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Zinc-Dependent Lysosomal Enlargement in TRPML1-Deficient Cells Involves MTF-1 Transcription Factor and ZnT4 (Slc30a4) Transporter

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Synopsis

Zn is critical for a multitude of cellular processes, including gene expression, secretion and enzymatic activities. Cellular Zn is controlled by Zn-chelating proteins and by Zn transporters. The recent identification of Zn permeability of the lysosomal ion channel TRPML1, and the evidence of abnormal Zn levels in cells deficient in TRPML1, suggested a role for TRPML1 in Zn transport. Here we provide new evidence for such a role and identify additional cellular components responsible for it. In agreement with the previously published data, an acute siRNA-driven TRPML1 knockdown (KD) leads to the buildup of large cytoplasmic vesicles positive for Lysotracker and Zn staining, when cells are exposed to high concentrations of Zn. We now show that lysosomal enlargement and Zn buildup in TRPML1-KD cells exposed to Zn are ameliorated by KD of the Zn-sensitive transcription factor MTF-1 or Zn transporter ZnT4. TRPML1 KD is associated with a buildup of cytoplasmic Zn and with enhanced transcriptional response of mRNA for metallothionein 2a (MT2a). TRPML1 KD did not suppress lysosomal secretion, but it did delay Zn leak from the lysosomes into the cytoplasm. These data underscore a role for TRPML1 in Zn metabolism. Furthermore, they suggest that TRPML1 works in concert with ZnT4 to regulate Zn translocation between the cytoplasm and lysosomes.

Keywords

Zinc; lysosomal storage disease; TRP channels; MTF-1; Slc30a4; ZnT4

Introduction

Transition metals, including Zn, are widely recognized for their toxic effects caused by acute and chronic exposures, albeit most of the transition metals are indispensable for proper cell function. Zn has the widest repertoire of known biological roles, which include a cofactor in several enzymes and DNA-interacting proteins [1, 2]. Zn deficiency, as well as excess, has been shown to be deleterious for cells [3, 4], prompting the need for tight regulation of its cellular levels. Such regulation is carried out by a system of transporters, chelating proteins and Zn-sensitive transcriptional responses [5–7].

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Zn enters the body through enterocytes, primarily through Zip4, a member of the Zip (Slc39a) family of Zn transporters [8, 9]. From the enterocyte cytoplasm, Zn moves into the bloodstream through a series of transporters including those belonging to the ZnT (Slc30a) family [6, 10]. In general, Zip transporters are responsible for influx of Zn into the cytoplasm, while ZnT transporters for its efflux. In the bloodstream, Zn is taken up by cells via Zip transporters, and by the endocytosis of Zn bound to plasma proteins. After entering the cytoplasm, Zn is chelated by cytoplasmic proteins, such as metallothioneins (MTs) [11], and extracted into organelles or across the cell membrane by ZnT transporters. For example, ZnT2 and ZnT4 transporters are localized in intracellular vesicular compartments, including lysosomes. They play important roles in vesicular Zn accumulation [12–14]. The reason for Zn chelation and extraction is twofold. First, high levels of Zn are damaging to cells [3, 4]. Second, a number of biological processes requiring Zn take place inside organelles, necessitating Zn transport across the organellar membranes [6, 15]. MTs also serve as Zn reservoirs, releasing chelated Zn under low-Zn conditions [5].

The balance between ZnTs, Zips and MTs is responsible for the net Zn flux and for the free cytoplasmic Zn concentration in the given cell type. Zn spikes, driven by variations in dietary Zn uptake, are read by Zn-responsive transcription factors including MTF-1, which binds cytoplasmic Zn through its Zn-finger motifs leading to its nuclear translocation [16]. Upon activation by cytoplasmic Zn, MTF-1 induces the transcription of genes coding for MTs and some ZnTs, such as ZnT1 and ZnT2 [17, 18]. This feedback system allows cells to rapidly quench spikes in free cytoplasmic Zn. The combined activity of Zn transporters and chelators keeps free Zn concentration in the nanomolar range.

The recent identification of Zn permeability through the lysosomal ion channel TRPML1 [19] raised the possibility that this ion channel is involved in Zn transport. This notion was later supported by the finding that Zn builds up in the lysosomes of TRPML1-knockdown cells [20]. TRPML1 is an ion channel residing in the later portions of the endocytic pathway due to the presence of lysosomal localization signals on its C- and N- terminals [21, 22]. TRPML1 is encoded by the *MCOLN1* gene, which was identified as the gene mutated in the lysosomal storage disease mucolipidosis type IV (MLIV) [23, 24]. MLIV is associated with the buildup of cytoplasmic storage bodies, motor dysfunction and developmental delays [25–27]. The recent characterization of TRPML1 activation by $PI(3,5)P_2$ [28] suggested its activation by delivery to the $PI(3,5)P_2$-rich lysosomes and late endosomes [24]. The discussion of TRPML1 function has been focused on its role in the fusion of vesicles in the endocytic pathway (reviewed in [24]), and, recently, lysosomal secretion [29]. At the same time, the evidence of Fe and Zn permeability through TRPML1 [19] raises an interesting possibility that it plays a role in regulating cellular transition metal levels.

Here, we sought to delineate the mechanism through which TRPML1 participates in cellular Zn homeostasis. We based our search on the assumption that if TRPML1 is directly involved in the regulation of Zn transport, then, cellular Zn trafficking and Zn dependent processes should change as a function of TRPML1 status. Our assays show that both of these assumptions are true, strongly suggesting that TRPML1 is involved in the regulation of cellular Zn homeostasis. We found that the effects of Zn in TRPML1 KD cells included a characteristic lysosomal enlargement. The Zn-sensing cytoplasmic transcription factor MTF-1 plays a key role in this process, as MTF-1 KD reversed Zn-dependent lysosomal enlargement in TRPML1 KD cells. Suppressing the expression of the vesicular Zn transporter ZnT4 eliminated the Zn-induced lysosomal enlargement and Zn retention in TRPML1-deficient cells. Pulse-chase experiments with extracellular Zn show that Zn clearing from vesicular structures including lysosomes is delayed in TRPML1 KD cell. However, Zn secretion was not decreased in these cells; instead, it was somewhat increased. We conclude that TRPML1 works in concert with ZnT4, to regulate lysosomal Zn

trafficking, perhaps by providing a lysosomal Zn leak pathway. This is supporting evidence of the novel role of TRPML1 in regulating cellular transition metals and TRPML1 as a new target in transition metals toxicity.

Experimental procedures

Cell Culture

HeLa cells were maintained in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 7% FBS, 100 μg/ml penicillin/streptomycin, and 5 μg/ml Plasmocin prophylactic (Invivogen, San Diego, CA). For siRNA and cDNA transfection, antibiotic free media was used. Metals were added directly to the DMEM. Zn (100 μM) was added to antibiotic free medium, containing serum, 24 hrs after transfection, for 48 hrs.

siRNA-mediated KD and plasmid transfection

ON-TARGET plus siRNA were designed as described previously [30, 31] and synthesized by Dharmacon (Lafayette, CO). The TRPML1 siRNA probe targeting the sequence 5'-CCCACATCCAGGAGTGTAA-3' in *MCOLN1* was used for all TRPML1 KDs. The MTF-1 siRNA (Cat number SASI_Hs01_00177112), ZnT2 siRNA (Cat number SASI_Hs01_00055662), and ZnT4 siRNA (Cat number SASI_Hs00225995) were from Sigma. Non-targeting control siRNA#1 (Sigma) was used as a negative control. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbag, CA) as described by manufacturer's protocol using 600 nM siRNA per 35 mm well. All KDs were confirmed using SYBR-green based qPCR. For DNA transfections, 2 μg of HA-tagged human ZnT2 or ZnT4 were used in parallel with 1 μg of GFP tagged TRPML1 constructs.

Reverse Transcriptase and Quantitative qPCR

RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using the GeneAmp RNA PCR system (Applied Biosystems, Carlsbad, CA) with oligo dT priming. qPCR was performed SYBR green (Fermentas, Glen Burnie, MD). The amount of cDNA loaded was normalized to starting RNA concentrations, with a final concentration of 9 ng (40 ng in ZnT experiments) of RNA loaded per experimental well. Six-point standard curves were generated for each primer using 1:2 dilutions of cDNA. cDNA for the following genes were amplified using the indicated primers (IDT, Coralville, IA).

