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FANCD2 protects against bone marrow injury from ferroptosis

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Abstract

Bone marrow injury remains a serious concern in traditional cancer treatment. Ferroptosis is an iron- and oxidative-dependent form of regulated cell death that has become part of an emerging strategy for chemotherapy. However, the key regulator of ferroptosis in bone marrow injury remains unknown. Here, we show that Fanconi anemia complementation group D2 (FANCD2), a nuclear protein involved in DNA damage repair, protects against ferroptosis-mediated injury in bone marrow stromal cells (BMSCs). The classical ferroptosis inducer erastin remarkably increased the levels of monoubiquitinated FANCD2, which in turn limited DNA damage in BMSCs. FANCD2-deficient BMSCs were more sensitive to erastin-induced ferroptosis (but not autophagy) than FANCD2 wild-type cells. Knockout of FANCD2 increased ferroptosis-associated biochemical events (e.g., ferrous iron accumulation, glutathione depletion, and malondialdehyde production). Mechanically, FANCD2 regulated genes and/or expression of proteins involved in iron metabolism (e.g., FTH1, TF, TFRC, HAMP, HSPB1, SLC40A1, and STEAP3) and lipid peroxidation (e.g., GPX4). Collectively, these findings indicate that FANCD2 plays a novel role in the negative regulation of ferroptosis. FANCD2 could represent an amenable target for the development of novel anticancer therapies aiming to reduce the side effects of ferroptosis inducers.

Keywords

FANCD2; Ferroptosis; Bone marrow; DNA damage; Iron; Lipid peroxidation

1. Introduction

Cell death plays an important role in tumorigenesis, tumor progression, and antitumor therapy. Many of the novel anticancer agents being developed target pathways involved in various types of regulated cell death such as apoptosis, necroptosis, and autophagic cell

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death [1–3]. However, bone marrow injury is one of the most common dose-limiting side effects of traditional cancer therapy [4]. It can lead to dose reductions and delays in therapy. Analysis of the cell death signaling pathway in bone marrow cells may help identify novel therapeutic strategies to reduce side effects from anticancer agents.

Ferroptosis is a recently identified regulated type of cell death in both cancer and non-cancer cells [5]. Unlike apoptosis and necroptosis, ferroptosis does not involve activation of caspase and mixed lineage kinase domain-like protein, respectively [6]. In contrast, iron accumulation and lipid peroxidation contribute to ferroptosis [6]. Pharmacologic induction of ferroptosis by small molecules such as erastin becomes an emerging strategy for the treatment of a variety of cancers, especially kidney cancer [7], leukemia [7,8], and hepatocellular carcinoma [9–12]. However, it is unclear whether ferroptotic stimuli cause injury in bone marrow cells.

In the present study, we provide the first evidence that Fanconi anemia complementation group D2 (FANCD2), a nuclear protein involved in the repair of DNA damage [13,14], protects against ferroptosis-mediated damage in bone marrow stromal cells (BMSCs). BMSCs are multipotent cells that support hematopoietic reconstitution and immunomodulation. Compared to FANCD2 wild-type BMSCs, FANCD2-deficient BMSCs were more sensitive to erastin-induced death with increased DNA damage. Mechanically, FANCD2 inhibited iron accumulation and lipid peroxidation during ferroptosis by both transcription-dependent and -independent mechanisms. Our findings not only reveal a novel molecular mechanism of the DNA damage response pathway during ferroptosis, but also provide a potent approach to reduce side effects from ferroptosis-targeted anticancer therapies.

2. Methods

2.1. Regents

The antibodies to SLC7A11, p-H2AX, and FTH1 were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody to GPX4 and FANCD2 was obtained from Abcam (Cambridge, MA, USA). The antibody to LC3 was purchased from Novus (Littleton, CO, USA). The antibody to actin was obtained from Sigma Aldrich (Milwaukee, WI, USA). Erastin, ferrostatin-1, and liproxstatin-1 were purchased from Selleck Chemicals (Houston, TX, USA), β-mercaptoethanol, 3-methladenine, *N*-acetyl-L-cysteine, and chloroquine were purchased from Sigma Aldrich (Milwaukee, WI, USA).

