Zinc serves as an important intracellular second messenger. Zinc deficiency leads to impaired immunity, growth retardation, poor wound healing, hair loss, diarrhea, delayed sexual maturation. On the other hand, zinc toxicity may lead to nausea, vomiting, diarrhea, altered copper and iron function, and reduced immune function. Given zinc’s importance, the levels and distribution of zinc are tightly regulated by the ZIP and ZnT family of zinc transporters, and their dysregulations lead to various pathological conditions. ZIP7 regulates cytosolic zinc levels by allowing the transport of zinc from the ER and other organelles. The importance of ZIP7 during development at the organismic levels is shown by the various phenotypic manifestation of ZIP7 deficiency. ZIP7-deficient mice are embryonic lethal, and the hypomorphic alleles or tissue-specific removal of Zip7 in mice blocks B cell development, the dermis, and death of intestinal progenitors. The loss-of-function of ZIP7 homolog in Drosophila and zebrafish also resulted in defects in wings and neurodevelopment. Therefore, ZIP7 is an evolutionarily conserved regulator critical for the proper development of multiple model organisms.

ZIP7 has been previously shown to regulate ER homeostasis, and ZIP7 removal triggered ER stress in the intestine, heart, and dermis. ZIP7 removal leads to zinc accumulation in ER, which inhibits disulfide isomerase and results in protein aggregation and ER stress. Consistent with these previous reports, we found that ZIP7 inhibition in cancer cells also triggered ER stress responses. Herpud1 is an ER membrane protein induced by ER stresses. Herpud1 facilitates the retrotranslocation of proteins from the ER to the 26S proteasome in the cytosol for proteolytic elimination. Interestingly, a recent study has shown that HERPUD1 protected cells from H2O2-induced cell death by regulating calcium flux by inositol 1,4,5-trisphosphate.
Besides HERPUD1, other ER stress-associated genes may also contribute to ferroptosis protection. For example, xCT is expected to enhance the cystine import and GSH production to boost the ferroptosis inhibition of GPX4. The UPR-mediated induction of xCT may also contribute to the ferroptosis protection phenotypes of the ZIP7 knockdown. It is also important to point out that ZIP7 cannot fully explain the effects of zinc on ferroptosis. It has been estimated that 3000 proteins, representing ~10% of encoded mammalian
Fig. 5 (See legend on next page.)
proteins, interact with zinc. Therefore, zinc may have much broader effects on other proteins and ferroptosis susceptibility.

Ferroptosis sensitivity is known to be associated with the various cancer cells which have developed resistance to chemotherapies or target therapeutics. Interestingly, the activation of ZIP7 is indicative of tamoxifen-resistance and indispensable for the growth of resistant ER+ breast cancer. Therefore, we found that activated ZIP7 is essential for ferroptosis, which may further connect the ferroptosis susceptibility to the treatment-resistant cells. Thus, ferroptosis may be used to target these ZIP7 activated tamoxifen-resistant ER+ breast tumors. The high expression/activation of ZIP7 may be used as biomarkers to identify tumor cells that are sensitive to these ferroptosis-targeting therapeutics.

Our results may have significant therapeutic implications. While triggering ferroptosis may have substantial therapeutic potential for human cancers, much remains unknown about how best to select tumors that would be most responsive. Our results indicate that ZIP7 activation in tamoxifen-resistant ER+ breast tumors may be susceptible to various ferroptosis-inducing therapies. Ferroptosis is involved in many pathological conditions, such as neurodegeneration, renal damages, liver fibrosis, cardiomyopathy, and ischemia-reperfusion injuries. Therefore, blocking ferroptosis using ZIP7 inhibitors may hold significant therapeutic potentials. Reciprocally, acute, and chronic zinc toxicity may result in many symptoms, including nausea, vomiting, loss of appetite, abdominal cramps, diarrhea, and headaches. Our results suggest that ferroptosis may contribute to the pathogenesis of these disease processes associated with excessive zinc.

