Expanded View Figures

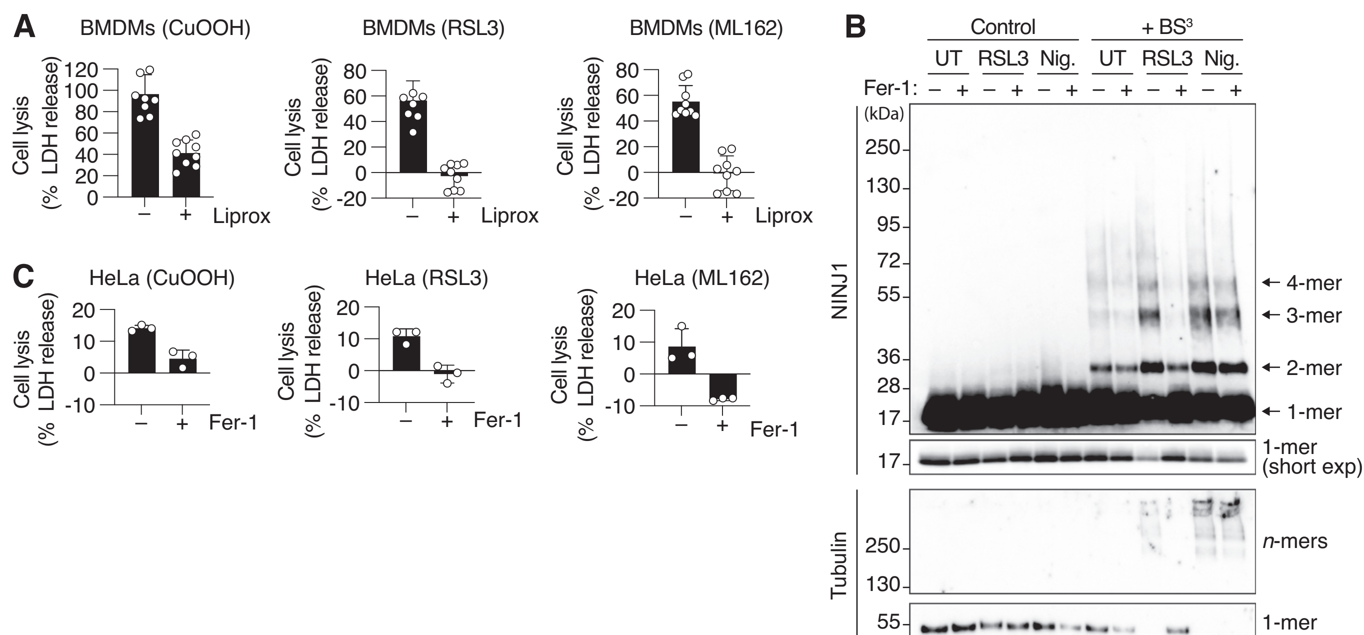

Figure EV1. Ferroptosis inhibitors specifically block ferroptosis in BMDMs.

(**A**) LDH release in WT BMDMs treatment with 1 mM CuOOH for 5 h, 5 μM RSL3 for 6 h, or 5 μM ML162 for 5 h. When indicated, 1 μM Liprox was added simultaneously with CuOOH, RSL3, or ML162. Liprox = Liproxstatin-1. (**B**) Western blot analysis of endogenous NINJ1 in WT BMDMs treated with 5μg/mL nigericin for 1.5 h or 5 μM RSL3 for 4 h followed by treatment with the membrane-impermeable crosslinker BS^3. Mixed supernatant and cell extracts were analyzed. FL, full length; Short exp = short exposure; 1-mer = monomer; 2-mer = dimer; 3-mer = trimer; 4-mer = tetramer and n-mer, higher-order oligomers. (**C**) LDH release in HeLa cells after treatment with 1 mM CuOOH for 3 h, 5 μM RSL3 for 3 h, or 5 μM ML162 for 3 h. When indicated, 25 μM Fer-1 was added simultaneously with ferroptosis and pyroptosis activators (**B**, **C**).
Data information: All graphs show the mean ± SD. Data are pooled from three independent experiments (**A**) or representative of two (**B**, **C**) independent experiments performed in triplicate.