2.2. Long-term bone marrow cultures

Long-term bone marrow cultures were established from the femurs and tibia marrow of FANCD2$^{+/+}$ and FANCD2$^{-/-}$mice as described previously [15,16]. Briefly, the contents of femurs and tibias were flushed into McCoy's 5A medium (Gibco, Gaithersburg, MD, USA) supplemented with 25% horse serum (Cambrex, Rockland, ME, USA) and hydrocortisone sodium hemisuccinate. Cultures were incubated at 33°C in 7% CO2. After four weeks, the horse serum was replaced with 25% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA). Finally, these cells were cultured in Dulbecco's Modified Eagle's Medium

(Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS and 100U/mL penicillin (Invitrogen, Grand Island, NY, USA) in a humidified incubator with 5% CO_2 and 95% air.

2.3. Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. CCK-8 uses WST-8 [(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium. monosodium salt)] to evaluate cell viability.

2.4. Quantitative reverse transcription-polymerase chain reaction (Q-PCR) analysis

Total RNA was extracted and purified from cultured cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The RNA was quantified by determining absorbance at 260 nm. One μg of total RNA from each sample was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) in a volume of 20 μl cDNA from cell samples was amplified with specific primers (SLC40A1:5′-CTTGCAGCAACTGTGTCACCGT-3' and 5′-CCATAGTCTCTGTCAGCCTGCT-3'; NRF2: 5′-TGGTCCGTGGACCAGTGTATAG-3′ and 5′-TGACGGCACCAAGCACATTACG-3'; FTH1: 5′-GCACACTCCATTGCATTCAGCC-3 5′-GCCGAGAAACTGATGAAGCTGC-3; FTL1: 5′- CCTGATTCAGGTTCTTCTCCATG-3 and 5′-CCTCGAGTTTCAGAACGATCGC-3'; HAMP: 5′-CAGATGGGGAAGTTGGTGTCTC-3′ and 5′-CAGCACCACCTATCTCCATCAAC-3'; TF: 5′-CAGTGGTCTCTGCTGACTCACA-3'; 5′-GTCACTGCCATTCGGAATCAGC-3'; TFRC: 5′-CAACCACTCAGTGGCACCAACA-3′ and 5′-GAAGTCCAGTGTGGGAACAGGT-3'; GPX4: 5′- CTTATCCAGGCAGACCATGTGC-3′ and 5′-CCTCTGCTGCAAGAGCCTCCC-3'; STEAP3: 5′-CTGGCTGATCACTGCAGATGAG-3′ and 5′-TCTTCAGCACCGCCAGTCTAAC-3'; HSPB1: 5′-TGAAGCACCGAGAGATGTAGCC-3′ and 5′-GCTCACAGTGAAGACCAAGGAAG-3′). The data was normalized to actin RNA (5′-CATTGCTGACAGGATGCAGAAGG-3′ and 5′-TGCTGGAAGGTGGACAGTGAGG-3′). Quantitative real time PCR was performed using ssoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) on the C1000 Touch Thermocycler CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Analysis was performed using the Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA, USA).

2.5. Western blot

Proteins in the cell lysate or supernatants were resolved on 4%–12% Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (pore size 0.22 μM) as previously described [17]. After blocking with 5% milk, the membrane was incubated for 2 h at 25°C or overnight at 4°C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies (1:3000) for 1 h at routine temperature, the signals were visualized using chemiluminescence (Pierce, Rockford, IL, USA).

2.6. Iron assay

Intracellular ferrous iron level was determined using an iron assay kit purchased from Sigma Aldrich (Milwaukee, WI, USA) according to the manufacturer's instructions.

