
Figures and figure supplements

Overburdened ferroptotic stress impairs tooth morphogenesis

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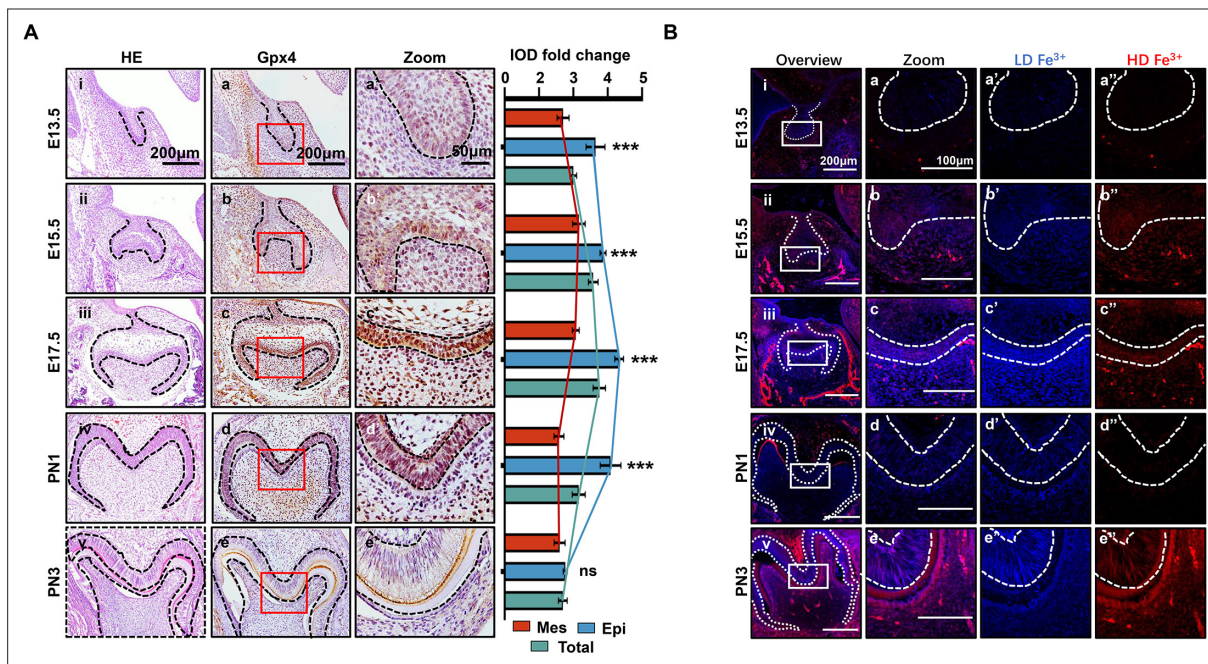


Figure 1. Spatiotemporal characterization of glutathione peroxidase 4 (Gpx4) expression and iron accumulation in tooth morphogenesis. **(A)** (i–v) HE staining for tooth germ in E13.5 to PN3, scale bars = 200 μ m. **(a–e)** Gpx4 expression detected by immunohistochemistry (IHC), scale bars = 200 μ m. **(a'–e')** Enlarged view of each Gpx4 staining, scale bars = 50 μ m; epithelia versus mesenchyme, $n=3$, *** $p<0.001$. **(B)** (i–v) Iron probe staining in for tooth germ in E13.5 to PN3, scale bars = 200 μ m. **(a–e)** Enlarged view of selected region, scale bars = 100 μ m, low concentration (**a'–e'**, blue) and high concentration of iron (**a''–e''**, red) are present, scale bars = 100 μ m. Epi, Epithelia; Mes, = mesenchyme.

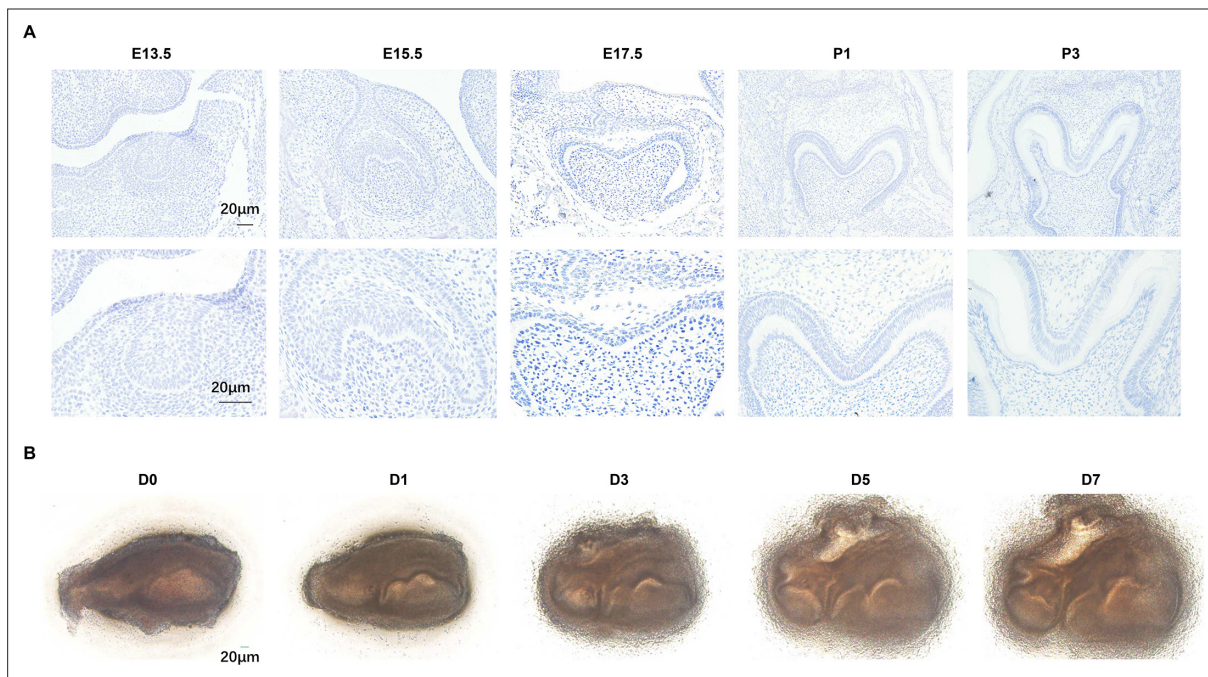


Figure 1—figure supplement 1. Negative control of IHC staining and the general view of ex vivo cultured tooth germ from D0 to D7. **(A)** Negative control of different developmental stage of molar germ in glutathione peroxidase 4 (Gpx4) immunohistochemistry (IHC) staining. **(B)** Ex vivo culture of tooth molar germ from D0 to D7. Scale bars = 20 μm.