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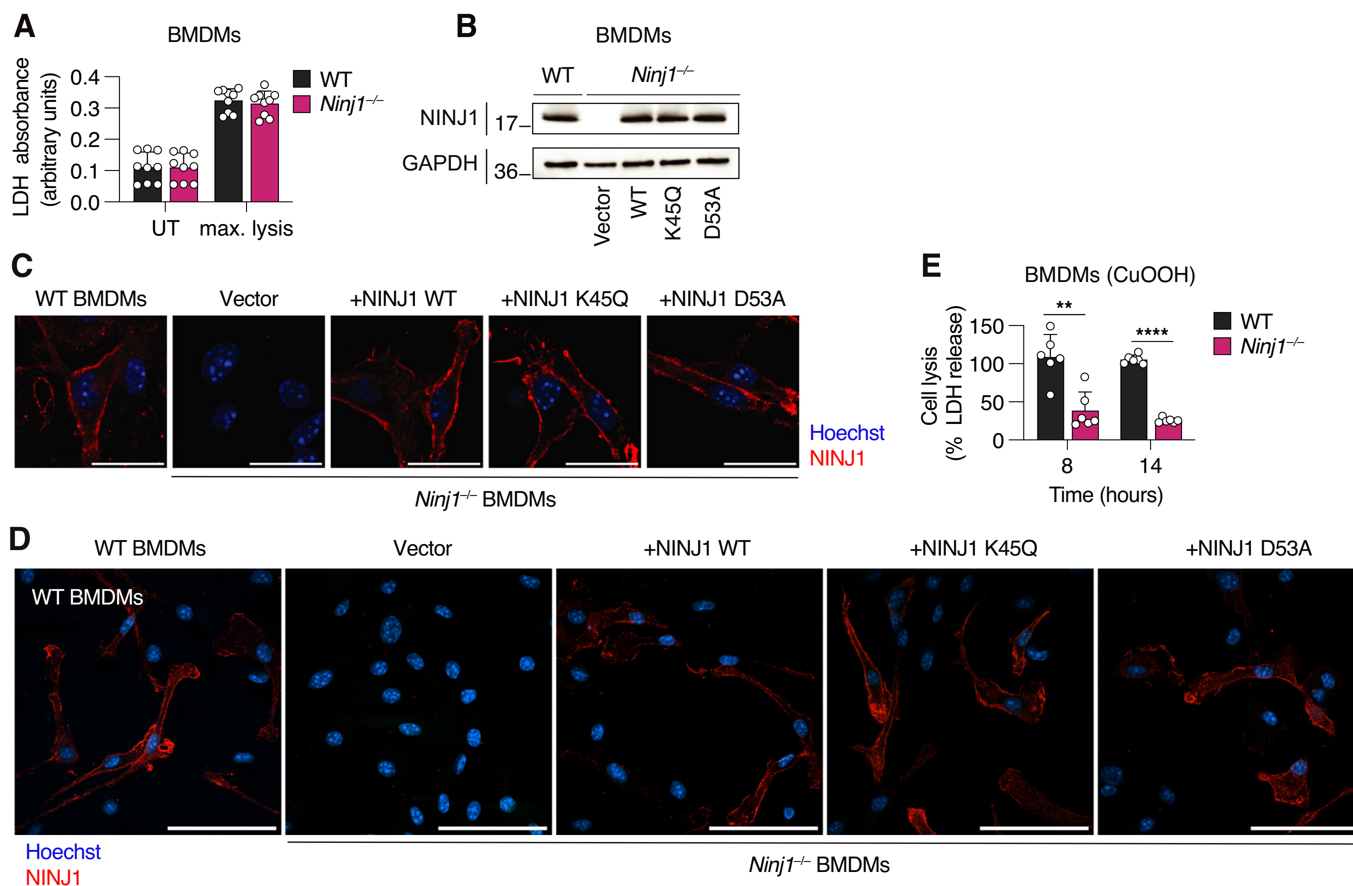

Figure EV2. NINJ1 filament formation is essential to induce cell lysis in ferroptosis.