2.7. Lipid peroxidation assay

The concentration of malondialdehyde, one of end products of lipid peroxidation, was assessed using a lipid peroxidation assay kit purchased from Sigma Aldrich (Milwaukee, WI, USA) according to the manufacturer's instructions.

2.8. Glutathione assay

The glutathione concentration in cell lysates was assessed using a glutathione assay kit purchased from Sigma Aldrich (Milwaukee, WI, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

Data are expressed as means ± SD. Significance of differences between groups was determined using two-tailed Student's *t*-test. A p-value < 0.05 was considered significant.

3. Results

3.1. FANCD2 inhibits erastin-induced DNA damage in BMSCs

We first assayed FANCD2 protein expression in response to treatment with erastin, the classical ferroptosis inducer [6]. Western blot analysis showed that both the unmodified (FANCD2-S) and the monoubiquitinated (FANCD2-L) FANCD2 forms were remarkably upregulated in BMSCs following erastin treatment for 24 h (Fig. 1A). In particular, erastin-induced FANCD2-L expression (Fig. 1B) was higher than FANCD2-S expression (Fig. 1C). Given that FANCD2-L is induced and important for repairing DNA damage [18], we next assayed whether erastin affects phosphorylated H2AX (p-H2AX, a marker of DNA damage [19]) expression in BMSCs from $FANCD2^{+/+}$ and $FANCD2^{-/-}$ mice. Indeed, the expression of p-H2AX was significantly induced in FANCD2 wild-type BMSCs following erastin treatment (Fig. 1A–D). Importantly, knockout of FANCD2 increased erastin-induced p-H2AX expression in FANCD2-deficient BMSCs (Fig. 1A–D). These findings indicate that inducible FANCD2 protects against erastin-induced DNA damage in BMSCs.

3.2. FANCD2 suppresses erastin-induced ferroptosis in BMSCs

To investigate the role of FANCD2 in the regulation of erastin-mediated cytotoxicity, we compared the cell viability of FANCD2-deficient and wild type BMSCs following dose-dependent erastin treatment (0.625−2.5 μM) for 24 h. CCK8 cell viability assay showed that FANCD2-deficient BMSCs were more sensitive to erastin-induced cell death compared with FANCD2 wild-type BMSCs (Fig. 2A, B), suggesting that FANCD2 plays a role in the protection against erastin-induced BMSC death.

Erastin has recently been demonstrated to induce autophagy, which contributes to ferroptosis in both cancer and non-cancer cells [20–22]. To characterize the role of FANCD2 in erastin-

induced cell death, we treated FANCD2-deficient and wild type BMSCs with erastin in the absence or presence of inhibitors for ferroptosis and autophagy. As expected, ferroptosis inhibitors (e.g., ferrostatin-1, liprostatin-1, β-mercaptoethanol, and *N*-acetylcys-teine) [5] reversed erastin-induced cell death in both FANCD2-deficient and wild type BMSCs (Fig. 2C). However, autophagy inhibitors (e.g., chloroquine and 3-methyladenine) [23] only inhibited erastin-induced cell death in FANCD2 wild-type BMSCs, but not FANCD2-deficient BMSCs (Fig. 2C). Tracking the conversion of microtubule-associated protein light chain 3 (LC3)-I to LC3-II is indicative of autophagic activity [24]. Moreover, western blot analysis showed that erastin-induced LC3-II expression was not affected by the loss of FANCD2 in BMSCs (Fig. 2D–E). These data suggest that FANCD2 inhibits erastin-induced ferroptosis, but not autophagy, in BMSCs.

