Lysosomal Zn 2+ release triggers rapid, mitochondria-mediated, non-apoptotic cell death in metastatic melanoma

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Cell Reports

Lysosomal Zn\(^{2+}\) release triggers rapid, mitochondria-mediated, non-apoptotic cell death in metastatic melanoma

Graphical abstract

Highlights

- TRPML1 is dramatically upregulated in metastatic melanoma cells
- Activation of TRPML1, instead of inhibition, induces selective melanoma cell death
- TRPML-specific synthetic agonists (ML-SAs) trigger a distinctive form of cell death
- ML-SAs exhibit potent \textit{in vivo} therapeutic efficacy in advanced melanoma mouse models

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In brief

Du et al. show that lysosomal TRPML1 is dramatically upregulated in metastatic melanoma cells and can be targeted by small molecules, TRPML-specific synthetic agonists (ML-SAs). ML-SAs show potent and selective cytotoxicity in melanoma cells while sparing normal cells. Additionally, ML-SAs exhibit potent \textit{in vivo} efficacy by suppressing tumor progression.
Lysosomal Zn\(^{2+}\) release triggers rapid, mitochondria-mediated, non-apoptotic cell death in metastatic melanoma

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SUMMARY

During tumor progression, lysosome function is often maladaptively upregulated to match the high energy demand required for cancer cell hyper-proliferation and invasion. Here, we report that mucolipin TRP channel 1 (TRPML1), a lysosomal Ca\(^{2+}\) and Zn\(^{2+}\) release channel that regulates multiple aspects of lysosome function, is dramatically upregulated in metastatic melanoma cells compared with normal cells. TRPML-specific synthetic agonists (ML-SAs) are sufficient to induce rapid (within hours) lysosomal Zn\(^{2+}\)-dependent necrotic cell death in metastatic melanoma cells while completely sparing normal cells. ML-SA-caused mitochondria swelling and dysfunction lead to cellular ATP depletion. While pharmacological inhibition or genetic silencing of TRPML1 in metastatic melanoma cells prevents such cell death, overexpression of TRPML1 in normal cells confers ML-SA vulnerability. In the melanoma mouse models, ML-SAs exhibit potent in vivo efficacy of suppressing tumor progression. Hence, targeting maladaptively upregulated lysosome machinery can selectively eradicate metastatic tumor cells in vitro and in vivo.

INTRODUCTION

Once metastasized, melanoma skin cancer responds poorly to the traditional anti-cancer options, calling for a need to develop novel treatment strategies (Narayana et al., 2013; Welsh et al., 2016). Lysosomes, the cellular hub in metabolism, protein degradation, and nutrient sensing, play an indispensable role in cell survival and growth in normal physiology (Lawrence and Zoncu, 2019). In the metastatic cancer, lysosomes are often “transformed” to be hypertrophic (Katheder et al., 2017; Kroemer and Jäättelä, 2005), actively contributing to tumor progression, not only in cancer cell proliferation and survival by providing nutrients via macromolecule degradation but also in cancer invasion and metastasis by secreting lysosomal hydrolases to digest the extracellular matrix (Finicle et al., 2018; Hämäläistö and Jäättelä, 2016). Due to a high energy demand of cancer cells, the function of lysosomes is often adaptively or maladaptively upregulated to meet the metabolic requirement of cancer cells (Kalunki et al., 2013; Piao and Amaravadi, 2016). Given the heavy dependence on lysosome machinery for hyper-proliferation and invasion, cancer cells are particularly sensitive to lysosome disruptions (Serrano-Puebla and Boya, 2018). Lysosome inhibitors and/or inducers of lysosome membrane permeability are reportedly effective in triggering cancer cell death (McAfee et al., 2012; Petersen et al., 2013; Tardy et al., 2006). However, broad-spectrum lysosome inhibition, such as inhibitors of vacuolar type ATPase (v-ATPase), may inevitably compromise the functions of normal cells and tissues (Pérez-Sayáns et al., 2009). Hence, it is more desirable to target specific lysosomal machineries that operate in normal physiology but are maladaptively upregulated in cancer.

Mucolipin TRP channel 1 (TRPML1/MCOLN1; ML1) is a Ca\(^{2+}\) and Zn\(^{2+}/\)Fe\(^{2+}\) dually permeable cation channel predominantly localized on the membranes of late endosomes and lysosomes (LELs) in all mammalian cell types (Cheng et al., 2010; Dong et al., 2008). The related ML2 and ML3 channels are also permeable to Ca\(^{2+}\), as well as heavy metal ions, but are more restrictively expressed (Cheng et al., 2010; Li et al., 2019). Using TRPML-specific synthetic agonists (ML-SAs) and synthetic inhibitors (ML-SIs), which bind directly to TRPML proteins in the atomic-resolution co-structures (Schmiege et al., 2017; Schmiege et al., 2021), we and others have demonstrated that TRPML channels, especially ML1, play essential roles in various lysosomal functions, which include lysosome movement, membrane trafficking, lysosomal exocytosis, lysosome biogenesis, and heavy metal homeostasis (Dong et al., 2008; Li et al., 2016; Minckley et al., 2019; Peng et al., 2020; Samie et al., 2013; Yu et al., 2020).

While a high level of ML1 expression may serve as a favorable prognostic marker for several types of cancer, RNA sequencing (RNA-seq) analysis revealed that the highest expression among them is in melanoma (WebLink, 2019). Although several recent
loss-of-function studies suggest that ML1 inhibition or knockdown could reduce cancer cell proliferation, the effects were marginal even over time courses of days (Jung et al., 2019; Shekoufeh Almasi et al., 2020; Xu et al., 2019). Whereas no gross growth phenotype is seen in Trpml1 knockout (KO) mice and type IV mucolipindosis patients (Venugopal et al., 2007), the Genome-wide Cancer Dependence Map showed that ML1 is dispensable for cancer cell survival (DepMap, https://depmaporg/portal/gene/MCOLN1?tab=overview). Consistent with these observations, we found that prolonged inhibition of ML1 using ML-SIs that are much more potent than the published ones barely inhibited the growth of multiple cancer cell lines (unpublished data). In the current study, we found that small-molecule activation, but not inhibition of ML1, induced selective cell death of metastatic melanoma in vitro and in vivo.

RESULTS

Lysosomal ML1 channels are upregulated in metastatic melanoma cells

We examined the protein expression levels of ML1 in normal skin tissues, nevus (benign), and metastatic melanoma using immunohistochemistry with a ML1-specific antibody (Sahoo et al., 2017). Much stronger immunoreactivities were seen in the metastatic melanoma tissues compared with normal skin and benign nevus (Figures 1A and 1B). Consistently, in two highly metastatic melanoma cell lines, MeWo and M12, which have been intensively studied for their high metastatic capabilities in vitro and in vivo (Du et al., 2017; Pietrobono et al., 2020; Shiku et al., 1976; Xie et al., 1997), quantitative real-time PCR and western blotting analyses revealed 2-to-4-fold increases in the mRNA and protein expression levels of ML1 compared with normal, non-cancerous immortalized human melanocytes (Figures S1A–S1C).

