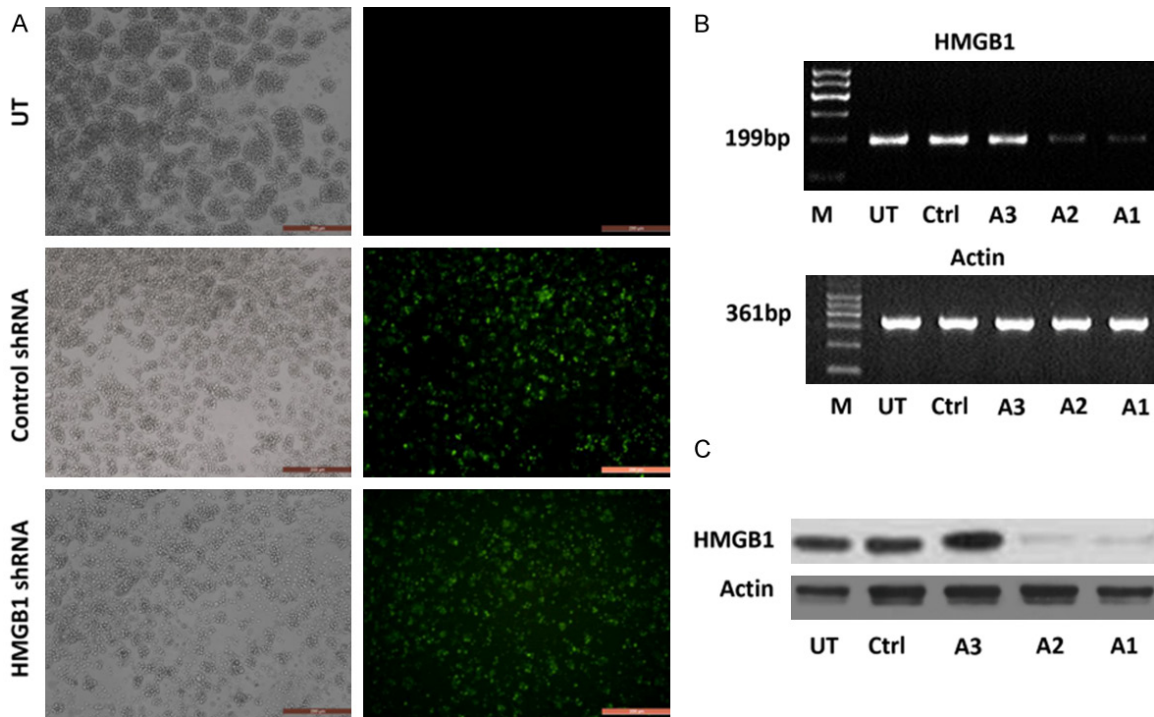
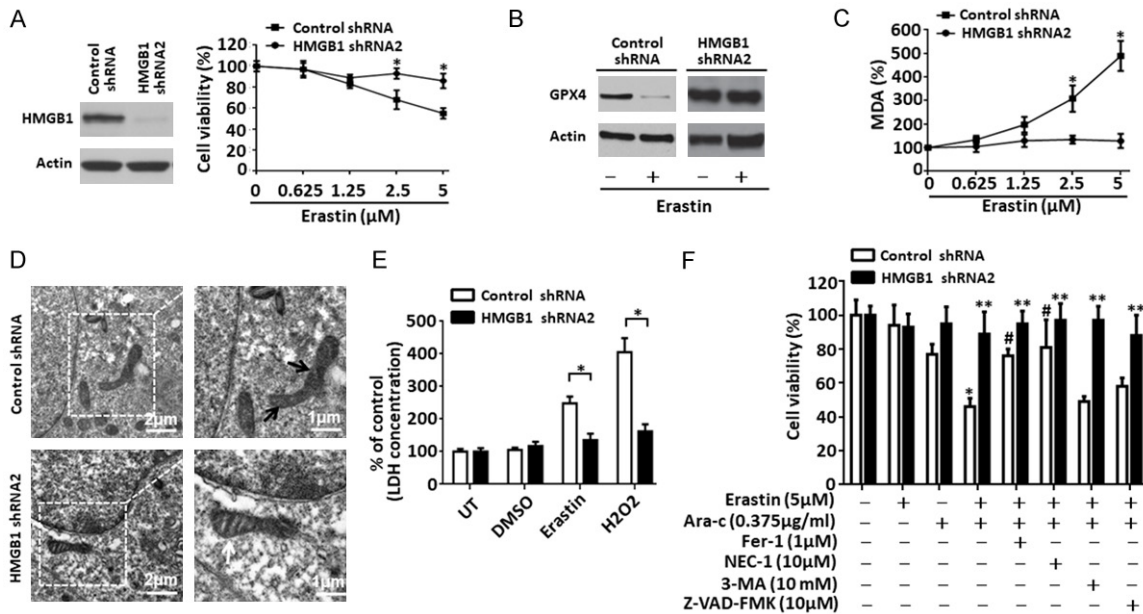