3.3. FANCD2 reduces iron accumulation and lipid peroxidation in ferroptosis

To determine the molecular mechanisms of action of FANCD2 in ferroptosis, we assayed iron accumulation and lipid peroxidation, two important signaling events in triggering ferroptosis. In particular, ferrous iron (Fe^{2+}) participates in the Fenton reaction, resulting in the generation of reactive oxygen species (ROS), which goes on to trigger ferroptosis [6]. Intracellular Fe^{2+} levels were significantly increased in FANCD2-deficient BMSCs with or without erastin treatment (Fig. 3A), suggesting that FANCD2 plays a role in the regulation of Fe^{2+} accumulation during ferroptosis. In addition to iron overload, erastin triggers glutathione (GSH) depletion, which causes lipid peroxidation in the induction of ferroptosis [7]. Erastin-induced GSH depletion was increased in FANCD2-deficient BMSCs (Fig. 3B). Moreover, the end products of lipid peroxidation such as malondialdehyde (MDA) were elevated in FANCD2-deficient BMSCs following erastin treatment (Fig. 3C). Thus, FANCD2 inhibits Fe^{2+} accumulation and lipid peroxidation in erastin-induced ferroptosis.

3.4. FANCD2 regulates ferroptosis-associated gene expression in BMSCs

Several genes have recently been identified to regulate ferroptosis by directly or indirectly targeting iron metabolism and lipid peroxidation [7,9,10,25–29]. To further define the role of FANCD2 in ferroptosis, the mRNA expression of 10 important genes (FTH1 [ferritin, heavy polypeptide 1], FTL1 [ferritin, light polypeptide 1], GPX4 [glutathione peroxidase 4], HAMP [hepcidin antimicrobial peptide], HSPB1 [heat shock protein family B (small) member 1], NRF2 [nuclear factor, erythroid 2 like 2], SLC40A1 [solute carrier family 40 member 1], STEAP3 [six-transmembrane epithelial antigen of prostate 3], TF [transferrin], and TFRC [transferrin receptor]) involved in iron metabolism and lipid peroxidation was assayed in both FANCD2-deficient and wild type BMSCs (Fig. 4A). Among them, loss of FANCD2 significantly inhibited erastin-induced mRNA expression of FTH1 (an inhibitor of ferroptosis by binding Fe^{2+} [30]) and STEAP3 (a metalloreductase capable of converting iron from Fe^{3+} to Fe^{2+}). In contrast, the mRNA expression of SLC40A1 (also termed ferroportin 1, the sole cellular efflux channel for iron), TF, and TFRC were significantly higher in FANCD2-deficient BMSCs following erastin treatment. The basic expressions of negative regulators of ferroptosis, inducing HAMP [31] and HSPB1 [32], but not NRF2 [10], were downregulated in FANCD2-deficient BMSCs.

GPX4 is a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation in ferroptosis [7]. The mRNA expression of GPX4 was mildly downregulated by FANCD2-deficient BMSCs (Fig. 4A). However, the protein expression of GPX4 was significantly inhibited in FANCD2-deficient BMSCs with or without erastin treatment (Fig. 4B and C). Consistent with mRNA assay (Fig. 4A), erastin-induced FTH1 protein expression was also suppressed FANCD2-deficient BMSCs (Fig. 4B and D). SLC7A11 is a major component of system X_c^-, which exchanges intracellular glutamate for extracellular cysteine to the synthesis of GSH [33]. The increased protein level of SLC7A11 in FANCD2-deficient BMSCs indicates a negative feedback of decreased GSH level (Fig. 4B–E).

Collectively, these findings confirm previous results that FANCD2 can regulate protein expression by both transcription-dependent and -independent mechanisms [34,35].