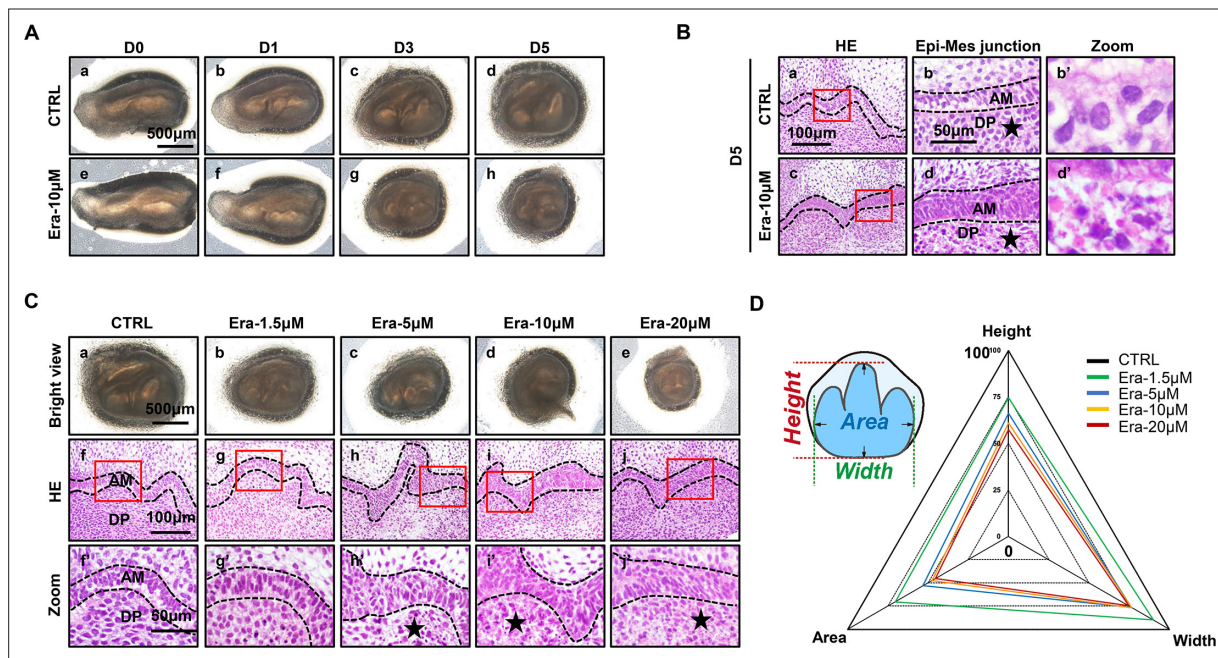


Figure 2. Erastin impairs tooth morphogenesis, especially within dental mesenchyme. **(A)** Gross anatomy of tooth germ cultured ex vivo for 5 d, scale bars = 500 µm. **(B)** **(a, c)** HE staining for tooth germ on day 5 (D5), scale bars = 100 µm. **(b, d)** High resolution of epi-mes junction papilla, scale bars = 50 µm; black stars point out necrotic-like cells (NLCs). **(b', d')** NLCs indicated by black stars. **(C)** **(a–e)** Gross anatomy of tooth germs in different concentrations of erastin on D5, scale bars = 500 µm. **(f–j)** HE staining of different concentration-treated tooth germ on D5. **(f'–j')** High-resolution view of epi-mes junction region of each tooth germ, scale bars = 50 µm. **(D)** Rada graph for calculation of height, width, and area in each tooth germ. Black dotted line outlines ameloblasts. AM, ameloblast; DP, dental papilla.