## HMGB1 regulates ferroptosis in leukemia



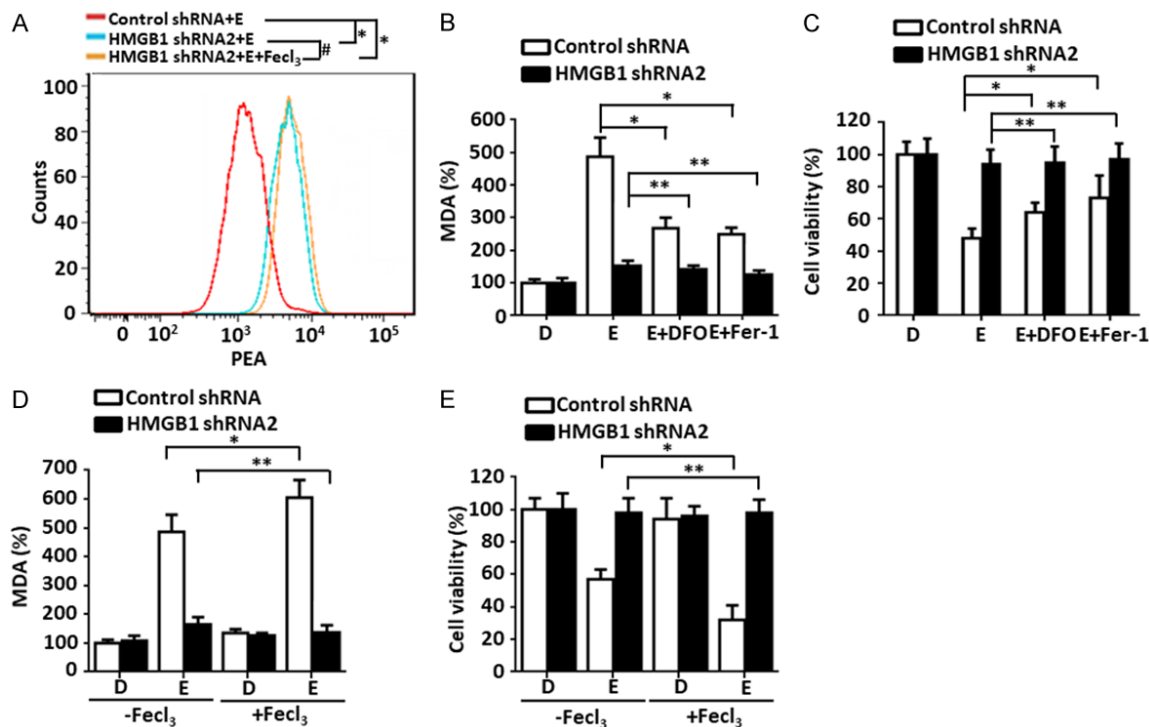
**Figure S1.** Verification of HMGB1 knockout effect. A. HL-60/NRAS<sup>O61L</sup> cells were incubated with HMGB1 shRNA and control shRNA transfection for 48 h according to the manufacturer's instructions and our previous study (multiplicity of infection [MOI] = 50) and visualized using a fluorescence microscope (magnification, 40 ×). Untransfected cells did not express the vector. B and C. After transfection with HMGB1 shRNA or control shRNA for 48 h, HMGB1 levels were, respectively, detected by RT-PCR and western blot analysis for HL-60/NRAS<sup>O61L</sup> cells. Actin was used as a loading control. M, marker; U, untreated; Ctrl, control; A1, HMGB1 shRNA1; A2, HMGB1 shRNA2; A3, HMGB1 shRNA3; Ctrl, Control shRNA.