To compare the channel activities of lysosomal ML1 in the cells, we performed patch-clamp recordings on chemically enlarged LELs (Dong et al., 2008) isolated from normal melanocytes, MeWo, and M12 cells. Whole-endolysosome ML1-mediated currents (I_{ML1}) were activated by ML-SA1 (20 μM), a synthetic agonist of ML1, in an immortalized human melanocyte (C), a MeWo cell (D), and a M12 cell (E). Currents were recorded with a ramp voltage protocol from –120 to +120 mV (only partial voltage ranges are shown). Pipette (luminal) solution was a standard external (Tyrode’s) solution adjusted to pH 4.6 to mimic the acidic environment of the lysosome lumen. Bath (cytoplasmic) solution was a K+-based solution (140 mM K-gluconate). Note that the inward currents indicate cations flowing out of endolysosomes.

(F) Mean current densities of whole-endolysosome I_{ML1} in normal melanocytes (n = 4), MeWo cells (n = 7), and M12 cells (n = 12). Each open circle represents one endolysosome/patch/cell; average data are presented as mean ± SEM; **p < 0.01.
Figure 2. Selective cytotoxic effects of ML-SAs on metastatic melanoma cells

(A–C) Dose-dependent effects of SA5 and SA8 on cell viabilities of normal melanocytes, MeWo, and M12 cells, which were measured using the CellTiter-Glo ATP assay 24 h after drug treatment. The average data represent mean ± SEM from three independent experiments, each with triplicates.

(D) PI staining of MeWo and M12 cells in the presence or absence of SA5 (3 μM). Normal melanocytes and MeWo cells were drug treated for 6 h, and M12 cells were drug treated for 3 h. Overlay phase contrast (Ph) images are also shown. Scale bar, 25 μm.

(E) The percentage of PI-positive cells in the presence of SA5 (3 μM, the left panels), as in (D), and SA8 (1 μM, right panels). Each open circle represents the average data from one batch of cells for each independent experiment.

(F and G) Dose-dependent effects of SA5/8 on the percentages of PI-positive MeWo (6 h) and M12 (3 h) cells.

(H) The effects of ML-SAs (3 μM SA5 or 1 μM SA8 for 12 h) on the cell viabilities of multiple cell types (n = 3 independent experiments).

(I) Overlay phase contrast and fluorescence images of PI-stained SA5 (3 μM)-treated MeWo (for 6 h) and M12 (for 3 h) cells in the presence or absence of ML-SIs (SI3 and SI4, 20 μM). Scale bar, 25 μm.

(legend continued on next page)
factors, may contribute to the much more dramatic upregulation in the lysosomal currents, relative to the mild increases in the total mRNA and protein expression levels.

Considering the established role of ML1 in activating transcription factor EB (TFEB), a master regulator of lysosome biogenesis (Li et al., 2019; Medina et al., 2015; Napolitano and Ballabio, 2016), we studied whether other lysosomal machineries were also upregulated in the metastatic melanoma cells. The protein expression level of lysosome-associated membrane protein 1 (LAMP1), a commonly used lysosomal marker (Sattig and Klumperman, 2009), was also increased by more than 2 folds in both MeWo and M12 cells compared with normal melanocytes (Figures S1B and S1D). In addition, the fluorescent intensity of Lyso-Tracker, a marker for lysosomal acidification (Tasdemir et al., 2008), was also increased (Figure S1G). It is worth noting that LAMP1 expression is reportedly elevated in aggressive brain tumors (Jensen et al., 2013). Taken together, these results suggest that lysosomal ML1 channels, or likely lysosome biogenesis in general, were upregulated in metastatic melanoma cells.

Selective cytotoxicity of ML-SAs to metastatic melanoma cells

We next investigated the roles of ML1 upregulation in the growth and survival of metastatic melanoma cells. No significant effects of ML-SI3 or ML-SI4 (0.1–100 μM for 12 h) on M12 or MeWo cell viability, measured by the cellular ATP level with the CellTiter-Glo ATP assay, were observed (Figure S2A). In contrast, both ML-SAs and ML-SA8 induced substantial cell death in M12 and MeWo cells while completely sparing normal melanocytes (Figures S1B and S1D). In addition, the fluorescent intensity of Lyso-Tracker, a marker for lysosomal acidification (Tasdemir et al., 2008), was also increased (Figure S1G). It is worth noting that LAMP1 expression is reportedly elevated in aggressive brain tumors (Jensen et al., 2013). Taken together, these results suggest that lysosomal ML1 channels, or likely lysosome biogenesis in general, were upregulated in metastatic melanoma cells.

ML-SA-induced cytotoxicity is dependent on the expression level of ML1

We next studied whether ML1 is required for ML-SA-induced cell death of metastatic melanoma cells by silencing the expression of ML1 using RNA interference (Kukic et al., 2013). In the siML1-transfected, ML1 knocking-down MeWo and M12 cells, in which whole-endolysosome Iₘ,ML1 was reduced by more than 80% (Figure 3A), ML-SA-induced cell death was significantly decreased (Figures 3B–3D), indicating that ML1 is required for ML-SA cytotoxicity in the metastatic melanoma cells. Note that knock down efficiency and genetic compensation (e.g., ML2/3 channels) might have contributed to the residual ML-SA cytotoxicity in the siML1-transfected cells.

We also investigated whether increasing the expression of ML1 in ML-SA-insensitive cells is sufficient to confer ML-SA vulnerability. Since HEK293 is not sensitive to ML-SA treatment (Figure S2D), we performed the test in the ML1-overexpressing HEK293 cells. In the Tet-On-HEK293-GCaMP7-ML1 stable cells (Zhang et al., 2019), in which ML1 expression was induced by doxycycline (Dox), cells became vulnerable to ML-SAs upon Dox application (Figure 3E). Likewise, HEK293 cells that were transiently transfected with EGFP-ML1 (HEK ML1 OE) also became sensitive to ML-SA treatment, and the vulnerability was diminished by ML-SIs (Figures 3F, 3G, S2E, and S2F). Hence, ML1 upregulation may have played an essential role in ML-SA cytotoxicity in metastatic melanoma cells.

ML-SA-induced cell death is a Zn²⁺-dependent process

Lysosomal ML1 is a non-selective cation channel that is permeable to Ca²⁺, as well as heavy metal ions such as Fe²⁺ and Zn²⁺ (Dong et al., 2008). Give the well-established role of Ca²⁺ in cell death (Orrenius and Nicotera, 1994), we first examined whether Ca²⁺ is required for ML-SA-induced cell death. Unexpectedly, BAPTA-AM, a membrane-permeable Ca²⁺ chelator (Garrity et al., 2016) that readily blocked ML-SA-evoked lysosomal Ca²⁺ release and TFEB nuclear translocation (Figures S3A and S3B), failed to prevent ML-SA’s cytotoxicity in M12 and MeWo cells. Surprisingly, BAPTA-AM, a membrane-permeable Ca²⁺ chelator (Garrity et al., 2016) that readily blocked ML-SA-evoked lysosomal Ca²⁺ release and TFEB nuclear translocation (Figures S3A and S3B), failed to prevent ML-SA’s cytotoxicity in metastatic melanoma cells (Figures 4A–4D). Likewise, EGTA-AM, another Ca²⁺ chelator, was also without effect (Figure S3C). Furthermore, cyclosporin A (CsA), a calcineurin inhibitor that reportedly blocks ML-SA-induced Ca²⁺-dependent TFEB nuclear translocation (Medina et al., 2015), or knocking down TFEB expression also failed to prevent ML-SA-induced cell death (Figures S3D and S3E). Hence, the well-documented ML1-Ca²⁺-TFEB pathway (Li et al., 2019; Napolitano and
Ballabio, 2016) might not play a dominant role in ML-SA-induced cell death.