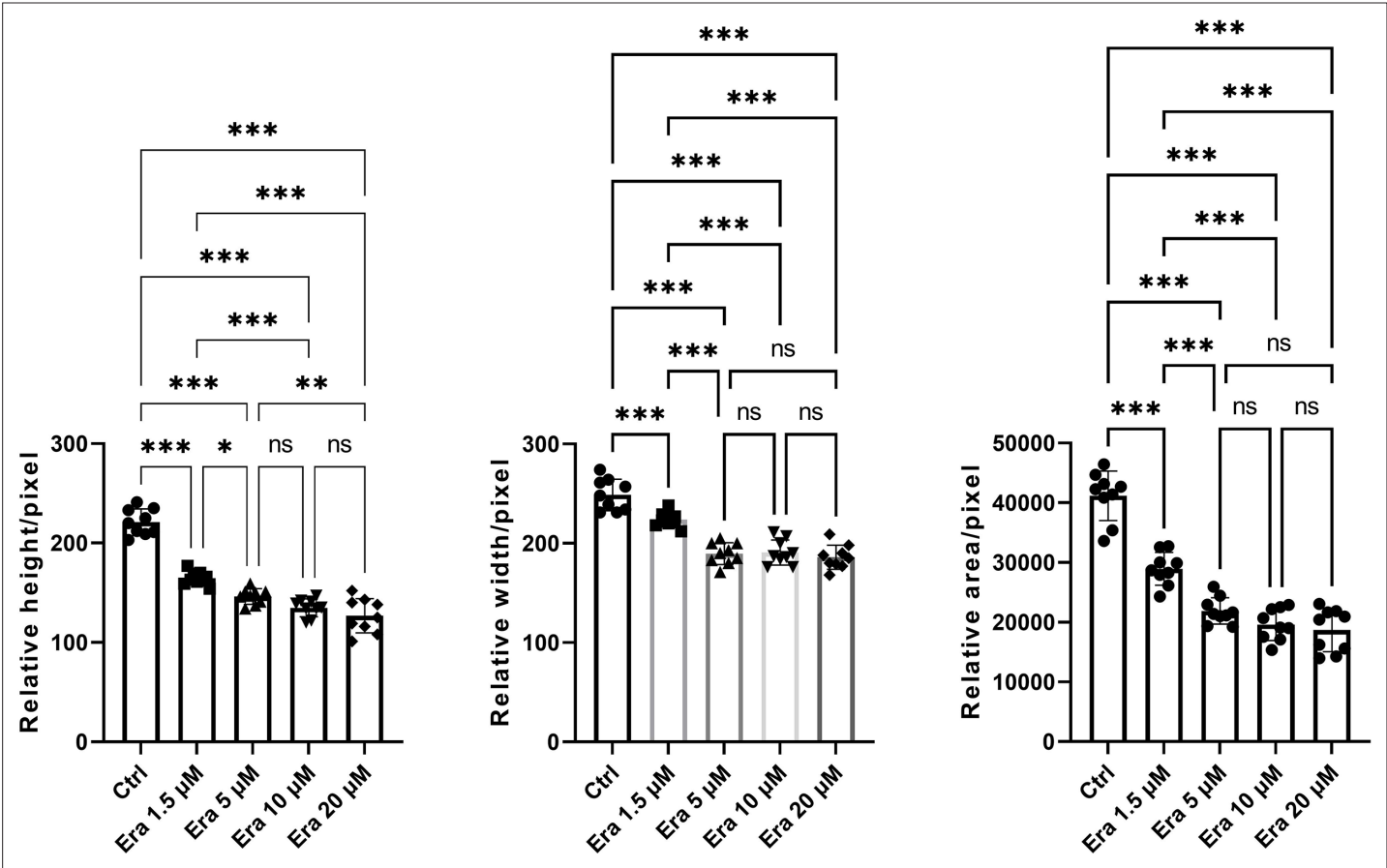


Figure 2—figure supplement 1. Original bar graphs of height, width, and area of erastin-treated tooth germ. *p<0.05, **p<0.01, ***p<0.001.

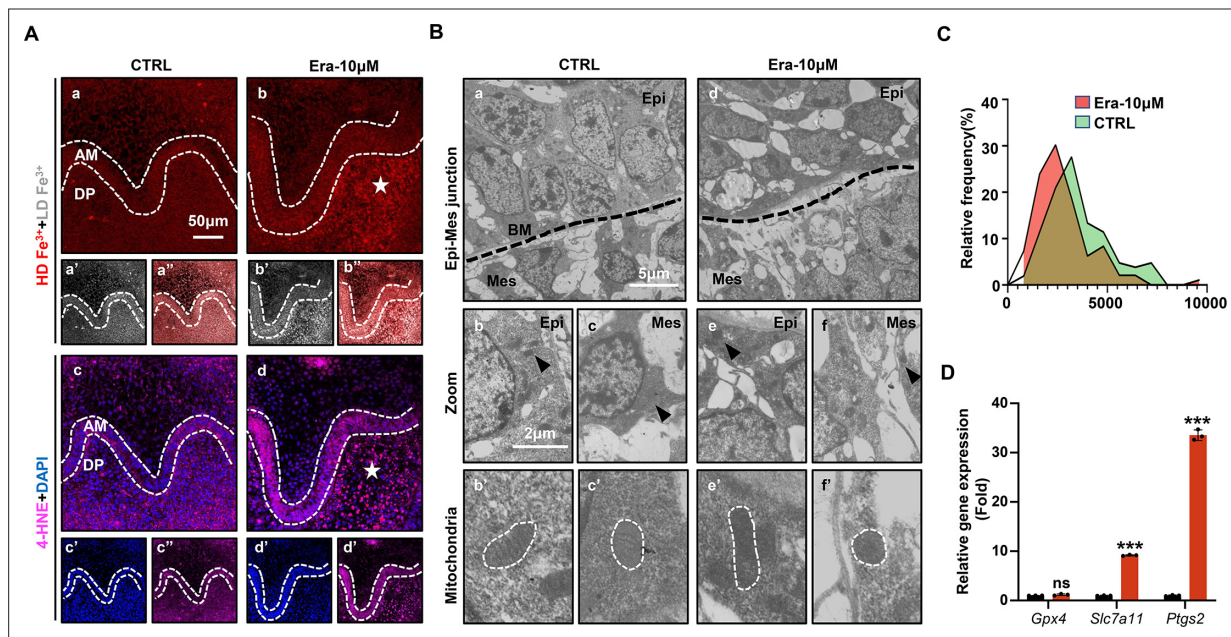


Figure 3. Ferroptosis is activated in erastin-treated molar germ. **(A)** (a, b) High-density Fe^{3+} (red) in CTRL and Era-10 μM of day 5 (D5); white star points out strong fluorescence signal of Fe^{3+} , scale bars = 50 μm . (a', b') Low-density Fe^{3+} (gray) and (a'', b'') merged view of iron probe staining. (c, d) Merged view of immunofluorescence (IF) staining of 4-hydroxy-2-nonenal (4-HNE) (magenta) and DAPI (blue), white star points out strong fluorescence signal of 4-HNE, scale bars = 5050 μm . (c', d') For DAPI and (c'', d'') 4-HNE. AM, ameloblast; DP, dental papilla; HD, high density; LD, low density. **(B)** Transmission electron microscope (TEM) scanning for CTRL and Era-10 μM on D5. (a, d) epi-mes junction area of CTRL and Era-10 μM on D5 are detected, scale bars = 5 μm . (b, c) Representative view of cells in epithelia and mesenchyme for CTRL, scale bars = 2 μm ; black arrow points out typical mitochondria in each region (b') for epithelia and (c') mesenchyme, outlined by the white dotted line. (e, f) Representative view of cells in epithelia and mesenchyme for Era-10 μM , scale bars = 2 μm ; black arrow points out typical mitochondria in each region (e') for epithelia and (f') mesenchyme, outlined by the white dotted line. **(C)** Relative frequency of the mitochondrial size in both groups. **(D)** Fold changes of gene expression in CTRL and Era-10 μM on D5 versus CTRL *** $p < 0.001$.