**Figure S2.** Depletion of HMGB1 (HMGB1 shRNA2) inhibits erastin-induced cell death and anticancer activity. A. HL-60/NRAS<sup>O61L</sup> cells were transfected with HMGB1 shRNA2 or control shRNA vector, HMGB1 expression was verified by western blot. Then, two groups of cells were stimulated with erastin at the indicated doses for 48 h. Cell viability was then assayed. ( $n = 3$ ,  $*P < 0.05$  versus the control group). B and C. HL-60/NRAS<sup>O61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector and then stimulated with erastin (5  $\mu$ M) for 48 h. GPX4 and intracellular

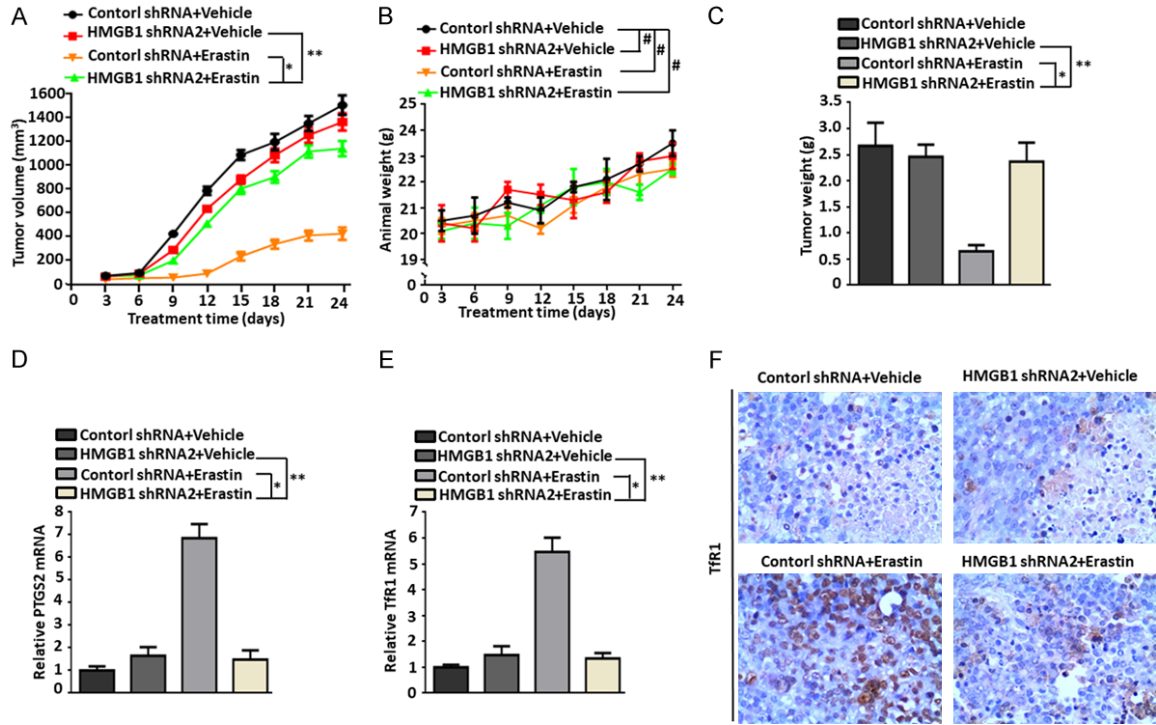
## HMGB1 regulates ferroptosis in leukemia