In contrast, N,N,N,N-Tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), a membrane-permeable, relatively specific, high-affinity Zn\(^{2+}\) chelator (Radford and Lippard, 2013) (Figure S4A), which exhibited no inhibitory effect on lysosomal Ca\(^{2+}\) release or TFEB nuclear translocation (Figures S3A and S3B), completely prevented ML-SA-induced cytotoxicity in M12 and MeWo cells.
Figure 4. Lysosomal Zn²⁺ release is required for ML-SA-induced cell death
(A) The effects of SA5 (3 μM) treatment on PI staining of MeWo (for 6 h) and M12 (for 3 h) cells that were pretreated with BAPTA-AM (10 μM) or TPEN (2.5 μM).
(B) The percentages of PI-positive cells as shown in (A). Each open circle represents one batch of cells. Note that the control groups of MeWo and M12 cells treated with ML-SA5 are re-plotted from Figure 2E, as they were from the same experiments.
(C and D) Cell viability analysis of MeWo (C) and M12 (D) cells treated with indicated chemicals for 12 h.
(E) PI staining of HEK293 and HEK293 transient expressing of EGFP-ML1 (HEK ML1 OE) cells under indicated drug treatments for 6 h. Phase contrast overlay images are shown together with the green (EGFP-ML1) and red (PI staining) fluorescence channels. White arrows point to the EGFP-positive cells that are also PI positive. Scale bar, 25 μm.
The effects of SA1 on GZnP3 imaging of MeWo cells transfected with GZnP3-ML1 or GZnP3-Rab7 in the presence of 100 mM Zn2+ were investigated. (Figures 4A–4D, S4A, and S4C). Likewise, 1,10-phenanthroline (10 mM), another zinc chelator (Yang et al., 2007) (Figure S4A), also significantly reduced ML-SA-induced melanoma cell death (Figure S4D). In the ML1-overexpressing HEK293 cells, TPEN, but not BAPTA-AM, diminished ML-SA’s cytotoxicity (Figures 4E–4G). Considering that ML1 is a Zn2+-permeable channel (Figure S4G), the collective results suggest that ML1’s Zn2+ permeability, but not its Ca2+ permeability, underlies ML-SA’s cytotoxicity.

**Activation of ML1 releases lysosomal Zn2+ to cause cell death in metastatic melanoma cells**

Lysosomes are vesicular Zn2+ stores and a major source for the free Zn2+ pool in the cytoplasm (Haase and Beyersmann, 1999; Palmer et al., 1996). A link between ML1 and lysosomal Zn2+ release has been recently established (Cuajungco et al., 2014; Minckley et al., 2019); in ML1-deficient cells, cellular Zn2+ is reportedly accumulated in the lysosomes (Eichelsoofer et al., 2010; Kukic et al., 2013). We hypothesize that ML-SA’s cytotoxicity is attributed to lysosomal Zn2+ release upon ML1 activation. FluoroZin-3-AM, a cell membrane-permeable Zn2+-sensitive fluorescent dye (K0 = 15 nM), can be used to monitor not only cytoplasmic [Zn2+] but also vesicular [Zn2+], due to its relative insensitivity to acidic pH (Deviney et al., 2005) (Figure S4H). In MeWo and M12 cells, the strongest FluoroZin-3 signal was detected in the vesicular compartments that were LysoTracker-positive (Figure 4H), suggesting that lysosomes are the major intracellular Zn2+ stores in MeWo and M12 cells. Notably, ML-SA5 significantly decreased vesicular FluoroZin-3 fluorescence in M12 cells (Figures 4I and 4J). In the HEK293 and MeWo cells that were transfected with ML1 tagged with GZNp3 (genetically encoded Zn2+ indicator, GZNp3-ML1), but not in the cells that were transfected with GZNp3-Rab7 (Minckley et al., 2019), ML-SA-induced increases in the cytoplasmic GZNp3 signal (Figures 4K and 4L). Taken together, these data suggest that ML-SAs activate lysosomal ML1 to induce Zn2+ release from lysosomal Zn2+ stores.

In the MeWo and M12 cells that were incubated with extracellular Zn2+, vesicular Zn2+ levels were found to be elevated (Figure 4M). In these cells, ML-SA5-induced cytotoxicity was synergetically increased (Figure 4N), suggesting that ML1 activation causes cell death via Zn2+ release from lysosomes in metastatic melanoma cells.

**ML-SAs induced mitochondrial swelling and dysfunction**

To explore the cell death mechanism(s) underlying ML-SA’s cytotoxicity, we tested a panel of established inhibitors for various cell death pathways, which included necrostatin-1 (Nec-1) for necroptosis (Degterev et al., 2005), pan-caspase inhibitor z-VAD-fmk for apoptosis (Cain et al., 1996), iron chelator deferoxamine (DFO) for ferroptosis (Dixon et al., 2012), and bafilomycin A1 (Baf-A1) and chloroquine (CQ) for autophagic cell death (Tasdemir et al., 2008). None of the tested cell-death inhibitors prevented ML-SA-induced cytotoxicity (Figures S5A–S5E), suggesting that ML-SAs induce a cell-death mechanism distinct from apoptosis, necroptosis, ferroptosis, or autophagic cell death.

Cytosolic Zn2+ overload is known to cause mitochondrial damage (Dineley et al., 2003; Sheline et al., 2000), which in turn may cause a rapid drop in cellular ATP levels, as was observed in ML-SA-treated MeWo and M12 cells (Figures 2B and 2C). Mitochondria staining (Gao et al., 2001) revealed that in MeWo and M12 cells, but not in normal melanocytes, mitochondria became swollen and fragmented within 0.5 to 1 hour post ML-SA treatment (Figures 2A–2C). Such morphological changes of mitochondria were not seen in cells that were co-treated with ML-SAs or TPEN (Figures 5D and 5E). Extensive mitochondrial swelling was also evident in the ML-SA-treated cells under the transmission electron microscopy (TEM) examination (Figures 5F and 5G). Tetramethylrhodamine methyl ester (TMRM) staining revealed that ML-SA5 treatment also caused a loss of mitochondrial membrane potential in M12 cells (Figure S6). Collectively, these results suggest that ML-SAs caused mitochondrial damage and cellular ATP depletion via ML1 activation and subsequent lysosomal Zn2+ release.