MCOLN1: forward: 5'-TCTTCCAGCACGGAGACAAC-3', reverse: 5'-AACTCGTTCTGCAGCAGGAAGC-3'.

MT2a: forward: 5'-AAGTCCCAGCGAACCCGCGT-3', reverse: 5'-CAGCAGCTGCACTTGTCCGACGC-3'.

MTF-1: forward: 5'-GCCATTTCGGTGCGATCACGAT-3', reverse: 5'-TTTCACCAGTATGTGTACGAACGTGAGT-3'.

ZnT2 (SLC30A2): forward: 5'-GCAATCCGGTCATACACGGGAT-3', reverse: 5'-CAGCTCAATGGCCTGCAAGT-3'.

ZnT4 (SLC30A4): forward: 5'- CACATACAGCTAATTCCTGGAAGTTCATCT-3', reverse: 5'- GCCTGTAACTCTGAAGCTGAATAGTACAT-3'.

All primers were designed to span exons and negative RT controls were tested to ensure amplification of cDNA only. qPCR was performed using the Standard Curve method on the 7300 Real Time System (Applied Biosystems). Reactions were run on the following parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 sec followed by

60°C for 1 min. All experimental samples were run in triplicate and normalized to a β-actin endogenous control.

Microscopy

Cells were seeded on glass coverslips and loaded with dyes for 15 min at 37°C in buffer containing, in mM: 150 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4, 1 g/L glucose. The loading was followed by 15 min washout in all cases except TSQ. Lysotracker Red DND-99, FluoZin-3,AM, TSQ (Sigma) were used at 0.1, 0.1, and 150 μM respectively. Confocal microscopy was performed using Leica TCS SP5 and BioRad 3000 confocal microscopes. Live cells were treated as above. For colocalization experiments, cells were fixed for 5 min in 4% paraformaldehyde at room temperature and permeabilized using 0.1% Triton ×100 for 5 min on ice. Following washout and blocking in BSA and goat serum, cells were treated with primary anti-HA antibody (Roche, 12CA5) overnight. Cells were then incubated in fluorescent-tagged secondary antibodies for 1 hr. Images were analyzed using ImageJ (Bethesda, MD).

β-Hexosamindase activity assay

Untreated control and TRPML1 siRNA-transfected cells were washed with 37°C PBS and 1 mL 37°C serum-free DMEM was added to each 35mm dish. For each sample, 100 μl of the supernatant was incubated with 400 μl 1mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma, N9376) for 1 hour at 37°C in 0.1M citrate buffer (pH 4.5) (Sigma, C2488). This volume was replaced with fresh 37°C 100 μl of DMEM to the dish. Samples were collected every hour for 4 hours. Reactions were stopped by adding 500 μl borate buffer (100 mM boric acid, 75 mM NaCl, 25 mM sodium borate, pH 9.8) and the absorbance was measured in a spectrophotometer at 405 nm. To determine total cellular content of β-hexosamindase, cells were lysed with 1mL 1% Triton X-100 and after a 10000g spin for 5 minutes at 4°C, 100 μl of the cell extracts were used for the enzyme activity reaction. Enzyme activity was determined as the amount of 4-nitrophenol produced per mg of protein (Bradford method). Absorbance was calibrated with different amounts of 4-nitrophenol (Sigma, N7660) in 0.1 M citrate buffer.

Zinc secretion assay

Control, TRPML1 and MTF1 siRNA-transfected cells were plated on a 12 well plate and after 48 hours, pulsed with 100 μM Zn for 3 hours, washed twice with warm PBS, and chased in 1mL DMEM per well. Duplicate time-points were collected for 0, 5, 15, 30 or 60 minutes. For each sample, 50 μl of supernatant was placed in a 96-well plate. Zn content was measured by incubating the supernatants with 10 μM cell-impermeant FluoZin-3 tetrapotassium salt (Molecular Probes, Invitrogen F-24194) for 15 minutes at 37°C. The 96-well plate was read via a FujiFilm FLA-5100 fluorescent image analyzer. After the last time point, cells were washed with PBS, 200 μl detergent solution was added to lyse the cells and fluorescence was normalized to total protein in each sample.

Statistical significance was calculated using a one-tailed, unpaired t-test with $p \leq 0.05$ considered significant. Data are presented as mean±S.E.M.

Results

TRPML1, Zn and lysosomal enlargement

Since Zn dyshomeostasis has been reported in TRPML1-KD cells [20], we sought to delineate mechanisms linking TRPML1 loss, Zn dysregulation, and vacuolar enlargement in TRPML1-KD cells. In order to test how TRPML1 loss affects cells exposed to Zn, we used TRPML1-specific siRNA described before [30, 31]. The siRNA-mediated KD resulted in

more than 85% KD of TRPML1 mRNA, as confirmed by qPCR with TRPML1 specific primers (Supplementary Fig S1). In the previous studies utilizing the same system, the same siRNA resulted in almost complete elimination of TRPML1 protein, within the same time scale [30, 31].

TRPML1 KD cells were previously reported to contain enlarged Zn-positive organelles [20]. We performed live-cell confocal analysis of control and TRPML1-KD HeLa cells stained with the Zn selective dyes TSQ and FluoZin-3,AM, in conjunction with the lysosomal dye Lysotracker. Fig 1A shows that, while Zn is barely detectable in Lysotracker-positive compartments in untreated control cells, such compartments displayed TSQ fluorescence in cells treated with 100 μM Zn for 48 hrs. We did not consistently detect enlarged Lysotracker-positive compartments in TRPML1-KD cells until these cells were treated with Zn. Following Zn treatment, large compartments positive for Zn (TSQ) and Lysotracker were abundant in TRPML1 KD cells (Fig 1A). Fig 1B shows similar analysis of Zn in control and TRPML1-KD cells, performed using Lysotracker and another Zn dye, FluoZin-3,AM. The enlarged Lysotracker-positive vesicles in Zn treated TRPML1-KD cells also displayed bright FluoZin-3 fluorescence, consistent with high concentrations of Zn. Interestingly, FluoZin-3 signal was also detected in Lysotracker-negative vesicles, consistent with our previous observations of non-lysosomal Zn pools in mammary cells [32, 33], which was more commonly observed in Zn-treated TRPML1-KD cells than in Zn-treated control cells (Fig 1B). The lysosomal enlargement trend in Zn-treated TRPML1-KD cells persisted over 96-hr period (not shown).

The fact that TRPML1-KD cells treated with Zn and stained with TSQ show cytoplasmic fluorescence (Fig 1A), while FluoZin-3,AM-stained cells do not (Fig 1B), can be explained by the nature of these dyes' fluorescence and interaction with Zn. In the cytoplasm, Zn is bound to proteins such as MTs and very little Zn is present in free ion form. MTs bind large amounts of Zn (up to 7 ions per molecule), with very high affinity (pM range) [34]. Both TSQ and FluoZin-3 are sensitive to Zn in mid- to high-nanomolar range. However, unlike FluoZin-3, which only fluoresces when bound to more labile Zn, TSQ fluoresces even when in a complex with metalloproteins such as MT-Zn [35, 36]. Therefore, the difference between TSQ and FluoZin-3 stains may reflect large amounts of Zn ions bound in Zn-treated TRPML1-KD cells to cytoplasmic proteins such as MT2a.

Using Lysotracker fluorescence images, we calculated the average gain in the lysosomal size in Zn-treated TRPML1-KD cells. The same Lysotracker concentration, laser intensity, pinhole and gain values were used throughout the experiments. The grey-scale Lysotracker images were filtered using 50% intensity threshold yielding binary black-and-white images, which were then treated using Watershed segmentation algorithm implemented as an ImageJ plugin (Supplementary Fig S2A). Large clusters of signal not resolved using this algorithm were manually eliminated (Supplementary Fig S2B). Next, the ImageJ "Analyze particles" algorithm was used, which yielded numbers and sizes of Lysotracker-positive particles in each image (Supplementary Fig S2C). For particle counting the following options were used: minimal size 0.09 μm^2, minimal circularity was 0.5. The same algorithm was used in all subsequent analysis in this paper.