4. Discussion

The dysregulated DNA damage response contributes to various types of regulated cell death such as apoptosis, necroptosis, and autophagy [36]. In the current study, we demonstrated that FANCD2, a critical DNA damage repair gene, plays a novel role in the inhibition of erastin-induced ferroptosis in BMSCs. The pathway through which FANCD2 inhibits ferroptosis involves transcriptional regulation of target genes, as well as transcription-independent functions of FANCD2, possibly reflecting distinct mechanisms of FANCD2 action under different stressors

FANCD2 contributes heterogeneously to Fanconi anemia, a genetic disorder characterized by chromosomal breakage, bone marrow failure, and hypersensitivity to DNA crosslinking agents, as well as cancer [37]. FANCD2 is monoubiquitinated both during the S phase and in response to various DNA damaging agents, including ionizing radiation and mitomycin C [13,38]. In this study, we showed that the expression of FANCD2, especially monoubiquitinated FANCD2, is significantly increased in response to erastin. Although it is clear from these observations that loss of FANCD2 increased erastin-induced DNA damage, it has not been determined how this process is coupled to the DNA-damage response or to S-phase progression in ferroptosis.

The original study concluded that ferroptosis is remarkably distinct from other types of regulated cell death such as apoptosis, necroptosis, and autophagic cell death [6]. In contrast, recent studies demonstrated that autophagy (a lysosomal degradation pathway) plays a critical role in promoting ferroptosis. In partially, nuclear receptor coactivator 4 (NCOA4) is a selective cargo receptor for the selective autophagic turnover of ferritin in ferroptosis [21]. Knockdown of NCOA4 inhibits ferroptosis, whereas overexpression of NCOA4 promotes ferroptosis [21]. We demonstrated here that FANCD2 inhibited ferroptosis in BMSCs in an autophagy-independent manner, although FANCD2 may play a role in autophagy in other cells [34]. Thus, FANCD2 plays dual roles in autophagy depending on the death stimulus, cell type, or context.

Our results indicate that loss of FANCD2 increased lipid peroxidation in ferroptosis. Several antioxidants (e.g., ferrostatin-1, liprostatin-1, 2-mercaptoethanol, and *N*-acetylcysteine)

prevent ferroptosis in FANCD2-deficient BMSCs. These observations are supported by studies performed on FANCD2$^{-/-}$ mice in tumorigenesis and radiotherapy. For example, antioxidants (e.g., tempol and resveratrol) reduce cancer incidence and hematopoietic defects in FANCD2$^{-/-}$ mice [39]. Intraoral mitochondrial-targeted GS-nitroxide (also termed JP4-039) has the ability to protect against radiation-induced normal tissue injury in tumor-bearing FANCD2$^{-/-}$ mice [16,40–43]. Lipid peroxidation in mitochondria promotes ferroptosis. Inhibiting mitochondrial iron uptake by CDGSH iron sulfur domain 1 (CISD1) protects against ferroptosis in hepatocellular carcinoma cells [28]. Unlike CISD1 [28], FANCD2 inhibited erastin-induced GPX4 protein degradation, suggesting that FANCD2 plays a transcription-independent role in the regulation of lipid peroxidation in ferroptosis. NRF2 is an emerging regulator of cellular resistance to ferroptosis in hepatocellular carcinoma cells [9,10]. However, FANCD2 has no significant effects on erastin-induced NRF2 expression in BMSCs. The recently identified acyl-CoA synthetase long-chain family member 4 (ACSL4) was required for production of 5-hydroxyeicosatetraenoic acid (5-HETE) to induce ferroptosis [27]. It remains unclear whether FANCD2 regulates the ACSL4-5-HETE pathway in ferroptosis.

Iron is a redox active metal involved in the regulation of both cell survival and death. Mammalian cells require sufficient amounts of iron to sustain enzymatic activities and cellular function. Excessive iron contributes to forms of cell death such as ferroptosis through producing ROS [44]. Disruption of iron metabolism plays a key role in the etiology of Fanconi anemia. Fanconi anemia patients receive deferiprone therapy after hematopoietic stem cell transplantation due to iron overload [45]. Iron metabolism is controlled by diverse genes at different levels [46]. FANCD2-deficient BMSCs exhibited iron overload with increased gene expression for iron uptake (e.g., TF, TFRC, and HSPB1) and decreased gene expression for iron storage (e.g., FTH1) and iron export (e.g., HAMP). Iron-chelation therapy may reduce ferroptosis-mediated injury in FANCD2-deficient BMSCs.