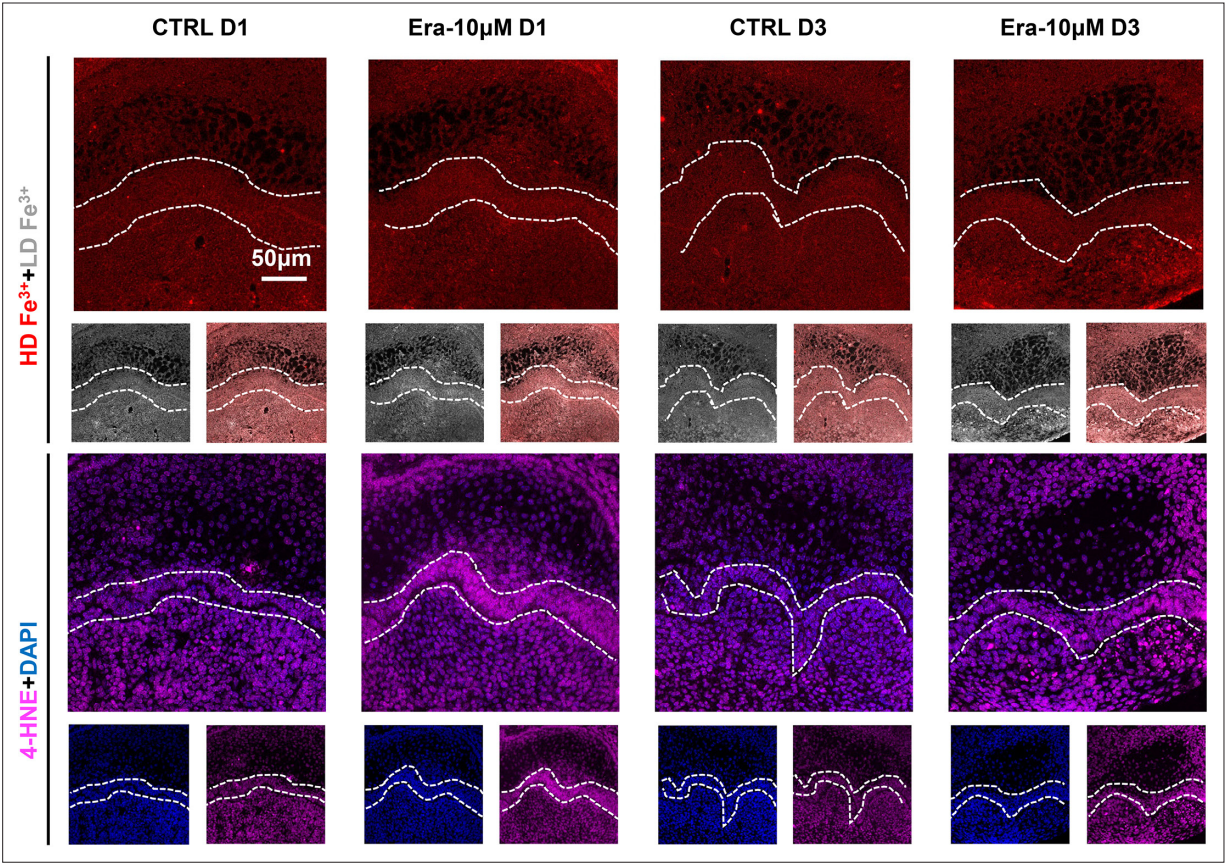


Figure 3—figure supplement 1. Results for iron accumulation and 4-hydroxy-2-nonenal (4-HNE) expression in Era-10 µM of D1 and D3. Scale bars = 50 µm.