**ML-SAs inhibit tumor progression in advanced melanoma mouse models**

To evaluate the therapeutic efficacy of ML-SAs in vivo, we engineered MeWo and M12 cells stably expressing both the fluorescent protein mCherry and the bioluminescent protein firefly luciferase (Fluc) (Du et al., 2017). The modified MeWo and M12 cells, referred to as MeWo-FmC and M12-FmC cells, exhibit similar cell morphology, proliferation, migration, and ML-SA sensitivity compared to the unmodified parental cells (data not shown). In a subcutaneous xenograft melanoma mouse model, in vivo bioluminescence imaging was performed weekly to monitor tumor growth. In MeWo-FmC-bearing mice that were randomly separated into vehicle versus ML-SA5-treated groups, intraperitoneal (i.p.) injection of ML-SA5 (5 mg/kg three times per week with drug administration starting one week post-tumor cell inoculation) resulted in a substantial reduction in tumor growth in vivo (Figures 6A–6C) without causing obvious systemic toxicity (Figure 6D). Meanwhile, i.p. injection of ML-SA5 (three times per week) into subcutaneous-tumor-bearing mice significantly prolonged animal survival post-tumor implantation (Figure S7).
Since M12 cells are primary melanoma cells derived from melanoma brain metastases (MBM) patients, we created a mouse model of MBM by implanting M12-FmC cells into mouse brain. In mice bearing MBM, single intracranial administration of ML-SA8 (10 mM/5 \( \mu L \)) was sufficient to markedly suppress MBM cell growth with a survival benefit (Figures 6 E–6G). Collectively, our results have demonstrated that pharmacological activation of ML1 using small-molecule agonists can mitigate metastatic melanoma progression in vivo.

**DISCUSSION**

We have demonstrated in the current study that in metastatic melanoma cells, the maladaptively upregulated lysosomal ML1 channels can be pharmaco logically targeted to induce a distinctive form of necrotic cell death by triggering lysosomal Zn\(^{2+}\) release to cause mitochondrial swelling/damage and rapid cellular ATP depletion (Figure 7). In contrast to ML-SA-induced cell death that occurs rapidly (within hours) in metastatic melanoma cells, ML1 inhibition was previously reported to exhibit a slow (in days) anti-proliferation effect in cancer cells (Jung et al., 2019; Shekoufeh Almasi et al., 2020; Xu et al., 2019). Whereas under physiological conditions, ML1-mediated lysosomal Zn\(^{2+}\) release is required for cellular homeostasis (Kukic et al., 2013), excessive activation of this pathway in metastatic cancer cells, such as MeWo, M12, and MDA-231-BrM2 cells, may induce such cell death (Figure 7). Hence, lysosomal ML1 channels are maladaptively upregulated in metastatic cancer cells, and this maladaptation, as a prognostic marker for advanced melanoma patients, can be targeted for selective eradication of metastatic melanoma cells without affecting normal cells.

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**Figure 5. ML-SAs cause mitochondrial swelling in metastatic melanoma cells**

(A) Live imaging of MitoTracker-loaded normal melanocytes (left panels, 60 min), MeWo (middle panels, 60 min), and M12 (right panels, 30 min) cells under DMSO (Ctrl) or SA5 (3 \( \mu M \)) treatment. Scale bar, 10 \( \mu m \).

(B and C) Statistical analysis of mitochondrial swelling in melanocytes, MeWo, and M12 cells that were treated with SA5 (3 \( \mu M \)) or SA8 (1 \( \mu M \)) for 30 or 60 min. Each open circle represents the average result from one batch of cells.

(D and E) The effects of ML-SIs and TPEN on mitochondrial swelling in SA5 (D) or SA8 (E)-treated MeWo and M12 cells. Note that the control groups of MeWo and M12 treated with SA5 or SA8 are re-plotted from (B) and (C), as they were from the same experiments.

(F) Representative transmission electron microscopy (TEM) images of M12 cells that were treated with DMSO (Ctrl) or SA5 (3 \( \mu M \)) for 30 min. M, mitochondria. Scale bar, 200 nm.

(G) Statistical analysis of mitochondrial size in control (n = 14 mitochondria from 3 cells) and SA5-treated cells (n = 22 mitochondria from 5 cells). All data are mean ± SEM; *p < 0.01.
Upon certain pathological challenges, such as in stroke and neurodegenerative disorders, increases in cytoplasmic chelatable Zn$^{2+}$ reportedly cause massive neuronal cell death (Koh et al., 1996). Unlike the cytosol, which keeps the free Zn$^{2+}$ concentration as low as 0.1 nM by multiple efflux and buffering mechanisms, intracellular vesicles such as lysosomes contain micromolar to millimolar concentrations of Zn$^{2+}$, functioning as vesicular Zn$^{2+}$ stores (Blaby-Haas and Merchant, 2014; Minckley et al., 2019; Palmiter et al., 1996). Cancer cells, due to their high metabolism and fast turnover of metal-binding macromolecules, tend to accumulate heavy metal ions in endolysosomal compartments (Chandler et al., 2016; Kroemer and Jäättelä, 2005; Pavlova and Thompson, 2016). Although cytosolic Zn$^{2+}$, as an essential trace element, is required for normal cellular functions, excessive Zn$^{2+}$ release from intracellular Zn$^{2+}$ stores reportedly inhibits mitochondrial functions, e.g., the electron transport chain, to cause cellular energy failure and subsequent cell death (Sheline et al., 2000). Although our results strongly suggest lysosomal Zn$^{2+}$ as the driving force in ML-SA-caused cell death, the possible contribution of Ca$^{2+}$ to the Zn$^{2+}$-dominant process cannot be excluded, as ML-SA application evokes lysosomal Ca$^{2+}$ release as well (Figure S3A) (Shen et al., 2012; Yu et al., 2020).