MDA levels were assayed ( $n = 3$ ,  $*P < 0.05$  versus the control group). D. Ultrastructural features of HL-60/NRAS<sup>Q61L</sup> cells with HMGB1 shRNA2 and control shRNA vector transfection plus erastin (5  $\mu$ M) treatment (white arrow, normal mitochondria; black arrow, shrunken mitochondria). E. HL-60/NRAS<sup>Q61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector and then stimulated with DMSO, erastin (5  $\mu$ M), and H<sub>2</sub>O<sub>2</sub> (50 mM) for 48 h. The LDH level in the culture medium was assayed. H<sub>2</sub>O<sub>2</sub> was used as a positive control. UT, untreated. ( $n = 3$ ,  $*P < 0.05$ ). F. HL-60/NRAS<sup>Q61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector and then stimulated with erastin (1.25  $\mu$ M) combined with cytarabine (Ara-C, 0.375  $\mu$ g/mL) in the absence or presence of the indicated inhibitors for 48 h. Cell viability was assayed. ( $n = 3$ ,  $*P < 0.05$  versus the erastin treatment only control shRNA group;  $#P < 0.05$  versus the erastin plus cytarabine treatment control shRNA group;  $**P > 0.05$  versus the untreated HMGB1 shRNA2 group). All experiments were conducted in triplicate, and the data are presented as the mean  $\pm$  SD. DFO, deferoxamine; Fer-1, ferrostatin-1; NEC-1, necrostatin-1; 3-MA, 3-methyladenine; Ara-C, cytarabine.



**Figure S3.** Depletion of HMGB1 (HMGB1 shRNA2) limits iron-mediated ROS generation and cell death. A. HL-60/NRAS<sup>Q61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector and then stimulated with erastin (5  $\mu$ M) with or without FeCl<sub>3</sub> (30  $\mu$ M, pretreated for 3 h) for 48 h. Then, cells were stained with PGSK, and the fluorescence profile of the stained cells was analyzed by flow cytometry. E, erastin. B and C. HL-60/NRAS<sup>Q61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector and then stimulated with erastin (5  $\mu$ M) combined with DFO (0.1 mM) and Fer-1 (1  $\mu$ M) for 48 h. Intracellular MDA levels and cell viability were assayed. D, DMSO; E, erastin. ( $n = 3$ ,  $*P < 0.05$ ,  $**P > 0.05$ ). D and E. HL-60/NRAS<sup>Q61L</sup> cells were transfected with HMGB1 shRNA2 and control shRNA vector, and then stimulated with erastin (5  $\mu$ M) with or without FeCl<sub>3</sub> (30  $\mu$ M, pretreated for 3 hours) for 48 h. Intracellular MDA levels and cell viability were then assayed. ( $n = 3$ ,  $*P < 0.05$ ,  $**P > 0.05$ ). Necrostatin-1 pretreatment was used in all experiments. All experiments were conducted in triplicate, and the data are presented as the mean  $\pm$  SD. D, DMSO; E, erastin; DFO, deferoxamine; DPL, diphenyleneiodonium chloride.

## HMGB1 regulates ferroptosis in leukemia



**Figure S4.** Knockdown of HMGB1 expression (HMGB1 shRNA2) inhibited anticancer activity of erastin in vivo. A-C. NOD/SCID mice were injected subcutaneously with HMGB1 shRNA2 HL-60/NRAS<sup>Q61L</sup> cells ( $1 \times 10^6$  cells/mouse) and treated with erastin (20 mg/kg i.v., twice every other day) starting at day seven for two weeks. Tumor volumes and animal weight were measured twice a week. At the termination of the experiments, all xenografts were removed and weighted ( $n = 4$  mice/roup,  $*P < 0.05$ ,  $**P > 0.05$ ,  $#P > 0.05$ ). D and E. qPCR analysis of PTGS2 and Tfr1 gene expressions in isolated tumors at the termination of experiments ( $*P < 0.05$ ,  $**P > 0.05$ ). F. Immunohistochemical staining of Tfr1 was performed with an isolated tumor at the termination of the experiments. All experiments were conducted in triplicate, and the data are presented as the mean  $\pm$  SD.