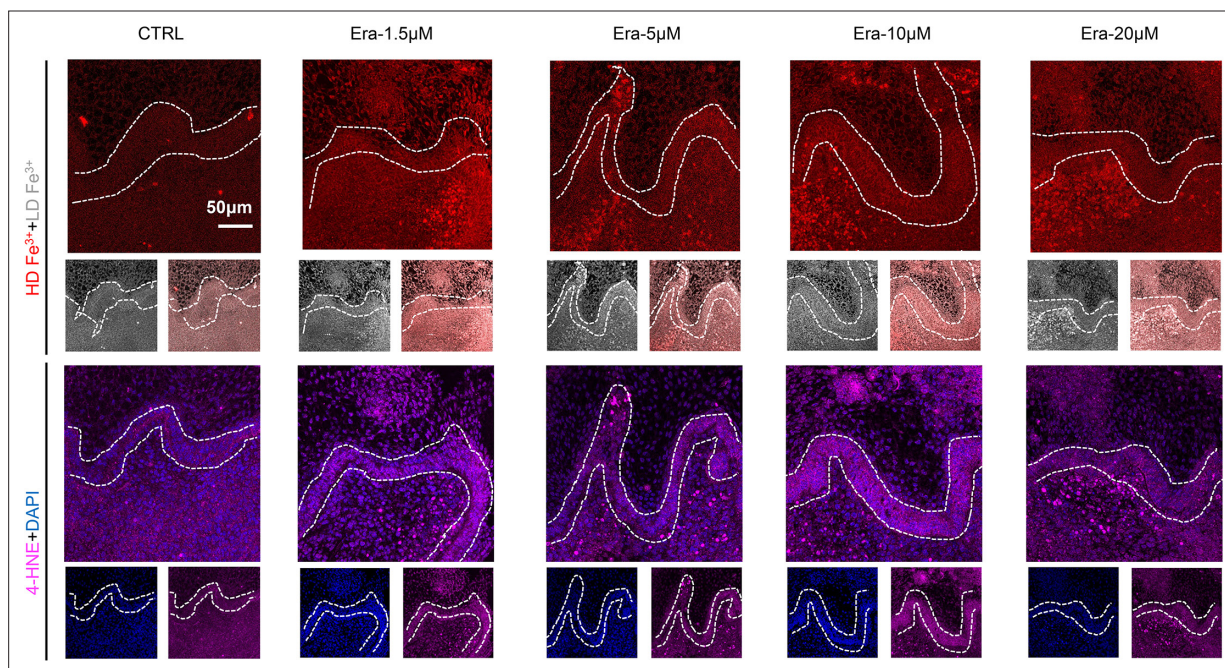


Figure 3—figure supplement 2. Results for iron accumulation and 4-hydroxy-2-nonenal (4-HNE) expression in Era-1.5 μM, Era-5 μM, Era-10 μM, and Era-20 μM of D5. Scale bars = 50 μm.

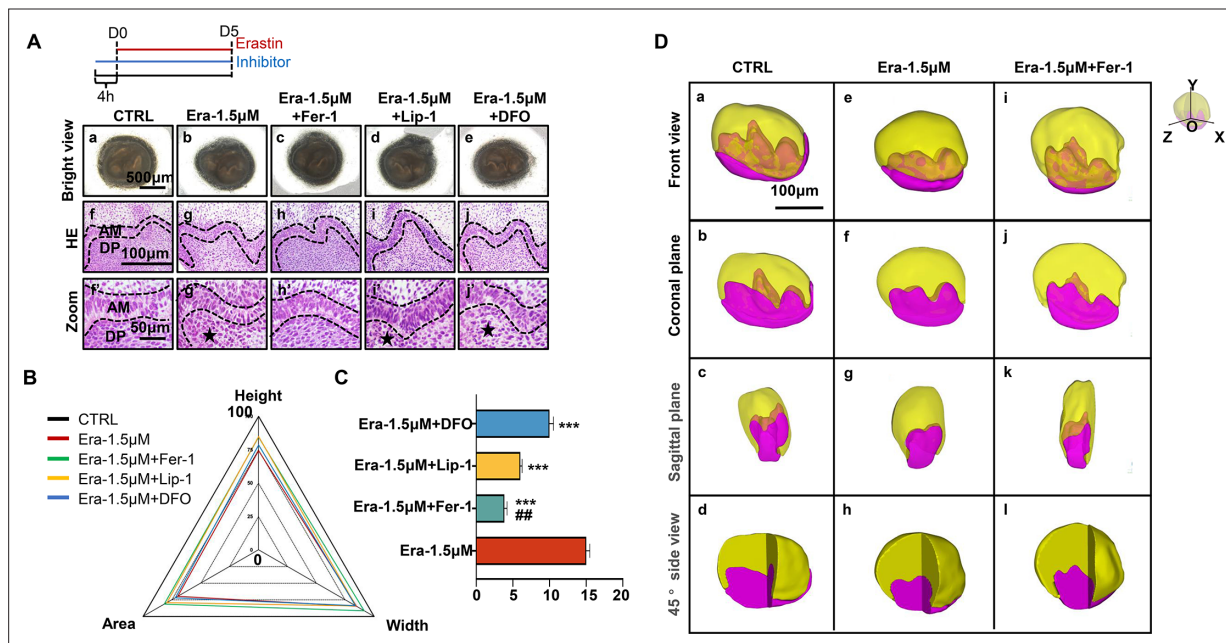


Figure 4. Ferroptotic inhibitors partially rescue erastin-impaired tooth organogenesis. **(A)** (a–e) Gross anatomy of tooth germs in the differently treated group, scale bars = 500 μm. (f–j) HE staining of differently treated tooth germ, scale bars = 100 μm. (f'–j') High-resolution view of epi-mes junction region of each tooth germ; black dotted line outlines ameloblasts, black star points out necrotic-like cells (NLCs). AM, ameloblast; DP, dental papilla; scale bars = 50 μm. **(B)** Rada graph for calculation of height, width, and area in each tooth germ. **(C)** Average number of NLCs in each group versus Era-1.5 μM, ***p<0.001, versus Era-1.5 μM + Lip-1, ##p<0.01. **(D)** 3D reconstructed view of tooth germ on day 5 (D5). (a–d) CTRL from the front view, coronal plane, sagittal plane, and 45° side view. (e–h) Era-1.5 μM and (i–l) Era-1.5 μM + Fer-1 are viewed the same way. Scale bars = 100 μm.

