



<b>Title</b>	<b>NAADP/TPC2/Ca(2+) Signaling Inhibits Autophagy</b>
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# NAADP/TPC2/Ca<sup>2+</sup> Signaling Inhibits Autophagy

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Nicotinic adenine acid dinucleotide phosphate (NAADP) is one of the most potent endogenous Ca<sup>2+</sup> mobilizing messengers. NAADP mobilizes Ca<sup>2+</sup> from an acidic lysosome-related store, which can be subsequently amplified into global Ca<sup>2+</sup> waves by calcium-induced calcium release (CICR) from ER/SR via Ins(1,4,5)P<sub>3</sub> receptors or ryanodine receptors. A body of evidence indicates that 2 pore channel 2 (TPC2), a new member of the superfamily of voltage-gated ion channels containing 12 putative transmembrane segments, is the long sought after NAADP receptor. Activation of NAADP/TPC2/Ca<sup>2+</sup> signaling inhibits the fusion between autophagosome and lysosome by alkalinizing the lysosomal pH, thereby arresting autophagic flux. In addition, TPC2 is downregulated during neural differentiation of mouse embryonic stem (ES) cells, and TPC2 down-regulation actually facilitates the neural lineage entry of ES cells. Here we propose the mechanism underlying how NAADP-induced Ca<sup>2+</sup> release increases lysosomal pH and discuss the role of TPC2 in neural differentiation of mouse ES cells.

Intracellular Ca<sup>2+</sup> mobilization plays an important role in a wide variety of cellular processes, and multiple second messengers are responsible for mediating intracellular Ca<sup>2+</sup> changes.<sup>1,2</sup> Nicotinic adenine acid dinucleotide phosphate (NAADP) is one of the most potent endogenous Ca<sup>2+</sup> mobilizing messengers. NAADP mobilizes Ca<sup>2+</sup> from acidic lysosome-related stores, which can be subsequently amplified by calcium-induced calcium release (CICR) from the endoplasmic reticulum (ER). It has been shown that many extracellular stimuli can induce NAADP production leading to Ca<sup>2+</sup> mobilization, which establishes NAADP as a second messenger.<sup>3</sup> Recently, 2 pore channels (TPCs) have been identified as a novel family of NAADP-gated calcium release channels in endolysosomes. The TPC2 forms NAADP receptors that release Ca<sup>2+</sup> from lysosomes, which can subsequently trigger global Ca<sup>2+</sup> signals via the ER.<sup>4-8</sup> Yet several recent papers suggest that NAADP binds to an accessory protein to activate TPC2.<sup>9,10</sup> The Ca<sup>2+</sup>-signaling pathway mediated by NAADP is ubiquitous and the functions it regulates are equally diverse, including fertilization,<sup>11,12</sup> receptor activation in lymphocytes,<sup>13</sup> insulin secretion in pancreatic islets,<sup>14</sup> hormonal signaling in pancreatic acinar cells,<sup>15</sup> platelet activation,<sup>16</sup> cardiac muscle contraction,<sup>17</sup> blood pressure control,<sup>18</sup> neurotransmitter release,<sup>19</sup> neurite outgrowth,<sup>20</sup> and neuronal differentiation.<sup>21</sup> Therefore, decoding the molecular mechanisms involved in this novel signaling pathway is important not only for scientific reasons but also has clinical relevance.

Autophagy, an evolutionarily conserved lysosomal degradation pathway, has been implicated in a wide variety of cellular processes, yet the underlying mechanisms remain poorly understood. Recent vigorous research efforts have led to the identification of the core molecular machinery for autophagy, powered by the discovery of 35 ATG genes via yeast genetics. However, even after extensive