In summary, our studies have identified a critical role of FANCD2 in the protection against ferroptosis in BMSCs. FANCD2 suppresses ferroptosis by modulation of iron metabolism and lipid peroxidation at multiple levels. Further functional studies are needed to define the interplay between FANCD2 and iron-responsive transcription factors (e.g., iron responsive element binding protein 2) in ferroptosis.

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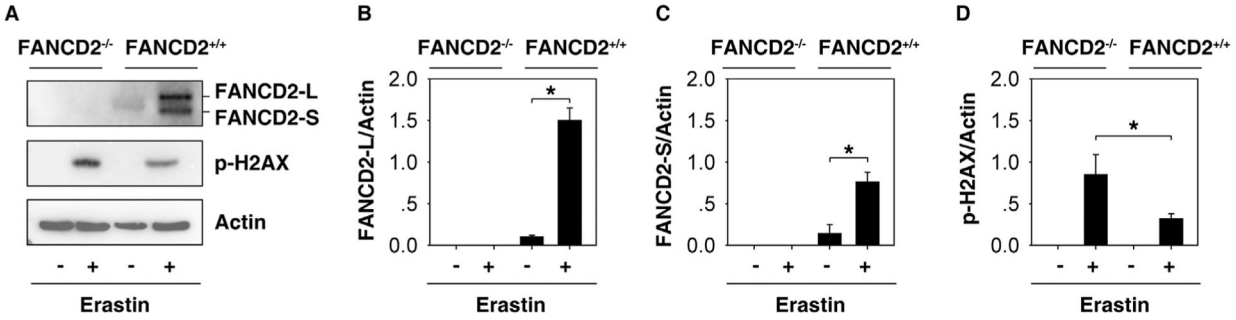

Fig. 1. FANCD2 inhibits erastin-induced DNA damage in BMSCs.

(A) FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs were treated with erastin (1.25 μM) for 24 h and expressions of indicated proteins were assayed by western blot. (B–D) In parallel, the relative expression level was quantified by densitometry analysis of bands and normalized to actin protein (n = 3, *P < 0.05).