(**A**) LDH absorbance in WT and *Ninj1*$^{-/-}$ BMDMs untreated (UT) or lysed with Triton X-100 to a final concentration of 0.01% (max. lysis). (**B**) Western blot of NINJ1 expression in WT or *Ninj1*$^{-/-}$ BMDMs transduced with a retroviral vector expressing WT mNINJ1 or different mNINJ1 mutants. Transduction with a GFP expressing vector was used as a control. Cell extracts were analyzed. GAPDH is a loading control. (**C**, **D**) Immunofluorescence confocal microscopy of NINJ1 (red) in WT or *Ninj1*$^{-/-}$ BMDMs complemented with WT or different NINJ1 mutants upon retroviral transduction. Scale bars: 20 μm (**C**) and 60 μm (**D**). (**E**) LDH release in WT and *Ninj1*$^{-/-}$ BMDMs upon treatment with 1 mM CuOOH for 8 or 14 h. Data information: All graphs show the mean ± SD. Data are pooled from three independent experiments performed in triplicate (**A**, **E**) or representative of two different experiments (**B**, **C**, **D**). Statistical analysis was done using Student's unpaired two-sided t-test. **** $P < 0.0001$, ** < 0.01.

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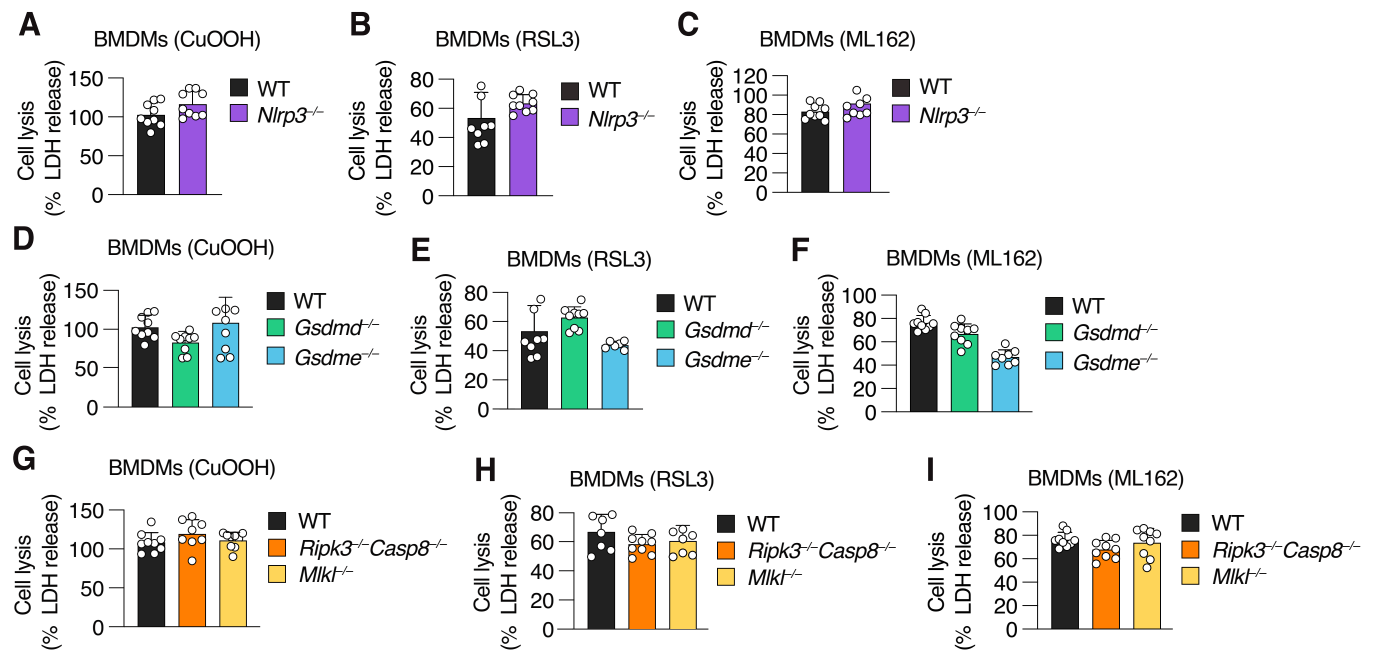

Figure EV3. NINJ1 is the only driver of cell lysis during ferroptosis.

(**A–I**) LDH release in WT and *Nlrp3*$^{-/-}$ BMDMs treated with 1 mM CuOOH for 5 h (**A**), 5 μM RSL3 for 6 h (**B**), or 5 μM ML162 for 5 h (**C**), WT, *Gsdmd*$^{-/-}$, and *Gsdme*$^{-/-}$ BMDMs treated with 1 mM CuOOH for 5 h (**D**), 5 μM RSL3 for 6 h (**E**) or 5 μM ML162 for 5 h (**F**) and WT, *Ripk3*$^{-/-}$/*Casp8*$^{-/-}$, and *Mlkl*$^{-/-}$ treated with 1 mM CuOOH for 5 h (**G**), 5 μM RSL3 for 6 h (**H**), or 5 μM ML162 for 5 h (**I**). Data information: All graphs show the mean ± SD. Data are pooled from three independent experiments performed in triplicate (**A–I**). Statistical analysis was done using Student's unpaired two-sided t-test (**A–C**) or one-way ANOVA (**D–I**).