Mitochondria have been implicated in several cell-death pathways, including apoptosis and ferroptosis (Gao et al., 2019). During ferroptosis, mitochondria become condensed and dysfunctional (Dixon et al., 2012). Remarkably distinct features, such as rapid (<1 h) mitochondrial swelling and fragmentation, occur during ML-SA-induced cell death. Although a causative role of mitochondrial swelling/damage in such cell death requires further investigation, the fast time course of mitochondrial swelling suggests that it is the cause of cellular ATP depletion and cell death. How lysosomal Zn$^{2+}$ release causes mitochondrial swelling and damage is not known. A recent study reported that Ca$^{2+}$ is transferred directly from lysosomes into mitochondria upon ML1 activation (Peng et al., 2020). It is likely that upon ML1 activation, lysosomal Zn$^{2+}$ may also rapidly flux into mitochondria through lysosome-mitochondria membrane contact sites. We hypothesize that certain Zn$^{2+}$-dependent enzymes or Zn$^{2+}$ regulated mitochondrial membrane proteins may function as the key mediators of ML-SA-induced cell death. For instance, inhibition of crucial enzymes in the ATP synthesis process, e.g., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), by Zn$^{2+}$ may become significant when the free cytosolic or mitochondrial [Zn$^{2+}$] is elevated (Beiersmann and Haase, 2001; Maret et al., 1999). Hence, Zn$^{2+}$...
release channels and transporters such as ML1 (Eichelsdoerfer et al., 2010; Kukic et al., 2013) must be tightly regulated within cells. ML1 upregulation might provide some growth advantage for metastatic cancer cells. However, this upregulation may render these ML1-upregulated cells a unique susceptibility to ML-SAs via the lysosome-initiated, mitochondria-mediated, necrotic cell death. Therefore, pharmacological activation of ML1 may serve as a potential therapeutic strategy for metastatic melanoma or other cancers, in which ML1 upregulation is a prognostic marker.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animal models
  - Cell culture
- **METHOD DETAILS**
  - Whole-endolysosome and whole-cell patch-clamp electrophysiology
  - RNA extraction and RT-qPCR
  - Western blotting
  - Immunohistochemistry (IHC)
  - *In vitro* cell death assay
  - Silencing RNA knockdown
  - Expression of ML1 in HEK293 cells
  - FluoZin-3, LysoTracker, and MitoTracker Imaging
  - Electron microscopy
  - GZnP3 Zn^{2+} imaging
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109848.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-TRPML1 (Mucolipin 1) Antibody | Alomone Labs | Cat# ACC-081; RRID:AB_10915894 |
| Mouse anti-human LAMP1 (H4A3) antibody | DSHB | Cat# h4a3; RRID: AB_2296838 |
| Mouse Anti-beta-Actin Antibody (AC-74) | Sigma-Aldrich | Cat# A5316; RRID: AB_476743 |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP | Fisher Scientific | Cat# 31460; RRID: AB_228341 |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP | Fisher Scientific | Cat# 31430; RRID: AB_228307 |
| **Chemicals, peptides, and recombinant proteins** | | |
| ML-SAs (SA1, SA5, SA8) | Shen et al., 2012; Sahoo et al., 2017; Yu et al., 2020; This paper; National Institutes of Health (NIH)/National Center for Advancing Translational Sciences (NCATS) Chemical Genomics Center & CalyGene Biotechnology Inc. | N.A. |
| ML-SIs (SI3, SI4) | Sahoo et al., 2017; National Institutes of Health (NIH)/National Center for Advancing Translational Sciences (NCATS) Chemical Genomics Center | N.A. |
| K-Gluconate | Sigma-Aldrich | P1847; CAS: 299-27-4 |
| NaCl | Sigma-Aldrich | S9888; CAS: 7647-14-5 |
| EGTA | Sigma-Aldrich | E4378; CAS: 67-42-5 |
| MgCl₂ | Sigma-Aldrich | 208337; CAS: 7786-30-3 |
| CaCl₂ | Sigma-Aldrich | C8106; CAS: 10035-04-8 |
| MgCl₂ | Sigma-Aldrich | 1374248; CAS: 7791-18-6 |
| ZnCl₂ | Sigma-Aldrich | 208086; CAS: 7646-85-7 |
| Glucose | Sigma-Aldrich | G6152; CAS: 50-99-7 |
| HEPES | Sigma-Aldrich | H3375; CAS: 7365-45-9 |
| KOH | Sigma-Aldrich | P250-500; CAS: 1310-58-3 |
| NaCl | Sigma-Aldrich | S9888; CAS: 10035-04-8 |
| KCl | Sigma-Aldrich | P9541; CAS: 7447-40-7 |
| MES | Sigma-Aldrich | M8250; CAS: 1266615-59-1 |
| NaOH | Fisher Scientific | S318-500; CAS: 1310-73-2 |
| Propidium iodide | Sigma-Aldrich | P4864 |
| Ethanol | Decon laboratories inc. | 2701; CAS: 64-17-5 |
| Hydrogen peroxide | Sigma-Aldrich | H1009; CAS: 7722-84-1 |
| Triton X-100 | Sigma-Aldrich | T8787; CAS: 9002-93-1 |
| NaF | Sigma-Aldrich | S7920; CAS: 7681-49-4 |
| Na-orthovanadate | Sigma-Aldrich | S6508; CAS: 13721-39-6 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D2650; CAS: 67-68-5 |
| Bovine Serum Albumin (BSA) | Sigma-Aldrich | A3059; CAS: 9048-46-8 |
| Paraformaldehyde (PFA) 4% in PBS | Fisher Scientific | J19943-K2 |
| Phosphate-buffered saline (PBS) | Fisher Scientific | 10010023 |
| HBSS, calcium, magnesium, no phenol red | Fisher Scientific | 14025092 |
| RIPA | Boston Bioproducts | BP-115 |

(Continued on next page)
| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Protease inhibitor cocktail | Sigma Aldrich | P8340 |
| Phosphatase inhibitor cocktail I | Sigma Aldrich | P0044 |
| LDS sample buffer (4x) | Invitrogen | NP0007 |
| Pierce BCA Protein Assay | Invitrogen | 23227 |
| PVDF membrane | Millipore | IPFL00010 |
| Tween-20 | Fisher Scientific | BP337-100; CAS: 9005-64-5 |
| SuperSignal West Atto Ultimate Sensitivity Substrate | Fisher Scientific | A38555 |
| LysoTracker™ Red DND-99 | Invitrogen | L7528 |
| MitoTracker Green FM | Invitrogen | M7514 |
| FluoZin-3, AM | Fisher Scientific | F24195 |
| BAPTA, AM | Fisher Scientific | B6769 |
| Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) | Fisher Scientific | T668 |
| N,N,N′,N′-tetraakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) | Cayman Chemical | 13340; CAS: 16858-02-9 |
| Deferoxamine (Radford and Lippard) | Cayman Chemical | 14595; CAS: 138-14-7 |
| Chloroquine | Cayman Chemical | 30708; CAS: 50-63-5 |
| z-VAD-FMK | Cayman Chemical | 14467; CAS: 161401-82-7 |
| Bafilomycin-A1 | Cayman Chemical | 11038; CAS: 88899-55-2 |
| Doxycycline | Sigma-Aldrich | D9891; CAS: 24390-14-5 |
| 1,10-Phenanthroline (hydrate) | Cayman Chemical | 28951; CAS: 5144-89-8 |
| Necrostatin-1 | Cayman Chemical | 11658; CAS: 4311-88-0 |
| EGTa, AM | Cayman Chemical | 20401; CAS: 99590-86-0 |
| Doxorubicin | Cayman Chemical | 15007; CAS: 25316-40-9 |
| Zinc Pyrithione | Cayman Chemical | 29154; CAS: 13463-41-7 |
| Lipofectamine 2000 | Invitrogen | 11668-027 |
| DMEM medium without phenol red | GIBCO | 21063029 |
| DMEM, high glucose | GIBCO | 11965092 |
| DMEM/F-12 | GIBCO | 11320033 |
| Ham’s F-10 Nutrient Mix | Fisher Scientific | 11550043 |
| Minimum Essential Media | Fisher Scientific | 11095080 |
| Trypsin-EDTA (0.05%), phenol red | GIBCO | 25300054 |
| Fetal bovine serum (FBS) | Gemini Bio-Products | 100-106 |
| Antibiotic-Antimycotic | GIBCO | 15240062 |
| Poly-D-lysine solution | Millipore | A-003-E |
| Poly-L-lysine hydrobromide | Sigma-Aldrich | P1399; CAS: 25988-63-0 |
| Vacuolin-1 | Sigma-Aldrich | 673000; CAS: 351986-85-1 |
| Ionomycin | Sigma-Aldrich | I9657; CAS: 56092-81-0 |
| Tissue-Tek® O.C.T. (Optimal Cutting Temperature) compound | Sakura Finetek | 4583 |
| Polyethylene glycol (PEG) 300 | Sigma-Aldrich | 1546423; CAS: 25322-68-3 |
| XenoLight D-Luciferin - K+ Salt Bioluminescent Substrate | PerkinElmer | 122799 |