Zn-treated TRPML1-KD cells showed a tendency towards increasing the number of larger Lysotracker-positive particles (Supplementary Fig S3). In Zn-exposed TRPML1-KD cells, lysosomes were approximately 80% larger than in Zn-exposed control cells or in untreated TRPML1-KD cells (Fig 2). Lysotracker-positive particles in Zn-treated TRPML1-KD cells averaged 176.15±12.50% (8 independent experiments, 3–5 images each, 1–3 cells on each image, 214 to 573 Lysotracker-positive particles in each experiment, $p<0.05$ relative to control) of the average size of individual lysosomal areas in Zn-treated control siRNA-

transfected cells (100±4%, 8 independent experiments, 3–5 images each, 1–3 cells on each image, 99 to 1100 Lysotracker-positive particles in each experiment). The use of threshold preserves the relative sizes of compartments, but makes evaluation of their absolute sizes unreliable. With this reservation in mind, the average size of Lysotracker-positive compartment in Zn-treated control cells was 2.09±0.30 μm^2 (statistics as above; two experiments were omitted from this analysis due to different microscope settings). In TRPML1-KD Zn-treated cell it was 3.56±0.51 μm^2. Beyond agreeing with the previous report on Zn buildup in TRPML1-KD cells [20], these data suggest that in the absence of TRPML1, a Zn-dependent process leads to the enlargement of Lysotracker-positive (ostensibly lysosomal) compartments.

Ni, used at the same concentration, did not induce lysosomal enlargement (Supplementary Fig S3), suggesting that this aspect of TRPML1 KD phenotype is Zn-specific.

These data confirm, in a different system, the previous findings on lysosomal enlargement and Zn retention in TRPML1-KD cells [20]. A logical question following observation of this phenomenon is the source and means of Zn buildup in the lysosomes of TRPML1-KD cells. Since Zn is retained by the lysosomes in TRPML1-KD cells, TRPML1 must be involved in the clearance of Zn from the lysosomes. Where does the Zn retained in TRPML1-KD cells come from? Zn retained by the lysosomes 1) may come through endocytosis, or 2) may be taken up by cells through cell membrane transporters and then exported into lysosomes across their membranes. In both cases TRPML1 dissipates Zn accumulation. The difference between the two scenarios is that the latter entails 1) a lysosomal Zn transporter that works in concert with TRPML1 to regulate Zn traffic between cytoplasm and lysosomes, and 2) dependence of the lysosomal Zn accumulation in TRPML1-KD cells on a cytoplasmic step.

TRPML1 and MTF-1

Cytoplasmic Zn is tightly regulated, and therefore, the expression of proteins involved in Zn maintenance is managed by Zn-dependent transcription factors such as MTF-1. Zn binding to MTF-1, followed by MTF-1 nuclear import leads to the expression of proteins involved in Zn handling, such as MTs. Their mRNA levels can thus be used to monitor cytoplasmic Zn levels.

Fig 3A shows a successful siRNA mediated knockdown of over 90% of MTF-1 mRNA in untreated cells. This effect had a clear physiological significance, as MTF-1 KD severely suppressed mRNA levels of MT2a, which is the most ubiquitous and best described MT, well known to be regulated by MTF-1 (Fig 3B). In unstimulated cells, MTF-1 siRNA decreased MT2a mRNA levels to 19.12±5.71% of control levels (n=3, $p<0.05$). This established a toolkit for testing the role of MTF-1 in TRPML1-dependent effects of Zn.

MTF-1 suppression eliminated the buildup of enlarged vacuoles in TRPML1 KD cells (Fig 2, Fig 3C, Supplementary Fig S4). In the double TRPML1/MTF-1–KD Zn-treated cells, the average lysosomal area was 101.78±7.06% (3 independent experiments, 3–4 images each, 1–3 cells on each image, 127 to 1035 Lysotracker-positive particles in each experiment) of its value in Zn-treated control cells, a statistically indistinguishable value. These data suggest that these TRPML1-dependent aspects of Zn phenotype in HeLa cells are mediated by MTF-1. The fact that suppression of a Zn-dependent step in the cytoplasm eliminates lysosomal enlargement and Zn retention in TRPML1-KD cells strongly suggests that the source of Zn retained in TRPML1-deficient lysosomes is not mediated through endocytosis, but is derived directly from a cytoplasmic route. This route is discussed below. We asked whether dysregulation of this route affects cytoplasmic Zn and which Zn transporters are involved in this process.

TRPML1 and MT2a

As discussed above, the presence of a cytoplasmic Zn- and TRPML1-dependent process suggests that the expression of Zn-dependent genes in cells exposed to Zn is also affected by TRPML1 activity. In this regard, MTF-1/Zn–dependent genes can be used as cytoplasmic Zn reporters. In order to test this idea, we first screened mRNA levels of a well-known MTF-1-dependent gene product, MT2a. Fig 4 shows that upregulation of MT2a mRNA in response to Zn is significantly augmented in TRPML1-KD cells, compared to cells transfected with control siRNA. In control cells, 48-h stimulation with Zn increased MT2a mRNA levels to 676.8±152.3% of control levels (n=3, $p<0.05$), while in TRPML1-KD cells, the response to Zn was significantly greater (2992.7±678.5% of control levels; n=3, $p<0.05$). We conclude that a Zn handling deficit in TRPML1-KD cells leads to a persistent dysregulation of Zn management.

It should be noted that, in our system, the difference in MT2a mRNA response is only seen under Zn exposure (Fig 4) and not under the basal conditions. Therefore, the upregulation of MT2a mRNA is a reaction to the change in cytoplasmic Zn buffering in the absence of TRPML1. Together with the MTF-1 KD assays, these experiments established that the lysosomal Zn buildup and enlargement in TRPML1-KD cells depend on a cytoplasmic step, and that TRPML1 loss increases cytoplasmic Zn (also consistent with the data shown in Fig 1. We propose that TRPML1 works in concert with a lysosomal Zn transporter to maintain normal Zn levels in the cytoplasm and in the lysosomes. The loss of such concerted activity leads to cytoplasmic and lysosomal Zn buildup. Next, we set out to identify such a transporter.

TRPML1 and ZnT4

Of the Slc30 (ZnT) family, ZnT2, ZnT3, ZnT4 and ZnT8 have been implicated in Zn loading within endocytic compartments [37–39]. However, ZnT3 and ZnT8 expression seems to be restricted to brain and pancreas [40, 41]. For this reason, ZnT2 and ZnT4 became the first target of our investigation. Recombinant ZnT2 and ZnT4 both partially colocalized with TRPML1 (Fig 5), indicating that they might work in concert with TRPML1. If either ZnT2 or ZnT4 are necessary for the TRPML1-dependent aspect of Zn homeostasis, then KD should affect the Zn phenotype in TRPML1-KD cells. The predicted direction of Zn transport through ZnT transporters is into the lysosomes. Therefore, a ZnT KD should negate the effects of TRPML1 KD. We chose to modulate ZnT levels using siRNA.

Fig 6A shows a qPCR confirmation of ZnT2 and ZnT4 KD (over 70% and 95% control mRNA levels in untreated cells, respectively). ZnT2 siRNA decreased ZnT2 mRNA levels to 29.88±21.31% of control levels (n=3, $p<0.05$), while ZnT4 siRNA decreased ZnT4 mRNA levels to 4.36±3.73% of control levels (n=3, $p<0.05$). siRNA-mediated KD of ZnT4, but not ZnT2 rescued lysosomal swelling and lysosomal Zn buildup in Zn-treated TRPML1 KD cells (Fig 6B,C). In the double TRPML1/ZnT4–KD cells exposed to Zn, the average lysosomal area fell to about 50% of the value reported in TRPML1-KD cells. It averaged 89.06±7.08% of the lysosomal size in control, untreated cells (4 independent experiments). In double TRPML1/ZnT2–KD cells, it remained largely the same as in TRPML1-KD cells exposed to Zn (167.22±15.71% of control cells, 4 independent experiments, $p<0.05$). The fact that ZnT4 KD abolished lysosomal enlargement under TRPML1 suppression has a clear physiological significance because it identifies the lysosomal Zn transporter that, in concert with TRPML1, forms a lysosomal “Zn sink,” which absorbs the cytoplasmic Zn (Fig 7). In direct agreement with this, Zn-treated TRPML1- and ZnT4-KD cells have large amounts of Zn within the cytoplasm compared to ZnT2 KD (Fig 6B). Whether or not this phenomenon is specific to HeLa cells remains to be established, but this is consistent with Zn

accumulation in lysosomes in ZnT4-overexpressing mammary cells [32]. We would like to note that the triple TRPML1/ZnT2/ZnT4 KD was extremely cytotoxic even at the control conditions, which precluded its analysis.