Materials and methods

Cell culture and reagents
MDA-MB-231, HT-1080, and RCC4 cells were obtained from Duke Cell Culture Facility, maintained at 37 °C with 5% CO2, and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with fetal bovine serum and penicillin/streptomycin. Cell lines were authenticated by Duke CCF using short tandem repeats testing and tested mycoplasma-free. The siRNAs were obtained from the following source: ZIP7 siRNA pool (Horizon, siGENOME SLC39A7 siRNA #M-007338-01-0005), ZIP7_1 (Horizon, #D-007338-03-0005), ZIP7_2 (Qiagen, FlexiTube siRNA, #SI04350367), HERPUD1 siRNA (Horizon, siGENOME D-020918-02-0005 (siHERPUD1_1) and D-020918-04-0005 (siHERPUD1_2)), ZnCl2 (Sigma, #39059), N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, Sigma, #P4413), ferric citrate (Sigma, #F3388), desferoxamine mesylate (DFO, Sigma, #D9533), erasin (Caymen Chemical, CAS 571203-78-6) and ZIP7 inhibitor (ZIP-4, MedChemExpress, #HY-114395 and 114395A).

Genome-wide RNAi screen
Qiagen Human whole-genome siRNA library v1.0 was applied to clear cell Renal cell carcinoma cell line RCC4 for this screen. In the library, there are at least 4 different siRNA sequences targeting each gene, two of which are pooled together (“A + B”, “C + D”) in a well of a 384-well microplate. In each well, 1 n mole of pooled siRNAs were reverse transfected to 1000 RCC4 cells with 0.05 μl Lipofectamine RNAiMAX (ThermoFisher, 13778030) and 4.95 μl Opti-MEM (ThermoFisher, 11058021) and culture in 37 °C, 5% CO2, a humidified incubator for 72 h. Then DMEM media contains 2 μM cystine (Gibco 210130-24, supplement with L-Glutamine 4 mM, L-cystine 2 µM, and L-Methionine 30 mg/l) with 10% diazolized fetal bovine serum (Sigma #F0392) is applied for 24 h in an incubator. Cell viability is measured by CellTiter-Glo luminescent cell viability assay (Promega, G7570) and normalized to the full media control on every plate.
the Illumina Hi-Seq 3000/4000 system at Sequencing and Genomic Technologies Shared Resource at Duke Center for Genomic and Computational Biology. The RNA-seq data were deposited into Gene Expression Omnibus (GEO) database with accession number GSE155437. To validate the differentially expressed genes uncovered from RNA-seq, cDNA was prepared using SuperScript II reverse transcriptase (Thermo Fisher Scientific #18064) with random hexamers. Quantitative RT-PCR was performed on the StepOnePlus platform (Applied Biosystems) using Power SYBR Green PCR Mix (Applied Biosystems, ThermoFisher Scientific). Primers used in the qRT-PCR were as follows: HERPUD1 (forward: 5’-TCTG GGAAGCTGTGTTGGGA; reverse: 5’-TTAGAACCAGC AAGCTCCTC), ZIP7 (forward: 5’-GGACAGGCTGACTACA GTGATACTCA; reverse: 5’-CTCCTGCGCTCTTCTGAA CC), and GAPDH (forward: 5’-GAGTCAACGGATTTGG TCGT; reverse: 5’-TTGATTTTGGAGGGATCTCG).

Cell viability assay
In general, cells were pretreated (metal chelator, metal, or inhibitors) or transfected with ZIP7 siRNA for 48–72 h and treated with erastin or cystine deprivation for an additional 18–24 h as indicated in each experiment. The cell viability was determined by CellTiter-Glo (Promega), or cell death was determined by CellTox-Green (Promega) according to the manufacturer’s instruction. The detailed conditions were described in the figure legends.

Western blotting and antibodies
To evaluate the knockdown efficiency of ZIP7, cell lysates were collected by RIPA buffer 48 to 72 h after siRNA transfection. The proteins were then separated by SDS-PAGE and blotted against ZIP7 (ProteinTech, #19429-1-AP), HERPUD1 (CST, #26730), HSPA5(CST, #3177), xCT (CST, #12691) or β-tubulin (Cell Signaling, CST, #2128 S) antibody as indicated. The blots were visualized by chemiluminescence and ChemiDoc Imager (Bio-Rad). The raw data for all blots are presented in supplemental Fig. 2.

Statistical analysis
All data were presented as mean ± SD. The statistical analysis was performed by Prism 8. One-way or two-way ANOVA test was used as indicated in the figure legends.

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