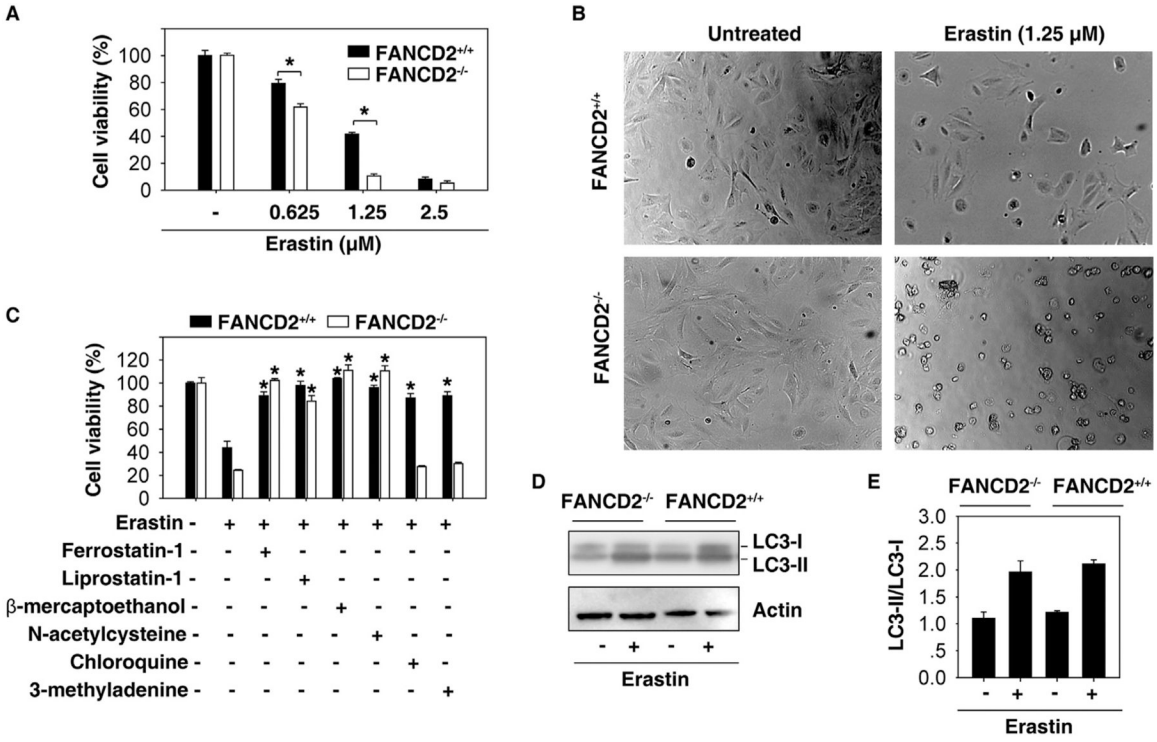

Fig. 2. FANCD2 suppresses erastin-induced ferroptosis in BMSCs.

(A) FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs were treated with erastin (0.625−2.5 (μM) for 24 h and cell viability was assayed (n = 3, *P < 0.05). (B) Interference contrast images of FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs with or without erastin (1.25 (μM) treatment for 24 h (C) FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs were treated with erastin (1.25 (μM) in the absence or presence of ferroptosis inhibitor (ferrostatin-1, 500 nM; liprostatin-1, 200 nM; β-mercaptoethanol, 50 (μM; *N*-acetylcysteine, 100 mM) or autophagy inhibitor (chloroquine, 10 μM; 3-methyladenine, 5 mM) for 24 h. Cell viability was assayed (n = 3, *P < 0.05 versus erastin treatment group). (D) Western blot analysis of LC3-I and LC3-II expression in FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs following erastin (1.25 (μM) treatment for 24 h. (E) In parallel, the LC3-II/I ratio quantified by densitometry analysis of bands.

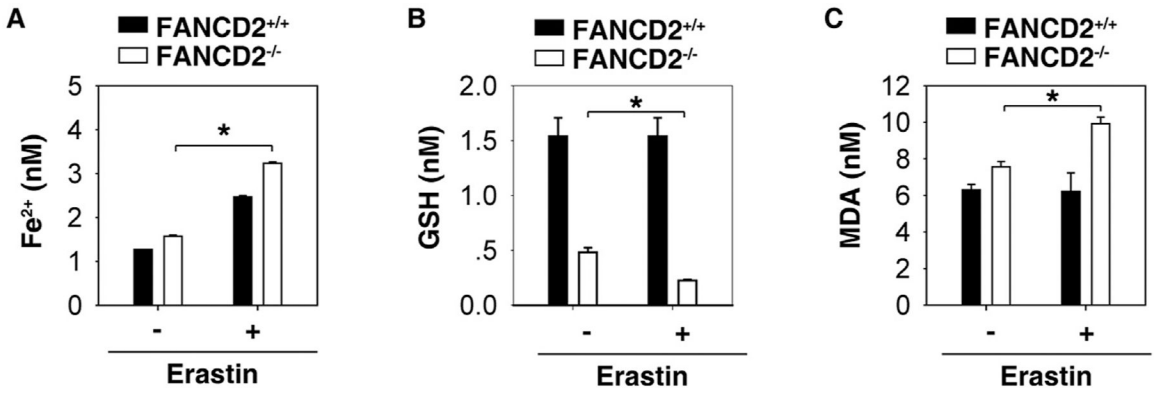

Fig. 3. FANCD2 reduces iron accumulation and lipid peroxidation in ferroptosis.