Zn secretion and lysosomal Zn leak in TRPML1-KD cells

The data described above suggest that ZnT4 loads lysosomes with Zn and that in the absence of TRPML1, Zn is trapped in the lysosomes. Furthermore, clearance of Zn from the lysosomes in TRPML1-KD cells was delayed, compared to control cells (Supplementary Fig S5). The reason for Zn retention in TRPML1-deficient lysosomes was the subject of our next set of experiments.

TRPML1 has been implicated in lysosomal secretion. Therefore, it is possible that suppression of the lysosomal secretion due to TRPML1 loss traps Zn in the lysosomes. Alternatively, it is possible that TRPML1 is lysosomal Zn channel, responsible for the Zn leak from lysosomes into the cytoplasm. The role of TRPML1 in Zn secretion was analyzed using two assays. The premise of this experiment was: if Zn retention in the TRPML1-KD cells is due to retarded secretion, then Zn secretion in TRPML1-KD cell will be significantly lower than in control cells. This is directly addressed in Fig 8A. We measured Zn release from Zn-preloaded control and TRPML1 KD-cells using FluoZin-3 tetrapotassium salt. Cells, grown on 12-well plates, were incubated in culture medium with 100 μM Zn for 3 hr (pulse). Next they were washed in PBS and placed into fresh culture medium (chase), whose samples were taken 0, 5, 15 and 60 min later. Samples were analyzed using FluoZin-3 (cell impermeable salt) fluorescence, which was normalized to the total protein content of the well containing the given sample of cells. Fig 8A shows that Zn content of the medium during the chase phase was not lower, but indeed higher in TRPML1-KD than in control cells.

Next, total constitutive lysosomal secretion was recorded using β-hexosaminidase release over four-hour time interval. The previously described protocol was used [42]. Fig 8B shows that there was no detectable difference in constitutive exocytosis of β- hexosaminidase between TRPML1-KD and control cells. These data are consistent with similar secretion rates, but higher lysosomal Zn content in TRPML1-KD cells. They argue against difference in secretion being the main cause of Zn retention in the lysosomes of TRPML1-KD cells.

Discussion

In the course of these studies, we found that, in agreement with the previously published report [20], there is a significant dysregulation of Zn handling in TRPML1-KD cells. Our findings provide further insight into this process by showing that TRPML1 loss affects processes beyond lysosomes, and by identifying some of the components of the lysosomal “Zn sink” responsible for export of the cytoplasmic Zn into the lysosomes.

We show that the lysosomal enlargement and Zn buildup are alleviated by the suppression of cytoplasmic transcription factor MTF-1. These data clearly establish that a cytoplasmic step is involved in the loading of lysosomes with Zn and their enlargement in TRPML1 KD cells exposed to Zn. They largely rule out endocytosis as the other source of lysosomal Zn retention. What is this step and what is the role of MTF-1 in this process?

We found that the loss of ZnT4 abolishes Zn buildup and lysosomal enlargement in TRPML1-KD cells exposed to Zn. The fact that ZnT2 does not appear to have an effect in our system is puzzling. It likely reflects the specifics of Zn handling by HeLa cells. ZnT2 expression requires several factors [17]. It is possible that ZnT2 expression, or its response to Zn, is defective in HeLa cells. Furthermore, it is possible that ZnT2 and TRPML1 do not

work in the same functional space. A more exciting possibility is that TRPML1 regulates localization, or activity of ZnTs, specifically ZnT2 (Fig 7). Accordingly, a dependence of ZnT2 activity and structural integrity on lysosomal pH was recently shown [43].

Finally, we demonstrate that Zn leak from the lysosomes into the cytoplasm is delayed in TRPML1-KD cells. It is unlikely and that such delay is due to problems with Zn secretion in TRPML1-KD cells, since there was no reduction in Zn secretion in these cells, compared with control cells (Fig 8). The lack of TRPML1 effect on β-hexosaminidase secretion in our system does not refute TRPML1's role in secretion. It is possible that TRPML1 is involved in another aspect of secretion, such as regulated secretion, which was beyond the scope of the present work.

Although MTF-1 was shown to regulate ZnT1 expression, no evidence of ZnT4 regulation by MTF-1 has been presented so far. It is important to remember that MTF-1 regulates MT expression and that MTs are indispensable for Zn transport. Several lines of evidence show that MTs deliver and load Zn on the transporters and that in the absence of MTs, Zn transport is severely compromised [44, 45]. Based on this, we suggest that, in our system, MTF-1 KD affected Zn transport into lysosomes due to the loss of MT2a, and possibly other MTs. This likely affected the loading of lysosomal Zn transporters with Zn. We hypothesize that, as previously suggested [46] MT2a transfers cytoplasmic Zn onto ZnTs, such as ZnT4, promoting its export into lysosomes. Such a lysosomal "Zn sink" may be a defense mechanism against deleterious Zn spikes during toxic levels of Zn exposure.

At the same time, MT2a mRNA response to Zn in TRPML1-KD cells is dramatically increased. This is consistent with increased cytoplasmic Zn levels in TRPML1 KD cells exposed to Zn. What is the role of TRPML1 in this process? The fact that TRPML1 loss leads to lysosomal Zn retention indicates that TRPML1 dissipates lysosomal Zn. TRPML1 may transport Zn from the lysosomes into the cytoplasm, as suggested by our data in Fig S5. It is important to remember that the upregulation of Zn chelation and extraction from cells persistently exposed to Zn is likely to lead to a drop in cytoplasmic Zn concentration after the initial spike induced by the addition of Zn. It is possible that TRPML1 function is to dissipate Zn accumulated in the lysosomes due to ZnT4 activity during Zn spike. TRPML1 loss would result in weak, but prolonged Zn leak through other transporters leading to low, but chronic Zn elevation, perhaps sufficient to chronically activate MTF-1.