Critical commercial assays

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| CellTiter-Glo® Luminescent Cell Viability Assay | Promega | G7571 |
| E.Z.N.A.® Total RNA Kit I | Omega Bio-tek | R6834-01 |

(Continued on next page)
| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SuperScript III One-Step RT-PCR System | Fisher Scientific | 12574026 |
| PowerUp SYBR Green Master Mix | Fisher Scientific | A25741 |
| Q5® High-Fidelity DNA Polymerase | New England Biolabs | M0491S |
| GoTaq™ master mixes | Promega | M7122 |
| MycoAlert™ Mycoplasma Detection Kit | Lonza | LT07-318 |
| Experimental models: Cell lines |        |            |
| pMEL/NRAS (Q61R) immortalized human melanocyte | A gift from Dr. D. Fisher, MGH | N.A. |
| Human MeWo cells | ATCC | HTB-65 |
| Human M12 cells | A gift from Dr. J. Sarkaria, Mayo Clinic; Du et al., 2017 | N.A. |
| Human MDA231BrM2a cells | A gift from Dr. J. Massague, Memorial Sloan Cancer Center Bos et al., 2009 | N.A. |
| Human skin fibroblast cells | Obtained from the Coriell Institute for Medical Research | clone GM05659 |
| Human: HEK293 cells | ATCC | CRL-1573 |
| Human: HeLa cells | ATCC | CCL-2 |
| Human SH-SY5Y cells | ATCC | CRL-2266 |
| TFEB-GFP Stable HeLa cells | Zhang et al., 2016; Zhang et al., 2019 | N.A. |
| Human HEK293-GCaMP7-ML1 cells | Zhang et al., 2019 | N.A. |
| Human M12-FmC cells | Du et al., 2017 | N.A. |
| Human MeWo-FmC cells | Du et al., 2017 | N.A. |
| Experimental models: Organisms/strains |        |            |
| Human paraffin-embedded tissue array (Tissue Microarray, TMA) | US Biomax | ME1004 |
| Athymic Nude Mouse | Charles River Laboratories | 490 Crt:NU(NCr)-Foxn1^+/+ |
| Oligonucleotides |        |            |
| GAPDH, forward primer | 5'-tgacccccacacgcttacgc-3' | N.A. |
| GAPDH, reverse primer | 5'-ggcgtggctgcgtgctgag-3' | N.A. |
| MCOLN1, forward primer | 5'-gactgggtcagcagtttc-3' | N.A. |
| MCOLN1, reverse primer | 5'-tgtctctctcctggagtc-3' | N.A. |
| Stealth siRNA human TFEB | Fisher Scientific | HSS111868 |
| MISSION® esiRNA targeting human MCOLN1 | Sigma-Aldrich | EHU062561 |
| Recombinant DNA |        |            |
| GZnP3-ML1 | A gift from Dr. Yan Qin, Denver University; Minckley et al., 2019 | N.A. |
| GZnP3-Rab7 | A gift from Dr. Yan Qin, Denver University; Minckley et al., 2019 | N.A. |
| EGFP-ML1 | Shen et al., 2012 | N.A. |
| ML1-L15/LAA-L57/LAA (abbreviated as ML1-4A) | Shen et al., 2012 | N.A. |
| Software and algorithms |        |            |
| MetaMorph 7.10 | Molecular Devices | https://www.moleculardevices.com/ |
| pClamp 10.7 | Molecular Devices | https://www.moleculardevices.com/ |
| OriginPro 2018 | Originlab | https://www.originlab.com/ |
| GraphPad Prism 8 | Graphpad | https://www.graphpad.com/ |
| Fiji/ImageJ | Fiji contributors | https://imagej.net/software/fiji |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Wanlu Du (wanludu@umich.edu).

Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal models
Athymic nude mice (females, 8 weeks old, Charles River Laboratories, Wilmington, MA) were used in this study. Mice were housed in standard cages at 21°C with a 12h light-12h dark cycle with access to food (standard laboratory chow) and water ad libitum. Based on pilot studies, with a power of 0.8 and \( p < 0.05 \), we calculated a sample size of between 5 and 11 mice per group. Mice were anesthetized using 2% isoflurane (inhale). For the subcutaneous (s.c.) tumor model, MeWo-FmC cells (1\( \sim \)1.5\( \times \)10^6 tumor cells/mouse) were suspended in 100 \( \mu \)L PBS and s.c. injected into immunocompromised nude mice. ML-SA5 (dissolved in 10% DMSO, 40% PEG300, and 50% PBS) (Yu et al., 2020) was administered to mice (5 mg/Kg) by intraperitoneal (i.p) injection 3 times a week until the end of experiments. Mice with one of the following conditions: 1) a tumor diameter measuring greater than 2 cm in any single dimension, 2) impairment in the normal movement, 3) ulceration that is greater than 1/2 the surface of the tumor area or has effusions, 4) infection, and 5) hemorrhage, were euthanized according to the Tumor Burden Policy for Rodents. For the MBM tumor model, M12-FmC cells were implanted stereotactically into nude mouse brains (0.5 \( \sim \)10^5 tumor cells/mouse) in the following co-ordinates: 2.2 mm lateral from bregma, 2.5 mm ventral from dura on the cranial suture. ML-SA8 (10 mM/5 \( \mu \)l) was administered on-site by a single intracranial injection. Mice were imaged for the success of tumor cell injection post-implantation and then periodically for tumor progression using in vivo bioluminescent imaging as described previously (Du et al., 2017). During tumor growth, we closely monitored the animal situation and strictly followed the Tumor Burden Policy for Rodents and the End-Stage Illness Scoring System for euthanizing the mice that reached a score of 6-11. All in vivo procedures were approved by animal protocol following the Institutional Animal Care Guidelines at the University of Michigan.

Cell culture
The pMEL/NRAS (Q61R) immortalized human melanocytes were cultured in Ham’s F-10 Nutrient Mix with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The patient-derived melanoma brain metastatic cells, M12 and MeWo, as well as HEK293, MDA231BrM2, HeLa, and SH-SY5Y cell lines, were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and 1% penicillin-streptomycin. Human fibroblasts were cultured in Minimum Essential Media supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were used at low passages with occasional testing for mycoplasma contamination by MycoAlert™ Mycoplasma Detection Kit.