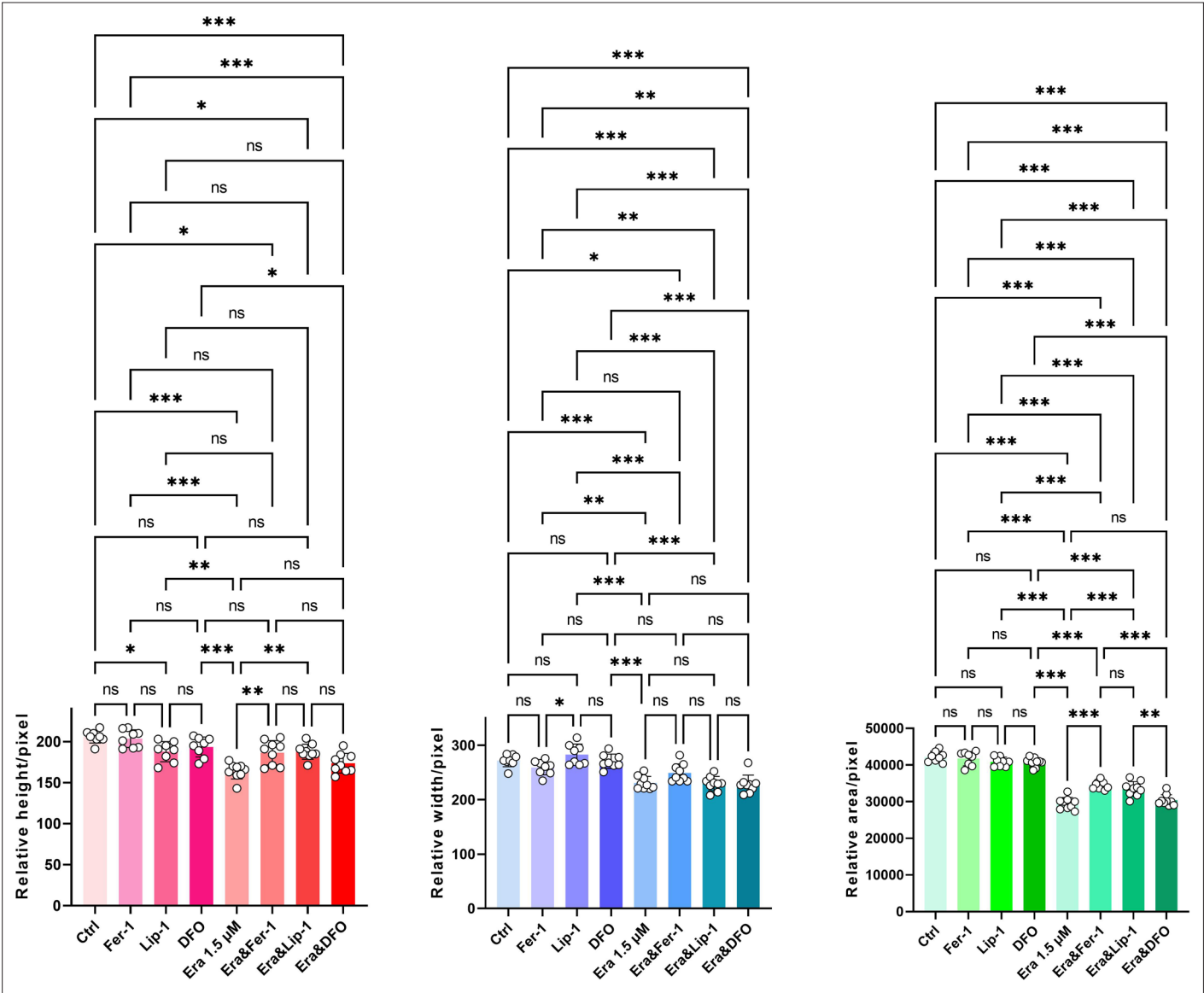


Figure 4—figure supplement 1. Original bar graphs of height, width, and area of tooth germ in rescue assay. *p<0.05, **p<0.01, ***p<0.001.

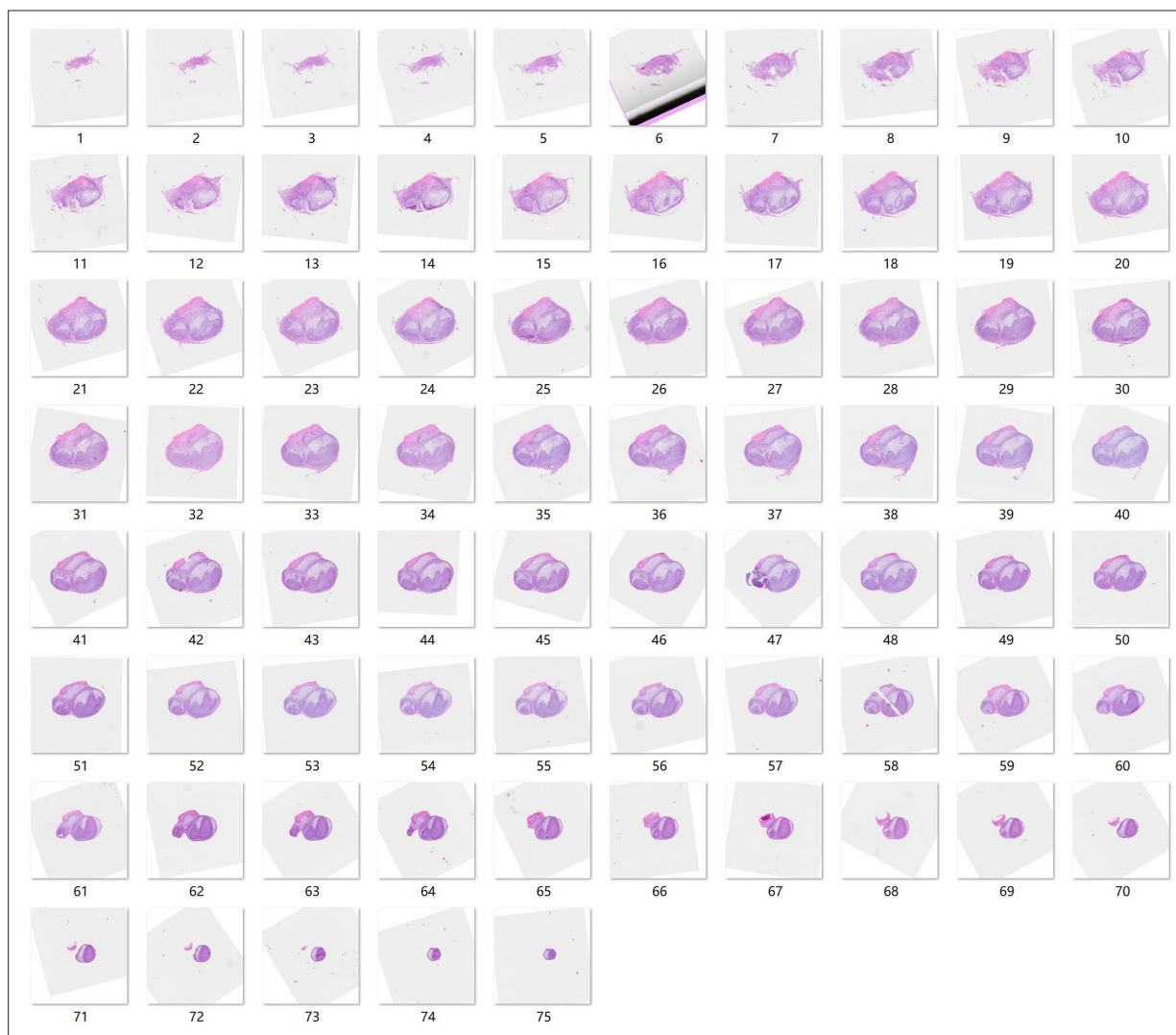


Figure 4—figure supplement 2. Source sequential HE slides for 3D reconstruction of CTRL.