The central finding of these studies are the identification of the source of Zn buildup in TRPML1-KD cells as cytoplasmic as well as the identification of components that are necessary for the TRPML1-deficient "Zn sink" to function properly: ZnT4 and cytoplasmic proteins, such as the transcription factor MTF-1. These components are involved in the cellular response to exposure to Zn against the background of the loss of a lysosomal ion channel whose function to date remains unknown. Our findings shed light on TRPML1 function and its importance for proper cell function and health. Since the loss of TRPML1 is the cause of MLIV, further studies will show whether or not dysregulation of cytoplasmic Zn in TRPML1-KD cells is a contributing factor in the key aspects of MLIV pathogenesis, such as the buildup of storage bodies or cell death. Beyond MLIV, the fact that TRPML1 is involved in Zn trafficking potentially impacts other neurodegenerative diseases. Zn is an essential transition metal that needs to be highly regulated and Zn dysregulation has been linked to neuronal death following a seizure or ischemic episode [47, 48], formation of β–amyloid plaques associated with Alzheimer's [49], and pancreatic β–cell degeneration [50]. It would be interesting to see the role of TRPML1 in those processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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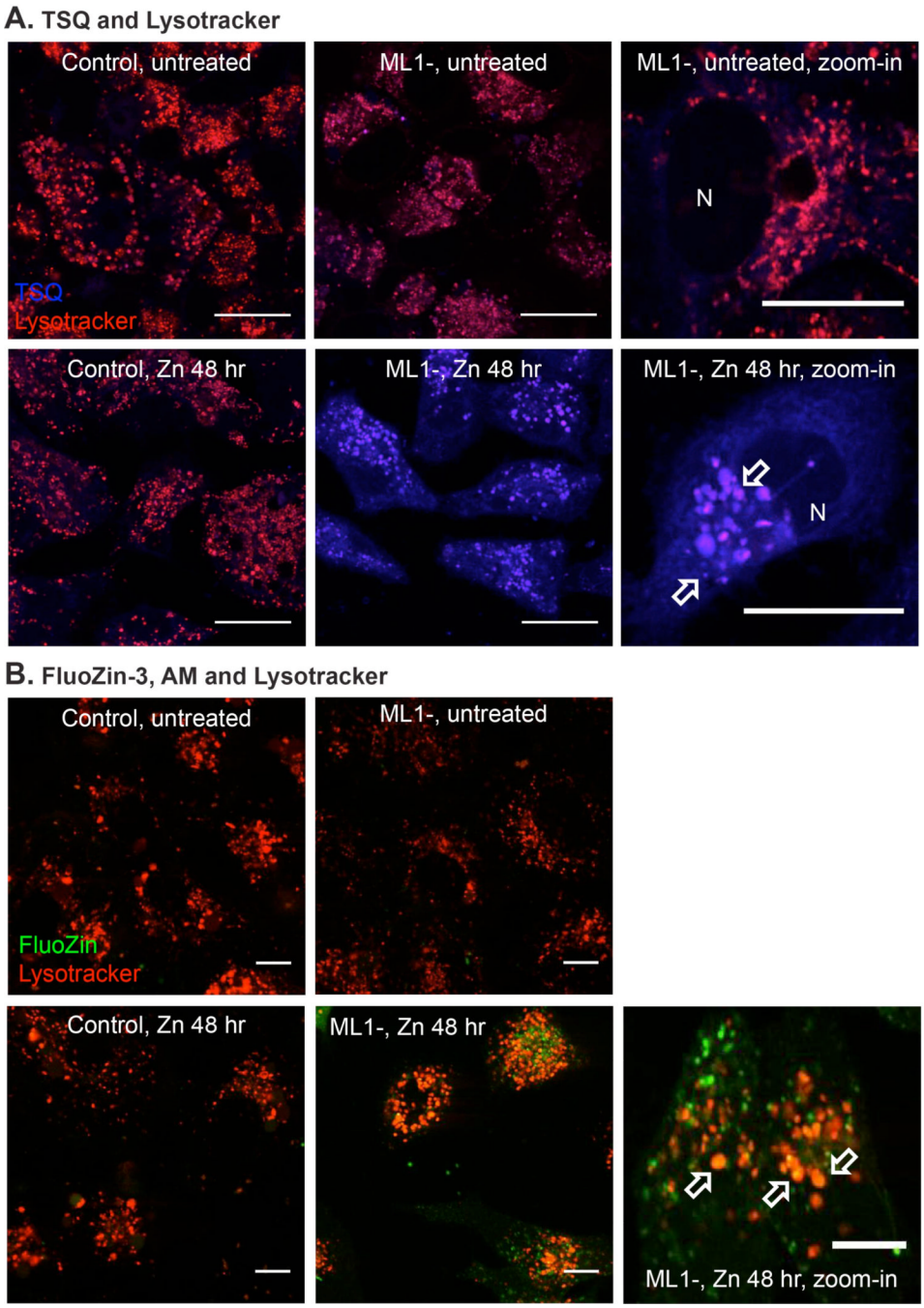

Fig 1. Zn accumulation and enlargement of Lysotracker positive compartments in TRPML1-deficient Zn treated cells

Confocal images of HeLa cells stained with Lysotracker and with TSQ (A) or FluoZin-3,AM (B). **A.** Cells costained with Lysotracker and TSQ. Control cells were transfected with scrambled siRNA. TRPML1 KD cells (ML1−) were transfected with TRPML1 siRNA, and treated with Zn 24 hrs later. Zn treatment took place over 48-hr period. N indicates nuclei; arrows point to enlarged Lysotracker and TSQ-positive compartments. Note enlargement of Lysotracker-positive compartments and presence of bright TSQ fluorescence inside them. **B.** Similarly treated cells costained with Lysotracker and FluoZin-3,AM. Each panel represents 3–5 individual experiments. Arrows point to

Lysotracker positive compartments showing bright FluoZin-3 fluorescence. Scale bars are 20 μm.

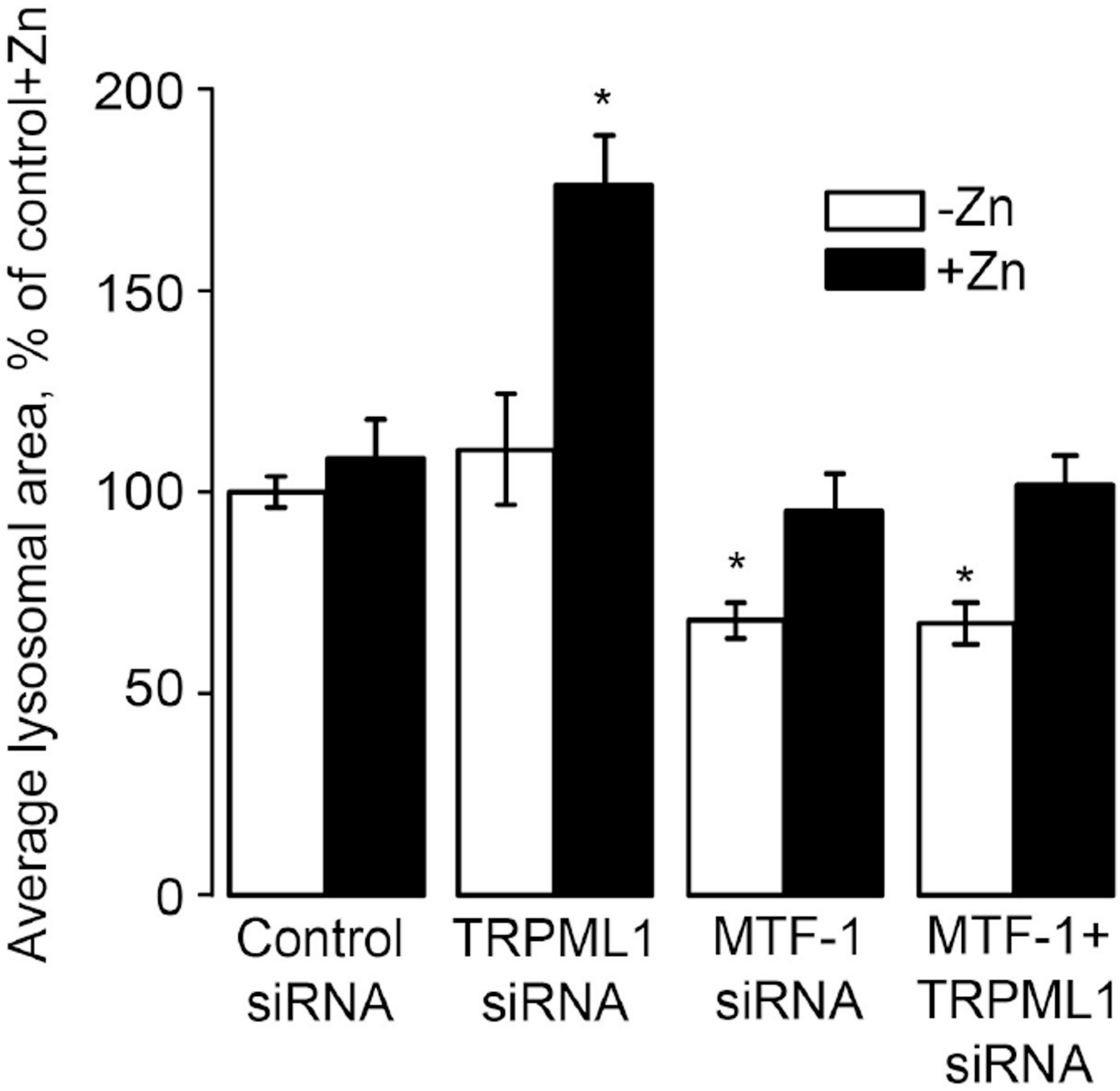

Fig 2. Statistical analysis of lysosomal enlargement in TRPML1-deficient, Zn-treated cells

Average lysosomal particle area in control, MTF-1 siRNA-transfected and TRPML1 siRNA-transfected cells under untreated (hollow) and 48 hr Zn-treated (shaded) conditions. Lysotracker confocal images of Fluozin-3,AM experiments were thresholded at 50% and particle size area calculated for each experiment using ImageJ. Represents 3–5 separate experiments. See main text for details of analysis and statistics. * represents $p<0.05$ relative to control sample. MTF-1+TRPML1 siRNA denotes cells transfected with mixture of MTF-1 and TRPML1 siRNA.