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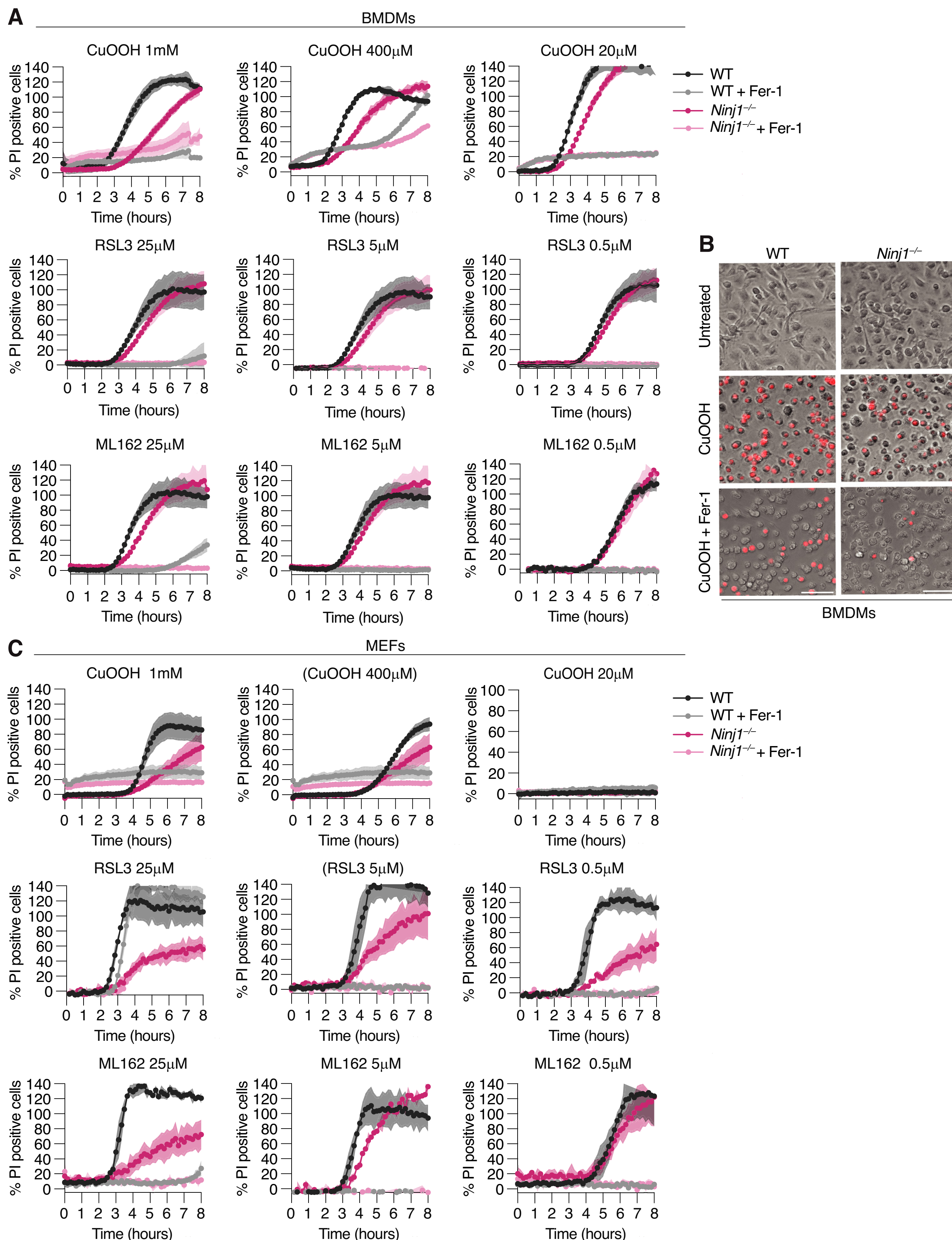
A
BMDMs
CuOOH 1mM
CuOOH 400μM
CuOOH 20μM
RSL3 25μM
RSL3 5μM
RSL3 0.5μM
ML162 25μM
ML162 5μM
ML162 0.5μM
% PI positive cells
Time (hours)
WT
WT + Fer-1
Ninj1−/−
Ninj1−/− + Fer-1
B
WT
Ninj1−/−
Untreated
CuOOH
CuOOH + Fer-1
BMDMs
C
MEFs
CuOOH 1mM
(CuOOH 400μM)
CuOOH 20μM
RSL3 25μM
(RSL3 5μM)
RSL3 0.5μM
ML162 25μM
ML162 5μM
ML162 0.5μM