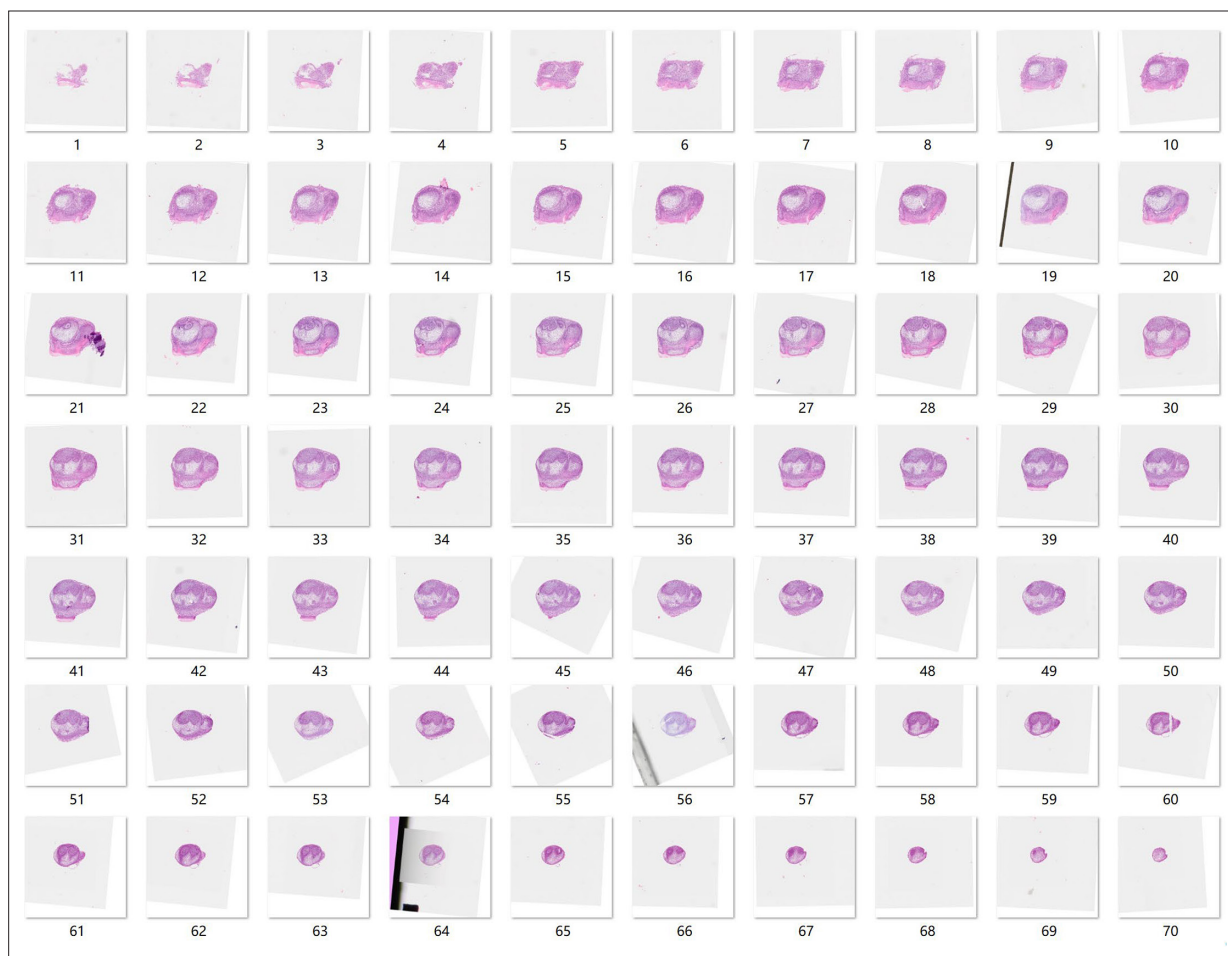


Figure 4—figure supplement 3. Source sequential HE slides for 3D reconstruction of Era-1.5 μ M.



Figure 4—figure supplement 4. Source sequential HE slides for 3D reconstruction of Era-1.5 μ M + Fer.