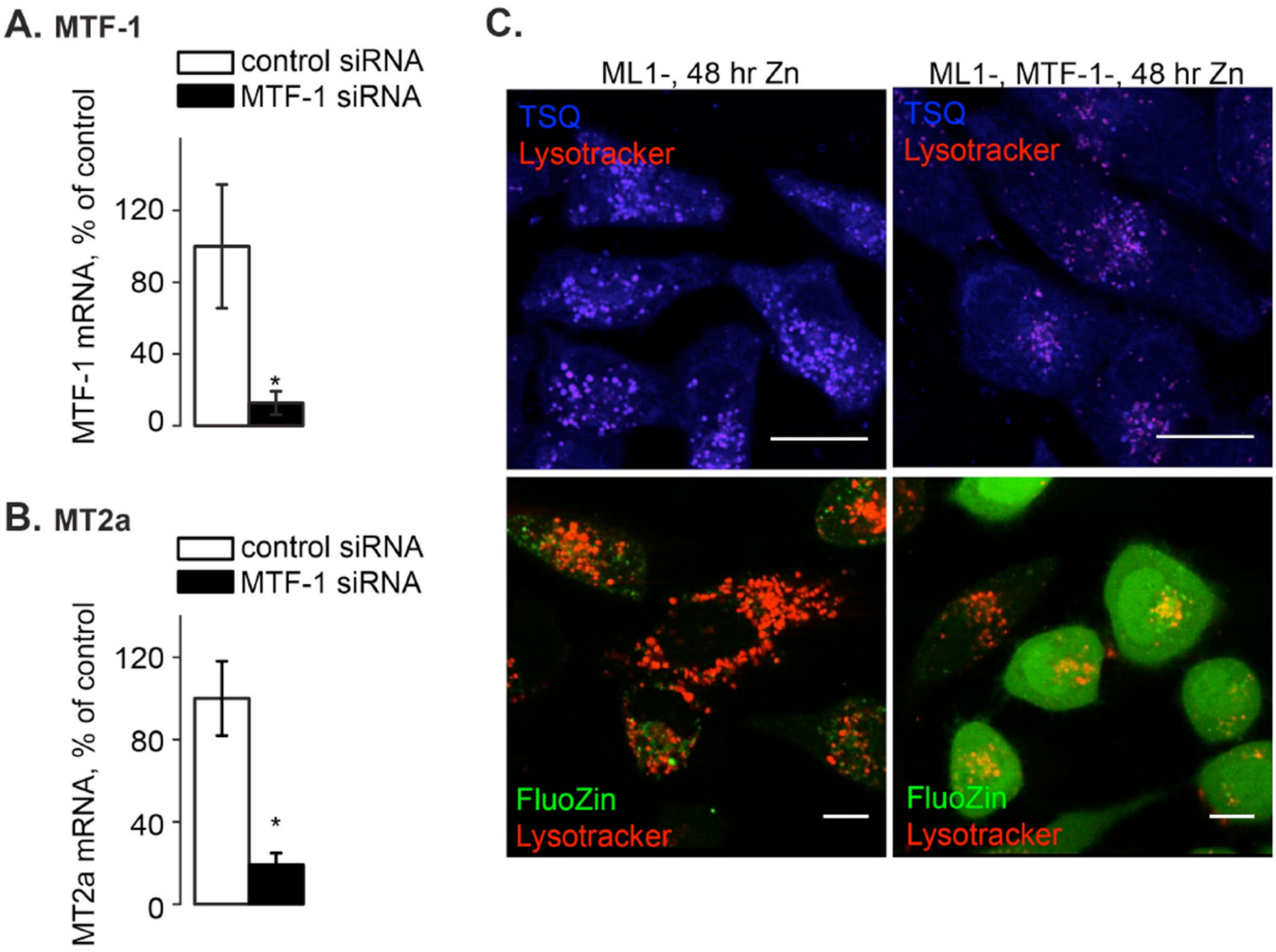

Fig 3. MTF-1 is involved in the enlargement of Lysotracker positive compartments in Zn-treated TRPML1 deficient cells

A. qPCR results demonstrating MTF-1 KD by siRNA. **B.** qPCR results showing MT2a suppression in cells treated with MTF-1 siRNA. Data points in Panels A and B represent 3 individual experiments, each performed in triplicate. * represents $p<0.05$. **C.** Confocal images of cells costained with Lysotracker and TSQ (top panels) or FluoZin-3 showing that Lysotracker positive compartments are no longer enlarged in Zn-treated cells when MTF-1 is suppressed alongside TRPML1. Represents 3 experiments. Scale bars are 20 μm.

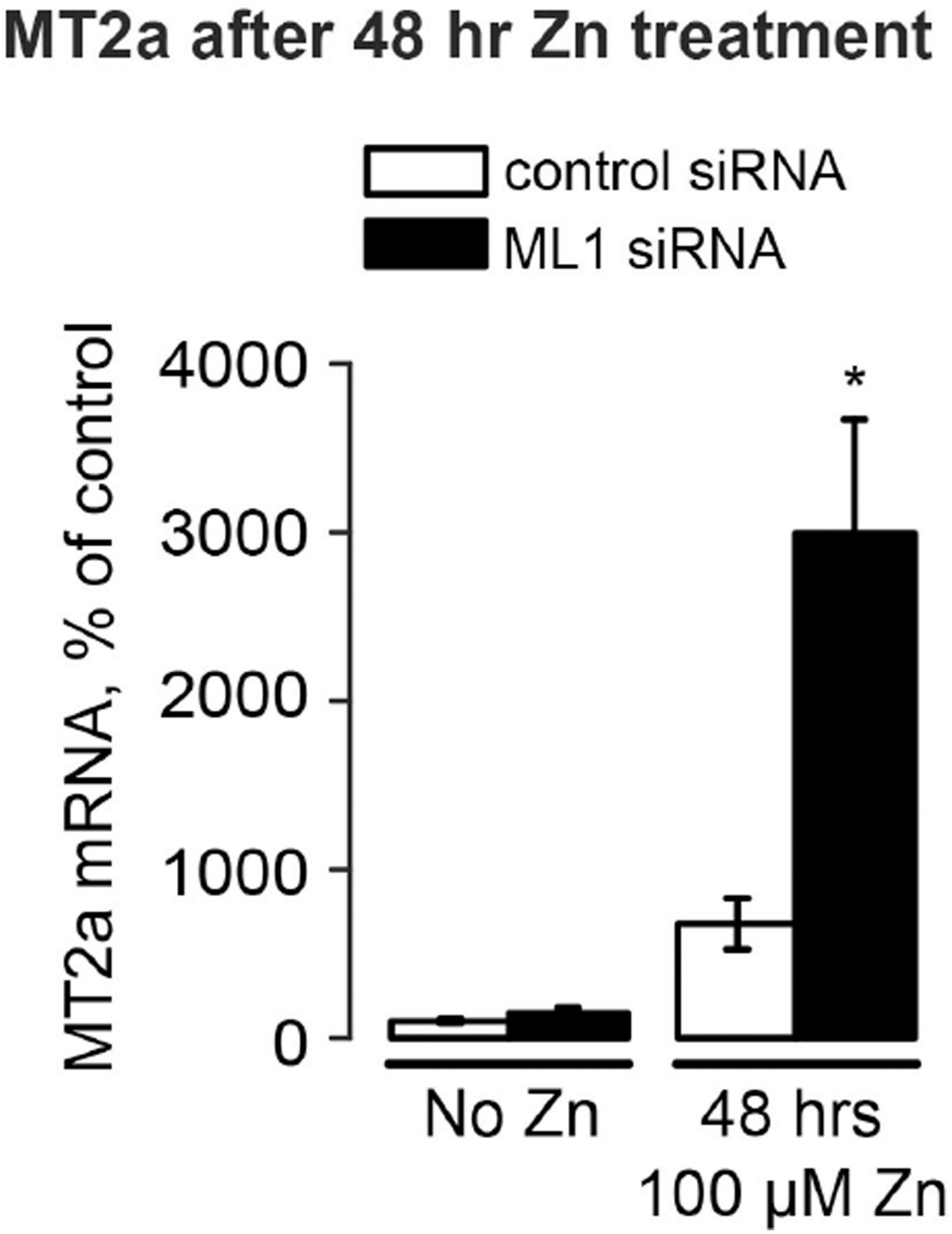

Fig 4. MT2a response to Zn changes is amplified in TRPML1-deficient cells

qPCR results showing augmented MT2a mRNA response to 48-hr long treatment with Zn in TRPML1 KD HeLa cells, compared to HeLa cells transfected with scrambled siRNA. Represents 3 individual experiments, each performed in triplicate. * represents $p<0.05$.