METHOD DETAILS

Whole-endolysosome and whole-cell patch-clamp electrophysiology
Whole-endolysosome patch-clamp experiments were performed in isolated enlarged endolysosomes as described previously (Dong et al., 2008; Zhang et al., 2019). Briefly, cells were treated with 1 \( \mu \)M vacuolin-1 overnight to selectively enlarge the size of late endosomes and lysosomes (LELs). A glass electrode was then used to break the plasma membrane to release the enlarged vacuoles. After formation of a giga-seal between the patch pipette and an enlarged endolysosome, voltage steps of several hundred millivolts with a millisecond duration were applied to break into the vacuolar membrane. All bath solutions were applied via a fast perfusion system that produced a complete solution exchange within a few seconds. Unless otherwise indicated, the bath (cytoplasmic) solution contained (in mM): 140 K-glucanate, 4 NaCl, 1 EGTA, 2 MgCl_2, 0.39 CaCl_2, and 20 HEPES (pH 7.2 adjusted with KOH; free [Ca^{2+}] approximately equal to 100 nM). The pipette (luminal) solution contained (in mM): 145 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 glucose, 10 HEPES, and 10 MES (pH 4.6 adjusted with NaOH). For whole-cell patch clamp recordings, the pipette (cytosolic) solution contained (in mM): 145 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). The bath (extracellular) solution
contained (in mM): 30 ZnCl₂, 110 NaCl, 10 HEPES, and 10 MES (pH 4.6 adjusted with HCl). Glass electrodes were pulled with resistance of 2–4 mΩ (whole-cell) or 9–11 mΩ (whole-endolysosome). Data were collected using an Axopatch 200B amplifier equipped with a Digidata 1440 controlled by Clampex 10.7 software. All experiments were conducted at room temperature (22°C). silenced MCOLN1, Luc7, and TFEB expression was induced in Tet-On HEK293-GCaMP7-ML1 cells (Zhang et al., 2019) 24h prior to experiments by adding 0.01 μg/mL doxycycline (Dox). For HEK ML1 OE cells, transient transfection of GFP-ML1 was performed in HEK293 cells using Lipofectamine 2000.

**FluoZin-3, LysoTracker, and MitoTracker Imaging**

Fluorescence and time-lapse imaging was conducted in a spinning-disk confocal imaging system composed of an Olympus IX81 inverted microscope, 10 ×, 20 ×, and 60 × Olympus objectives, a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor), a temperature controller, and MetaMorph Advanced Imaging acquisition software v.7.7.8.0. Fluoro-3-AM and LysoTracker were loaded according to the manufacturer’s instructions. Briefly, Fluoro-3-AM (1 mM stock solution in DMSO) was diluted to a final concentration of 3 μM in respective culture mediums. A 1 mM stock solution of LysoTracker RED DND-99 was diluted to a final concentration of 0.5 μM in respective culture mediums. Cells were loaded with the dye-containing buffer for 30 min before image collection. For time-lapse imaging of PI fluorescence (Videos S1 and S2), cells were challenged with DMEM supplemented with 10 μg/mL PI, and images were taken at an interval of 5 or 10 min for M12 cells and MeWo cells, respectively. For MitoTracker live imaging, cells were loaded with MitoTracker and treated with ML-SAs (1h for melanocytes and MeWo cells, 30 min for M12 cells). For most inhibitor experiments, drugs were pretreated for 30 min. Images were acquired and analyzed with MetaMorph Advanced imaging acquisition software v.7.7.8.0 and ImageJ (NIH).
EM was performed at the Microscopy Core at the University of Michigan. Briefly, M12 cells were plated in the conventional TEM cell monolayer consumables (4 Thermofluor 8mm coverslips and a 12-well culture plate). Cells were treated with DMSO or SA5 (3 μM) for 30 min, fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, and then processed for Epon embedding (Electron Microscopy Sciences, 14,120). Sections of 70 nm were mounted onto Formvar-coated nickel grids (Electron Microscopy Sciences, FF200-Ni) and double contrasted with 2% uranyl acetate (Electron Microscopy Sciences, 22400) for 5 min and 3% lead citrate (Electron Microscopy Sciences, 17810) for 5 min. Grids were imaged using a JEM 1400plus TEM imaging system with an AMT XR81M-B camera. Mitochondria were morphologically identified. Images were analyzed using ImageJ.

GZnP3 Zn²⁺ imaging
GZnP3 imaging was performed in HEK293 and MeWo cells that were transiently transfected with GZnP3-ML1 or GZnP3-Rab7 as described previously (Minckley et al., 2019). The fluorescence intensity at 488 nm was recorded with an EasyRatioPro system (Photon Technology International, Inc. New Jersey, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Average data are presented as means ± standard errors of the mean (SEMs). Statistical comparisons were made by using Student’s t test or one way ANOVA. Differences were considered significant at *p < 0.05, ** p < 0.01, *** p < 0.001. Survival curves were compared using the Log-rank test. All data analyses were conducted using Origin Pro 2019 or GraphPad Prism 8.0.
Supplemental information

Lysosomal Zn$^{2+}$ release triggers rapid, mitochondria-mediated, non-apoptotic cell death in metastatic melanoma

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Figure S1. Lysosomal ML1 channels are upregulated in metastatic melanoma cells. Related to Figure 1.

(A) RT-qPCR analysis of ML1 mRNA expression in normal melanocytes, MeWo, and M12 cells. Each open circle represents the average result from one batch of cells. (B) Western blotting analysis of ML1 and LAMP1 protein expression. β-actin served as the loading control. (C, D) Normalized ML1 and LAMP1 protein expression levels in normal melanocytes, MeWo, and M12 cells. (E) ML-SA1 (SA1), ML-SA5 (SA5), and ML-SA8 (SA8) dose-dependently activated $I_{\text{ML1}}$. (F) $I_{\text{ML1}}$ was activated and inhibited by ML-SA and ML-SI compounds, respectively, in a cytoplasmic-side-out endolysosomal patch from a MeWo cell. (G) LysoTracker staining of normal melanocytes, MeWo, and M12 cells. Scale bar = 10 μm. Right panels show the relative fluorescence intensity of LysoTracker in melanocytes (n=19 randomly selected cells), MeWo cells (n=31), and M12 cells (n=24). Average data are presented as the mean ± SEM; cells are randomly selected for analysis from at least three independent experiments; ** $p < 0.01$, *** $p < 0.001$. 
A

Cell viability (%) vs. SI3 and SI4

B

Time (h) 0.5 1 2 3 6 9 12

MeWo
SA8 (1µM) SA5 (3µM)

M12
SA8 (1µM) SA5 (3µM)

Melanocyte
SA8 (1µM) SA5 (3µM)

C

Cell viability (%) vs. SA5 (µM)

D

PI Positive Cells (%) vs. Time (h)

E

HEK

SA5 (µM)

F

PI Positive Cells (%) vs. Inhibitors
Figure S2. Small-molecule activation of lysosomal ML1 channels induces cell death in metastatic melanoma cells. Related to Figure 2.