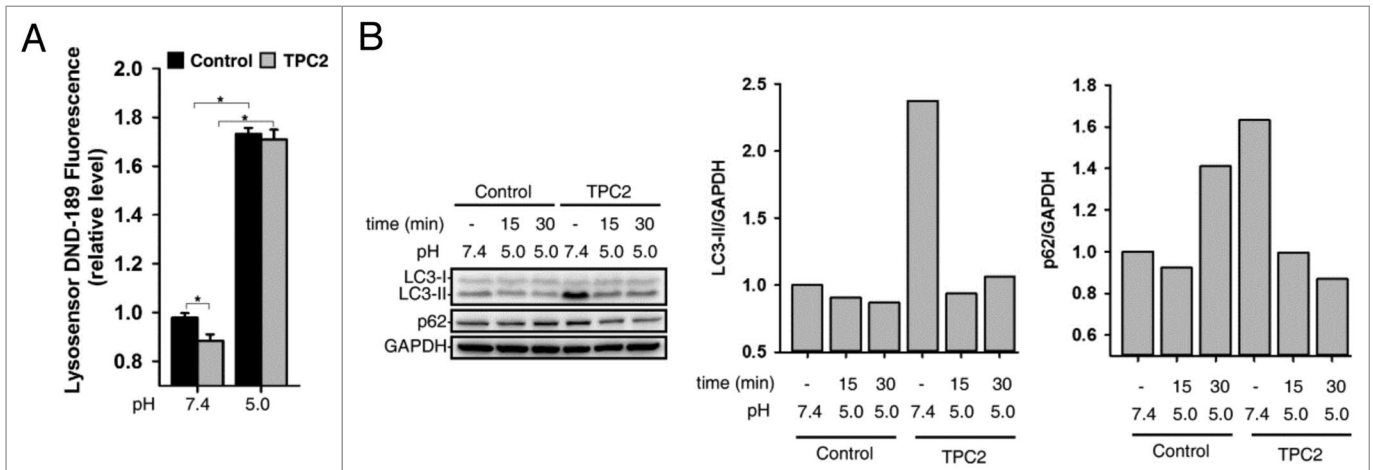
research, the regulation and mechanisms of autophagy induction, autophagosome formation and maturation, and autophagosomal-lysosomal fusion remain elusive in mammalian cells.<sup>22-27</sup> Since the completion of autophagy depends on lysosomal activity, any defect in autophagosomal-lysosomal fusion can lead to the accumulation of autophagosomes, ultimately damaging cells or resulting in cell death. Despite that autophagosomal-lysosomal fusion is poorly understood, many popular autophagy modulators, e.g., bafilomycin and hydroxychloroquine, actually inhibit this process by targeting lysosomal activity. Hydroxychloroquine has even been applied in several human anticancer clinical trials. Yet most of these inhibitors either lack specificity or potency.<sup>28,29</sup> Thus, in order to identify potent and specific modulators of autophagy for future human disease therapy, it is essential to fully understand the molecular mechanisms underlying this process.

Intracellular Ca<sup>2+</sup> has already been established as one of the regulators of autophagy induction, either positively or negatively depending upon the context of time, space, Ca<sup>2+</sup> source, and cell state.<sup>30-32</sup> Yet, the effects of Ca<sup>2+</sup> on autophagosomal-lysosomal fusion have not been determined. Thus, we examined the role of the NAADP/TPC2/ Ca<sup>2+</sup> signaling in this process in mammalian cells. We found that overexpression of a wildtype, not an inactive mutant, TPC2 in HeLa cells that lack detectable level of endogenous TPC2 protein inhibited autophagosomal-lysosomal fusion. Treatment of TPC2 overexpressing cells with a cell permeant-NAADP agonist, NAADP-AM, further inhibited fusion, whereas Ned-19, a NAADP antagonist, promoted fusion. Likewise, TPC2 knockdown in mouse embryonic stem (ES) cells promoted autophagosomal-lysosomal fusion during early neural differentiation. ATG5 knockdown abolished TPC2-induced accumulation of autophagosomes, but inhibiting mTOR activity had no effect