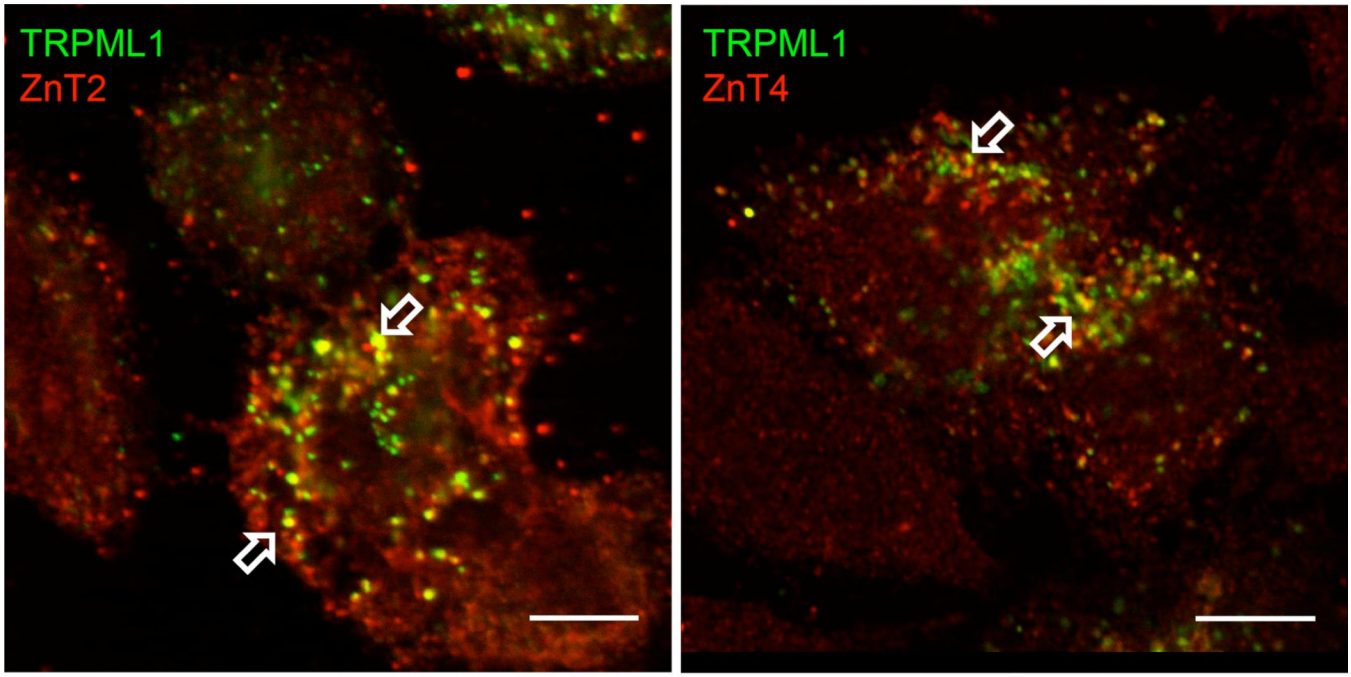

Fig 5. ZnT2 and ZnT4 colocalize with TRPML1

Confocal images of HeLa cells transfected with HA-tagged ZnT2 or ZnT4 constructs (in red) in parallel with a GFP-tagged TRPML1 construct (in green). Panel represents 3 separate experiments, 3 images per experiment, per condition. Merged images showing colocalization (yellow) of the HA- and GFP-tagged constructs. Cells were transfected for 24 hrs and not treated with Zn.

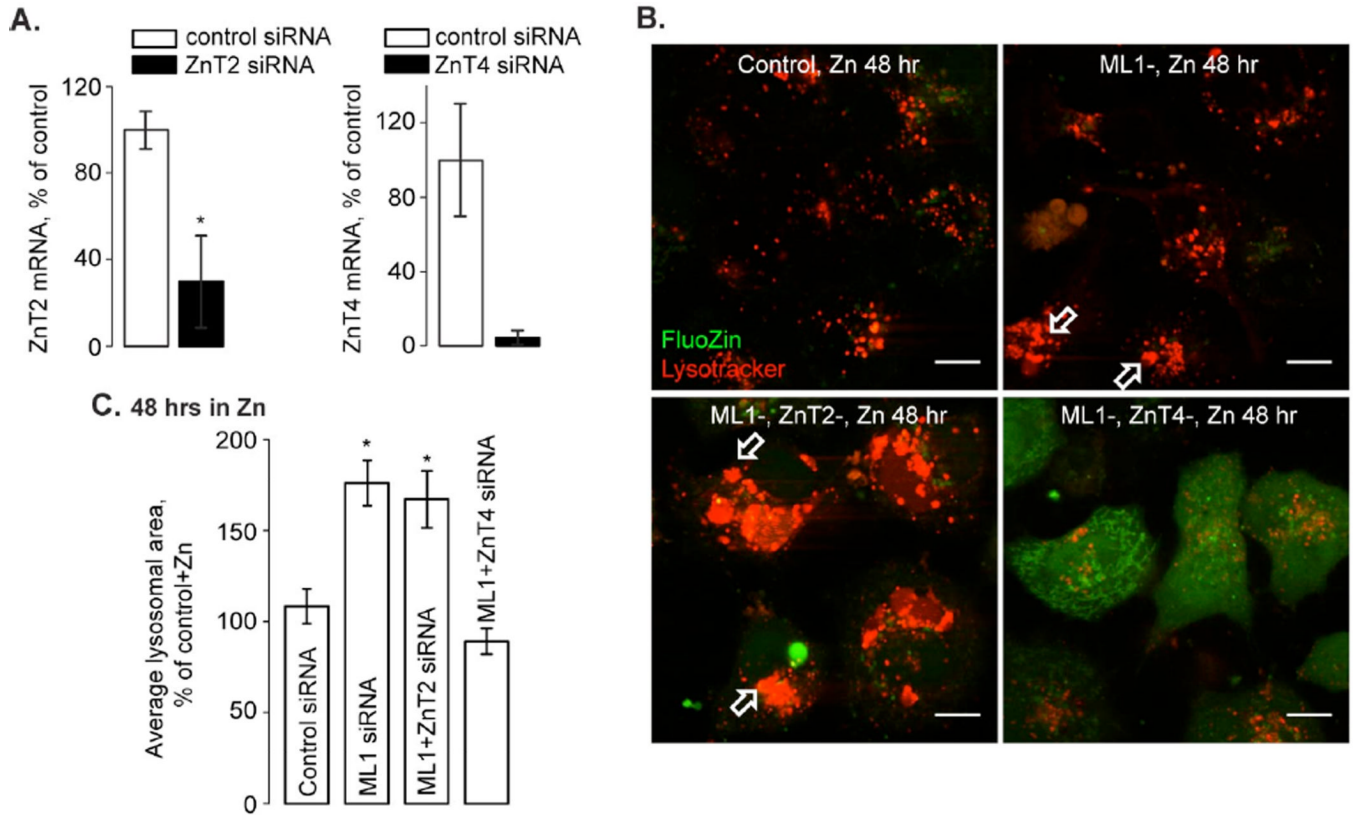

Fig 6. ZnT4 knockdown rescues enlarged lysosomes under TRPML1 suppression

A. qPCR results showing ZnT2 and ZnT4 KD by siRNA. Data points represent 3 individual experiments, each performed in triplicate. * represents $p<0.05$. **B.** Confocal images of HeLa cells treated with 100 μM Zn for 48 hrs, and stained with Lysotracker (red) and FluoZin-3,AM (green). Cells were transfected with different siRNA constructs and treated with Zn 24 hrs later. Merged confocal images show localization of Zn and lysosomes in cells transfected with control siRNA, TRPML1 siRNA (ML1−), TRPML1 and ZnT2 siRNA, or TRPML1 and ZnT4 siRNA. Represents 3 individual experiments, 3 images per experiment, per condition. Note enlarged lysosomes present in Zn treated TRPML1-deficient cells with and without ZnT2. Note the marked reduced size of lysosomes in ZnT4 KD cells, even under TRPML1 suppression. Scale bars are 20 μm.