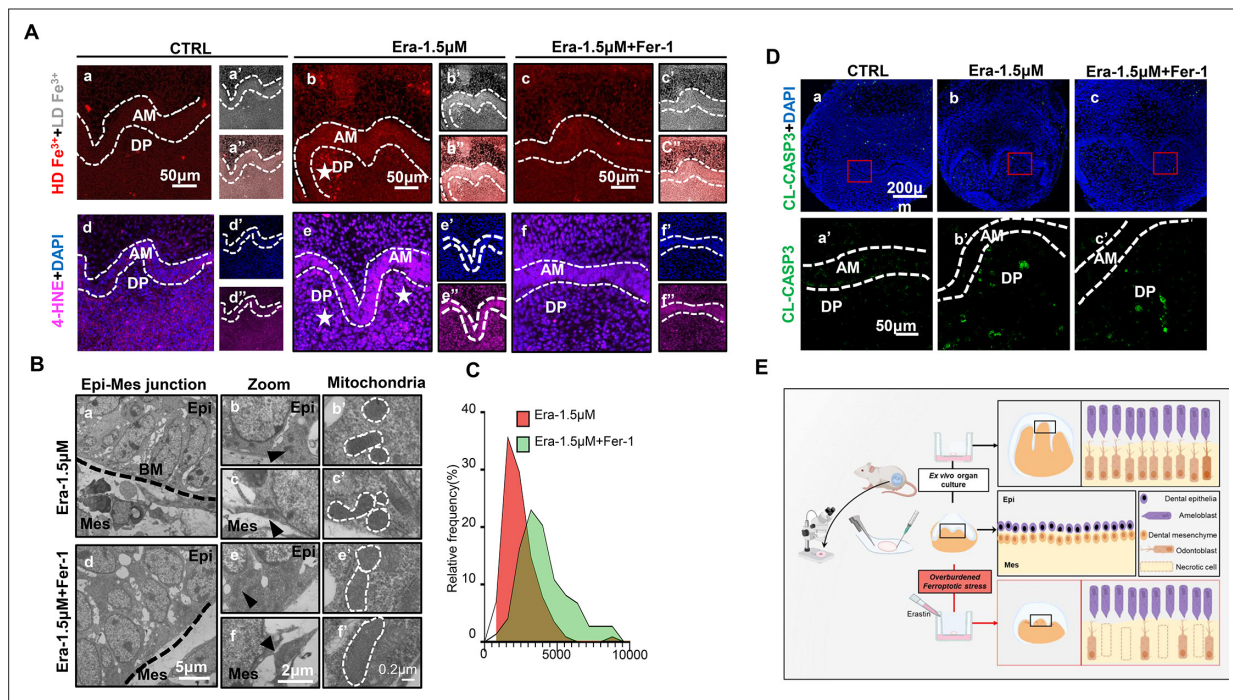


Figure 5. Ferroptosis is the dominant cell death type contributing to erastin-impaired tooth morphogenesis. **(A)** (a–c) High-density Fe^{3+} (red) in CTRL, Era-1.5 μM , and Era-1.5 μM + Fer-1 of day 5 (D5), white star points out strong fluorescence signal of Fe^{3+} , scale bars = 50 μm . (a'–c') Low-density Fe^{3+} (gray) and (a''–c'') merged view of iron probe staining. (d–f) Merged view of immunofluorescence (IF) staining of 4-hydroxy-2-nonenal (4-HNE) (magenta) and DAPI (blue), white star points out strong fluorescence signal of 4-HNE, scale bars = 50 μm . (d'–f') DAPI and (d''–f'') 4-HNE, AM, ameloblast; DP, dental papilla; HD, high density; LD, low density. **(B)** Transmission electron microscope (TEM) scanning of Era-1.5 μM and Era-1.5 μM + Fer-1 on D5. (a, d) epi-mes junction area of Era-1.5 μM and Era-1.5 μM + Fer-1 on D5 is detected, scale bars = 5 μm . (b, c) Representative view of cells in epithelia and mesenchyme for Era-1.5 μM , scale bars = 2 μm ; black arrow points out typical mitochondria in each region (b') for epithelia and (c') mesenchyme, outlined by the white dotted line. (e, f) Representative view of cells in epithelia and mesenchyme for Era-1.5 μM + Fer-1, scale bars = 2 μm ; black arrow points out typical mitochondria in each region (e') for epithelia and (f') mesenchyme, outlined by the white dotted line. **(C)** Relative frequency of the mitochondrial size in both groups. **(D)** (a–c) Expression of CL-CASP3 (green) in CTRL, Era-1.5 μM , and Era-1.5 μM + Fer-1 on D5, scale bars = 200 μm . (a'–c') Enlarged view of CL-CASP3 in each group, scale bars = 50 μm . **(E)** Schematic model illustrates the overburdened ferroptotic stress impaired tooth morphogenesis in ex vivo organ culture model.

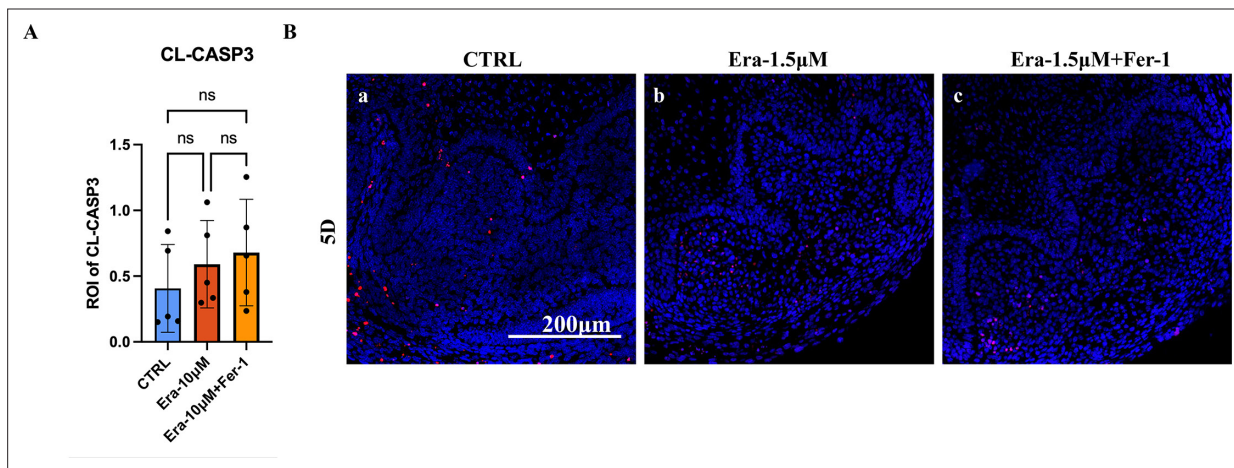


Figure 5—figure supplement 1. Activation of apoptosis indicated by CL-CASP3 and TUNEL staining. **(A)** Mean influence intensity of ROI (Region of Interest) of CL-CASP3 in CTRL, Era-1.5 μ M, and Era-1.5 μ M + Fer-1. Both Era-1.5 μ M and Era-1.5 μ M + Fer-1 showed slightly increased CL-CASP3 activation than that in CTRL but showed no statistical differences. **(B)** Results of TUNEL assay in each experimental group. DNA damage detected by TUNEL assay is similar to that of CL-CASP3, which indicated that apoptosis is not significantly activated in erastin-treated tooth germ. Scale bars = 200 μ m.