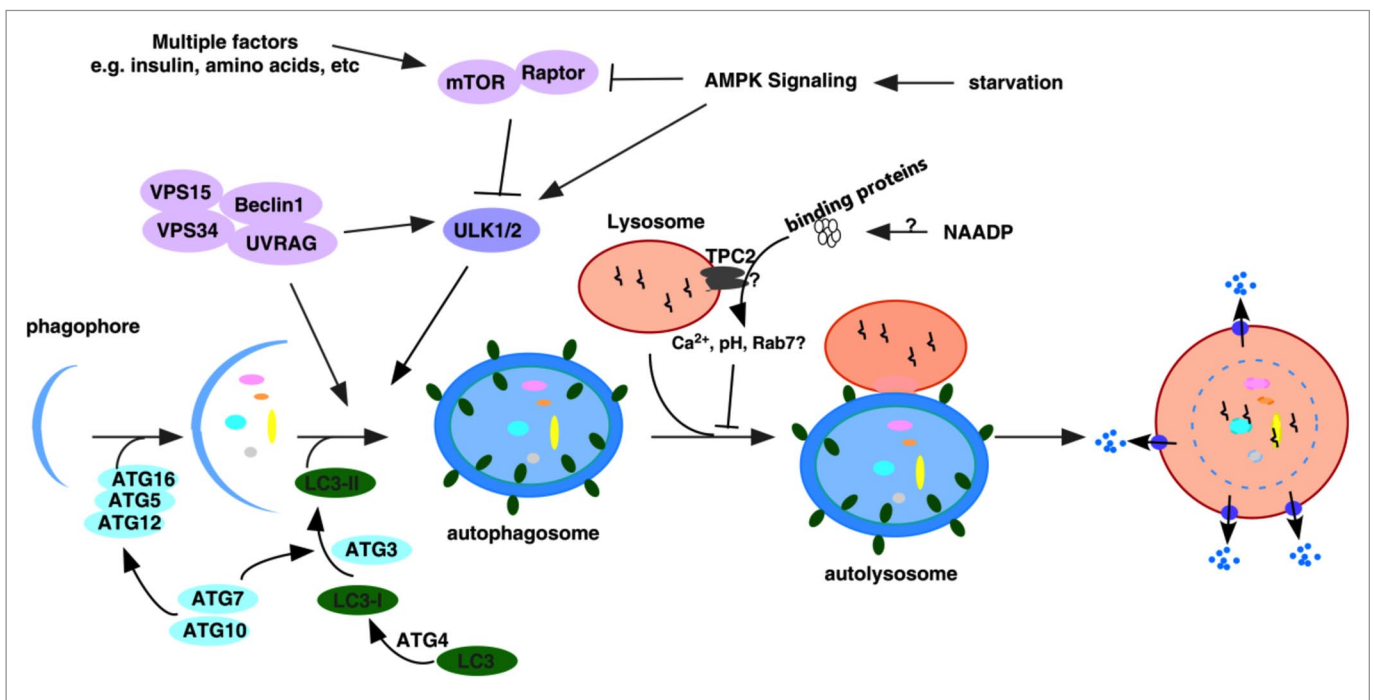
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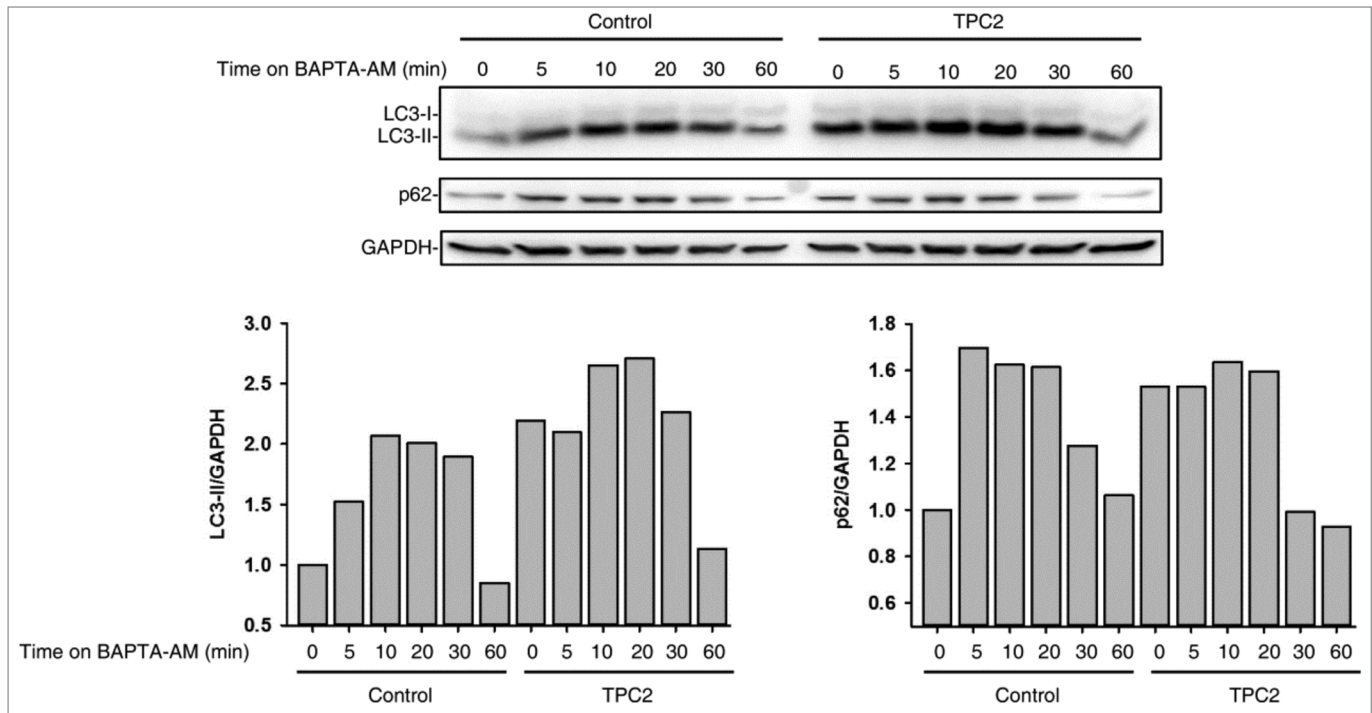
**Figure 1.** TPC2 signaling inhibited autophagy by increasing lysosomal pH in HeLa cells. **(A)** TPC2 overexpression induced an increase of lysosomal pH in HeLa cells as determined by microplate reader measurement of Lysosensor DND-189 stained cells. Extracellular acidification decreased lysosomal pH in both control and TPC2 overexpressing cells. **(B)** Extracellular acidification reversed the accumulation of LC3-II and p62 in TPC2 overexpressing HeLa cells. The densitometric analyses of LC3-II and p62 represent data from 2 independent experiments.