FANCD2$^{+/+}$ and FANCD2 $^{-/-}$ BMSCs were treated with erastin (1.25 (μM) for 24 h. The intracellular levels of Fe^{2+} (A), GSH (B), and MDA (C) were assayed (n = 3, *P < 0.05).

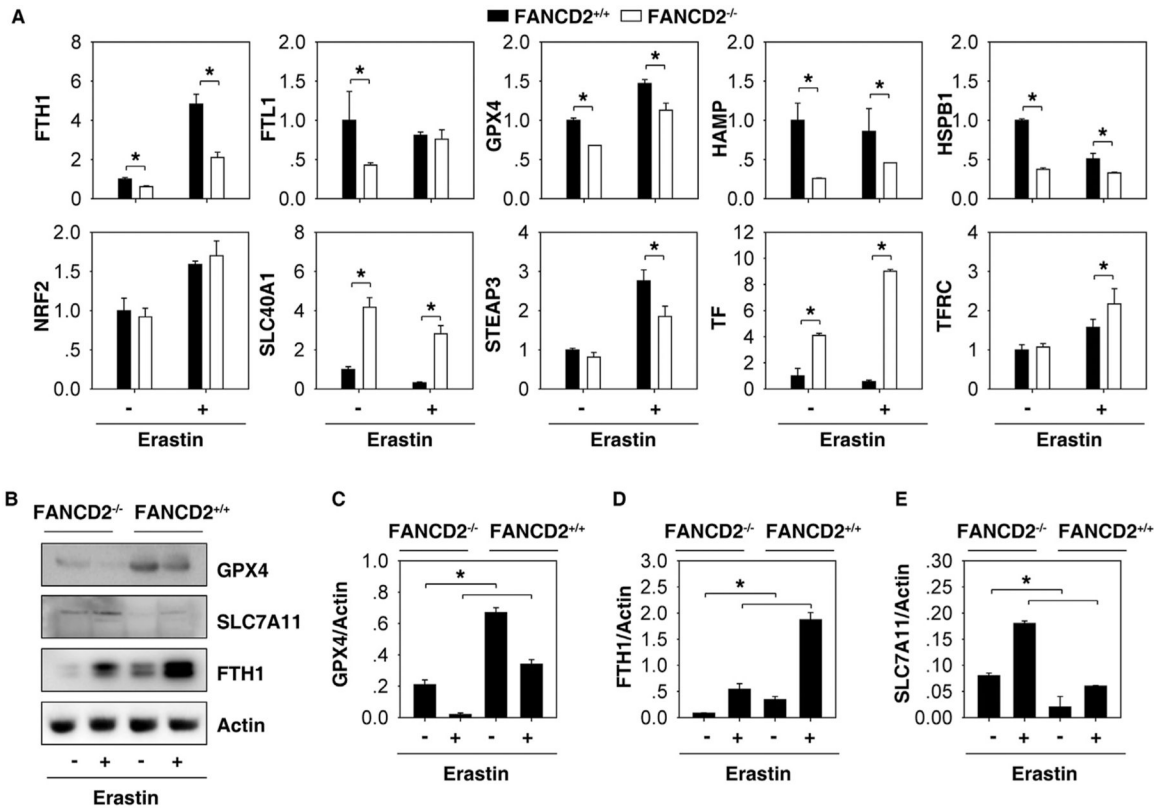

Fig. 4. FANCD2 regulates ferroptosis-associated gene expression in BMSCs.

(A) FANCD2$^{+/+}$ and FANCD2$^{-/-}$ BMSCs were treated with erastin (1.25 μM) for 24 h. Expressions of the indicated genes were assayed by Q-PCR (n = 3, *P < 0.05). (B) Western blot analysis of GPX4, SLC7A11, and FTH1 expression in FANCD2$^{+/+}$ and FANCD2 $^{-/-}$ BMSCs following erastin (1.25 μM) treatment for 24 h (C–E) In parallel, the relative expression level quantified by densitometry analysis of bands and normalized to actin protein (n = 3, *P < 0.05).