Figure EV4. NINJ1 controls plasma membrane permeabilization during ferroptosis in macrophages and fibroblasts.

(**A**) Percentage of propidium iodide (PI, Mw = 668 Da) uptake in WT and *Ninj1*$^{-/-}$ BMDMs over time (0–8 h) after treatment with different concentrations of CuOOH, RSL3, or ML162. (**B**) Representative images showing PI uptake (red) in WT and *Ninj1*$^{-/-}$ BMDMs left untreated or after treatment with 1 mM CuOOH for 5 h. Scale bar: 200 μm. (**C**) Percentage of propidium iodide (PI, Mw = 668 Da) uptake in WT and *Ninj1*$^{-/-}$ MEFs over time (0–8 h) after treatment with different concentrations of CuOOH, RSL3, or ML162. When indicated, 25 μM Fer-1 was added simultaneously with ferroptosis activators (**A–C**). Data information: All graphs show the mean ± SD. Data are representative of three (**A**, **C**) or two (**B**) independent experiments performed in triplicate.

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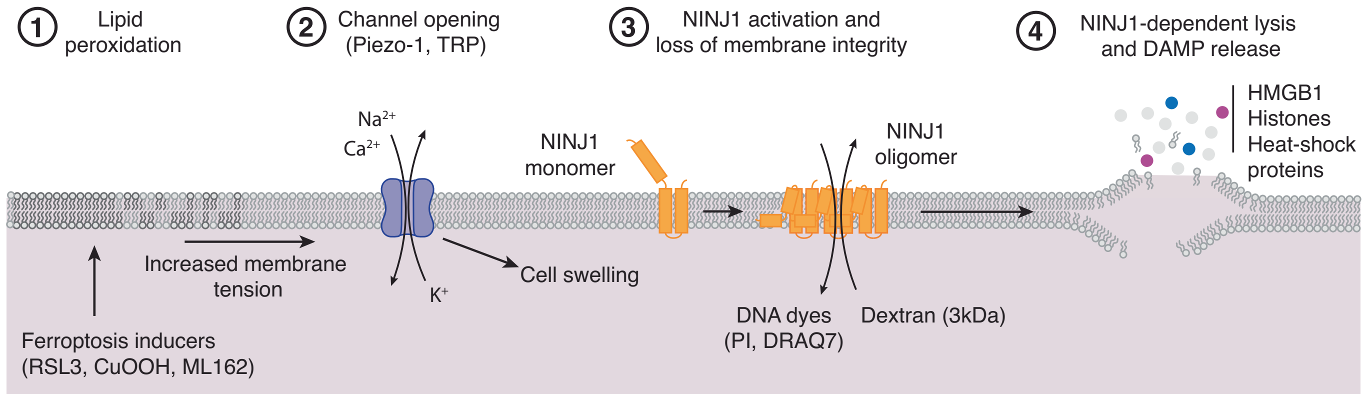

Figure EV5. Model for ferroptosis-associated cell lysis.

(1) Ferroptosis inducers promote the formation of lipid peroxides at the plasma membrane which cause and increase in membrane tension. (2) Mechanosensitive ion channels (Piezo-1 and TRP channels) open is response to increased membrane tension, resulting in the entry of calcium and sodium ions and the release of potassium ions. These non-selective ion fluxes cause an increase in the cell volume and swelling (3) Following this step, NINJ1 is activated, possibly because of ion disbalance, swelling or a yet unrecognized signal, and oligomerizes to form amphipathic filaments. Initially these filaments cause only small lesions that allow the entry and exit of small molecules, such as DNA-binding dyes. (4) Eventually NINJ1 lesions cause complete membrane rupture and cell lysis. As a consequence of NINJ1-dependent PMR, ferroptotic cells start to leak intracellular content among them many DAMPs that can drive inflammation.

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