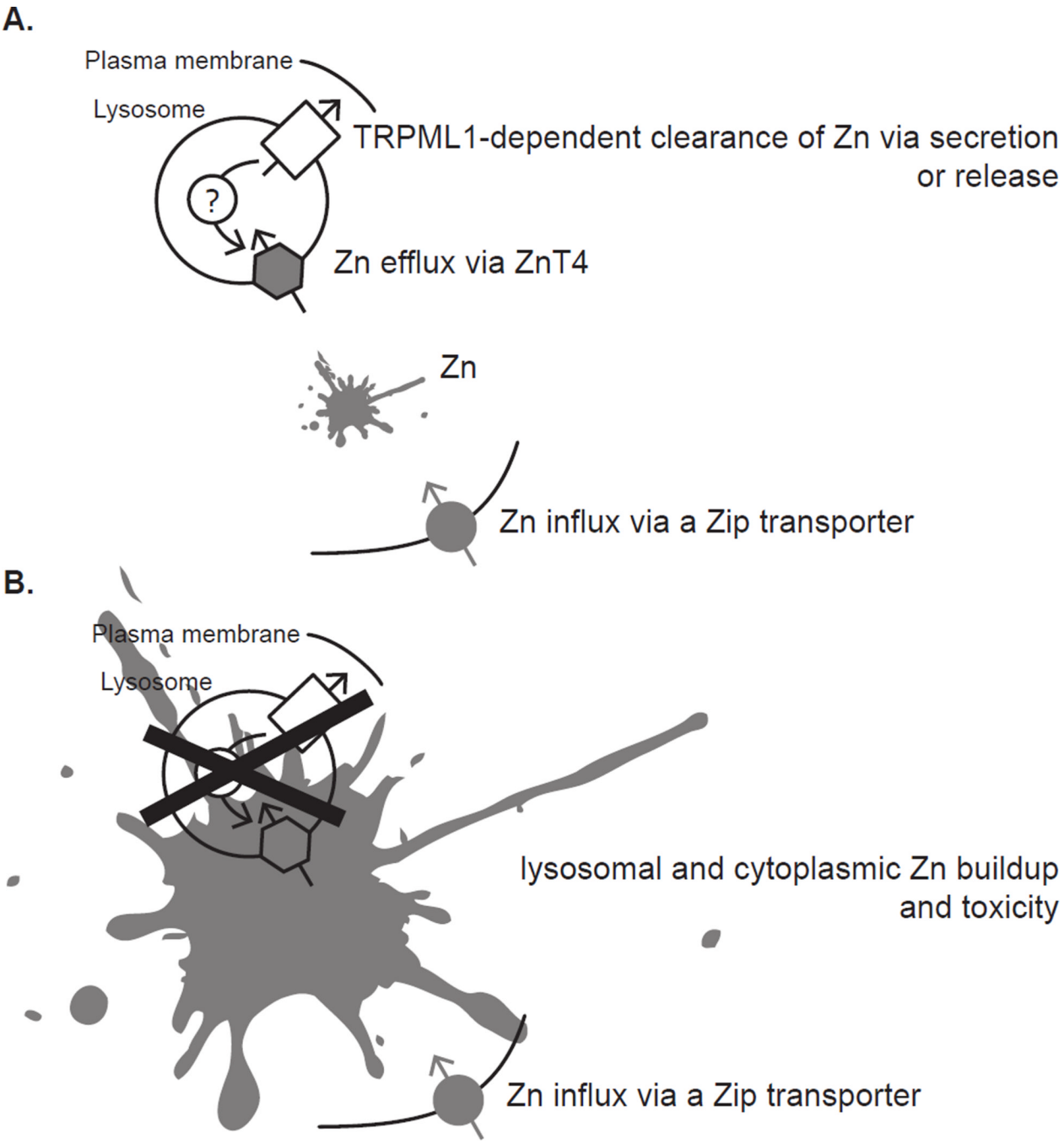

Fig 7. A model of "Zn sink"

A. Under the normal conditions, Zn entering the cytoplasm is sequestered into lysosomes by ZnT4 (and perhaps ZnT2 in some tissues). Lysosomal Zn is released or secreted via a TRPML1-dependent process. TRPML1 may also regulate ZnT activity (denoted by "?"). **B.** The loss of "Zn sink" function leads to lysosomal and cytoplasmic Zn buildup.

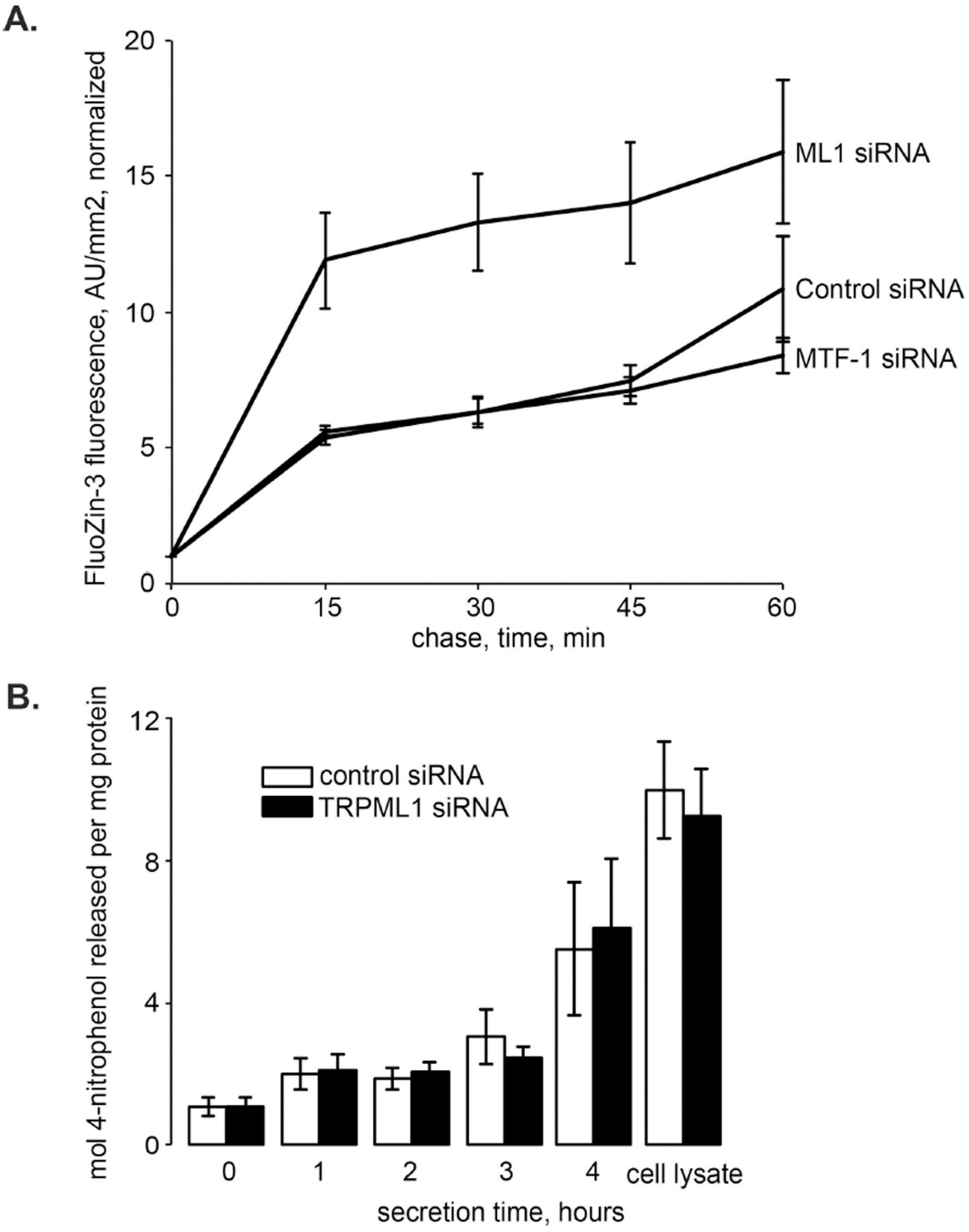

Fig 8. Zn clearance and secretion in TRPML1-KD cells

A. Zn secretion in control and TRPML1-KD cells. Cells were loaded in 100 μM Zn for 3 hours, washed and Zn secretion was analyzed as described in the text. FluoZin-3 fluorescence was expressed in arbitrary units per illumination/light collection area and normalized to the values in control cells exposed to Zn, which were taken as 1. Data represent 3 individual experiments, performed in duplicate. **B.** β-hexosaminidase secretion.