(A) Cell viability analyses of MeWo and M12 cells treated with ML-SI3 (0–100 μM) or ML-SI4 (0–100 μM) for 12 hrs. (B) Overlay phase contrast (Ph) and fluorescence images of PI-stained indicated cells in the presence of SA5 (3 μM) or SA8 (1 μM) for indicated time periods. Scale bar, 25 μm. Time-dependent effects of SA5/8 on PI-positive cell percentages are also shown in the right panels. (C) Effects of SI4 (20 μM, 12h) on SA5-induced cytotoxicity in M12 cells. (D) The effects of SA5 and SA8 on the percentages of PI-positive HEK293 cells. (E, F) The effects of ML-SIs on SA8-induced cell death in ML1-overexpressing HEK293 cells. All data are presented as the mean ± SEM; * p < 0.05; ** p < 0.001.
Figure S3. Ca\(^{2+}\) and TFEB are not required for ML-SA-induced cytotoxicity in metastatic melanoma cells. Related to Figure 3.

(A) The effects of BAPTA-AM (20 μM) and TPEN (10 μM) pre-treatment on SA5-evoked lysosomal Ca\(^{2+}\) release, measured by GCaMP7 Ca\(^{2+}\) imaging (F470) in HEK293-GCaMP7-ML1 cells. SA5 (3 μM) was bath applied in a low or 'zero' Ca\(^{2+}\) (free [Ca\(^{2+}\)] < 10 nM) external solution. BAPTA-AM and TPEN were added to the cells 30 min prior to SA5 treatment. Ionomycin served as a positive control to induce the maximal Ca\(^{2+}\) response. (B) The effects of BAPTA-AM (20 μM) or TPEN (10 μM) treatment on SA5 (1 μM/30 min)-induced TFEB nuclear translocation in HeLa cells stably expressing TFEB-GFP. Scale bar, 10 μm. (C) Cell viability analysis of M12 cells that were treated with SA5 for 12h in the presence or absence of EGTA-AM (20 μM) or TPEN (2.5 μM). (D) Cell viability analysis of M12 cells that were treated with SA5 for 12h in the presence of Cyclosporin A (CsA, 10 μM) or TPEN (2.5 μM). (E) Cell viability analysis of control, scramble or siTFEB-transfected M12 cells that were treated with various concentration of SA5 for 24h. Left panels show the knockdown effect of siTFEB on TFEB protein expression.
**SA5 (µM)**

Cell Viability (%)

- No chelator
- 1,10-PT
- +BAPTA-AM +TPEN

**MeWo**

**Ph PI**

**M12**

**Ctrl**

**Ph PI**

**HEK**

**SA8**

**FluoZin-3 Lysotracker**

**ML1-4A-expressing HEK293 cells**

**Whole-Cell**

+ SA1 (20 µM)

Tyrode's

Zn²⁺ (30 mM)

pH 4.6

**FluoZin-3**

**Lysotracker**

Ctrl

Baf-A1

n.s.

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***

***

n.s.
Figure S4. Zn²⁺ chelation prevents ML-SA-induced cytotoxicity in metastatic melanoma cells. Related to Figure 4.

(A) Representative images of FluoZin-3 staining in M12 cells that were incubated with Zinc Pyrithione (PYZ, 3 μM/1hr), PYZ+TPEN (10 μM), and PYZ+1,10-PT (50 μM). Right panels show the statistical analyses of relative FluoZin-3 intensities of M12 cells that were treated with PYZ (n=49 cells), PYZ+TPEN (n=50 cells), or PYZ+1,10-PT-treated (n=15 cells). (B) PI staining of MeWo and M12 cells that were pretreated with BAPTA-AM (10 μM) or TPEN (2.5 μM) in the presence of SA8 (1 μM) treatment. (C) The percentage of PI-positive cells as shown in (B). Each open circle represents one batch of cells. Note that the control groups of MeWo and M12 cells treated with ML-SA8 are re-plotted from Figure 2J, as they were from the same experiments. (D) Cell viability analysis of SA5-treated M12 cells in the presence or absence of 1,10-PT (10 μM). (E) PI staining of ML1-overexpressing HEK293 cells (HEK293 ML1 OE) upon SA8 treatment (1 μM) for 6h in the presence or absence of BAPTA-AM (10 μM) or TPEN (2.5 μM). Note that left two panels are controls replotted from Figure S2E, as they were from the same experiments. (F) The percentages of PI-positive HEK293 cells as shown in (E). (G) Whole-cell ML1 currents in ML1-4A-transfected HEK293 cells in the presence of Tyrode’s or high Zn²⁺ solutions. (H) FluoZin-3 and LysoTracker staining of M12 cells treated with Baf-A1 (0.5 μM, 1h). Right panels show the relative fluorescence intensity of FluoZin-3 or LysoTracker. Scale bar = 5 μm. Each open circle represents one individual cell. All data are presented as the mean ± SEM; * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure S5. Lack of effects of known cell death inhibitors on ML-SA5-induced cytotoxicity. Related to Figure 5.

(A) Cell viability analysis of M12 cells that were treated with various concentration of SA5 for 12h in the presence or absence of necrostatin-1 (Nec-1, 20 μM). (B) Cell viability analysis of M12 cells that were treated with SA5 (3 μM; 12h) in the presence or absence of pan-caspase inhibitor z-VAD-fmk (20 μM). Doxorubicin (DOX, 3 μM; 12h) was used to show the positive control effect of z-VAD-fmk. (C) Cell viability analysis of MeWo cells that were treated with various concentration of SA5 for 12h in the presence or absence of DFO (200 μM). Right panels show the positive-control effects of DFO on cell viability of HeLa cells treated with Erastin (30 μM, 24h). (D) Cell viability analysis of MeWo cells that were treated with SA5 (3 μM, 12h) in the presence or absence of chloroquine (CQ, 20 μM). (E) Cell viability analysis of M12 cells that were treated with various concentration of SA5 for 12h in the presence or absence of Baf-A1 (0.5 μM). All data are presented as the mean ± SEM; *** p < 0.001.
M12

Control

SA5

Relative TMRM Fluorescence Intensity

0
1
2

***

Ctrl SA5
Figure S6. ML-SA5 treatment causes a loss of mitochondrial membrane potential in metastatic melanoma cells. Related to Figure 5.

Representative images of DIC and TMRM staining of M12 cells treated with DMSO (control) or SA5 (3 μM/0.5h). Scale bar = 10 μm. Right panel, TMRM staining shows reduced mean pixel intensity after SA5 treatment. All data are presented as the mean ± SEM; *** $p < 0.001$. 
Days after tumor implantation

Survival Percentage (%)
Figure S7. Survival analysis of mice bearing MeWo-FmC subcutaneous tumors. Related to Figure 6.

Nude mice were s.c. injected with $1.5 \times 10^6$ MeWo-FmC cells. One week later, tumor-bearing mice were randomly assigned to vehicle ($n=6$ mice) vs. SA5 treated group ($n=7$). Vehicle or ML-SA5 was administrated to mice through i.p. injection 3 times every week until the end of experiment. Survival curves were compared using the Log-rank test, $p < 0.01$. 