**Figure 2.** Schematic of the NAADP/TPC2/ $\text{Ca}^{2+}$  pathway-mediated inhibition of autophagosomal-lysosomal fusion in mammalian cells. NAADP, likely via accessory protein(s), activates TPC2 to trigger  $\text{Ca}^{2+}$  release from lysosomes. This is accompanied with lysosomal pH increase, which subsequently prevents the recruitment of Rab-7 to autophagosomes, thereby inhibiting the fusion between autophagosome and lysosome.

on it. Instead, overexpression of TPC2 alkalinized lysosomal pH, and lysosomal re-acidification abolished TPC2-induced autophagosome accumulation (Fig. 1). Interestingly, TPC2 overexpression had no effect on general endosomal-lysosomal degradation but prevented the recruitment of Rab-7 to autophagosomes. Taken together, our data demonstrate that TPC2/NAADP/ $\text{Ca}^{2+}$  signaling alkalinizes lysosomal pH to suppress the later stage of autophagy progression (Fig. 2).<sup>7,8</sup>

Upon withdrawal of self-renewal stimuli, mouse ES cells spontaneously enter neural lineages in monolayer adherent monoculture.<sup>33</sup> Interestingly, the expression of TPC2 was significantly decreased in ES cells during their initial entry into neural progenitors, which was accompanied by the gradual induction of autophagy. TPC2 knockdown accelerated mouse ES cells entry into early neural lineages, whereas TPC2 overexpression in ES cells markedly inhibited it. We speculate that TPC2 downregulation during



**Figure 3.** The effects of BAPTA-AM on the accumulation of LC3-II and p62 in control and TPC2 overexpressing HeLa cells. Cell lysates were harvested at indicated time points after BAPTA-AM (20  $\mu$ M) treatment in both control and TPC2 overexpressing HeLa cells, and analyzed for expression of LC3, p62, and GAPDH by western blot analyses. The densitometric analyses of LC3-II and p62 represent data from 2 independent experiments.

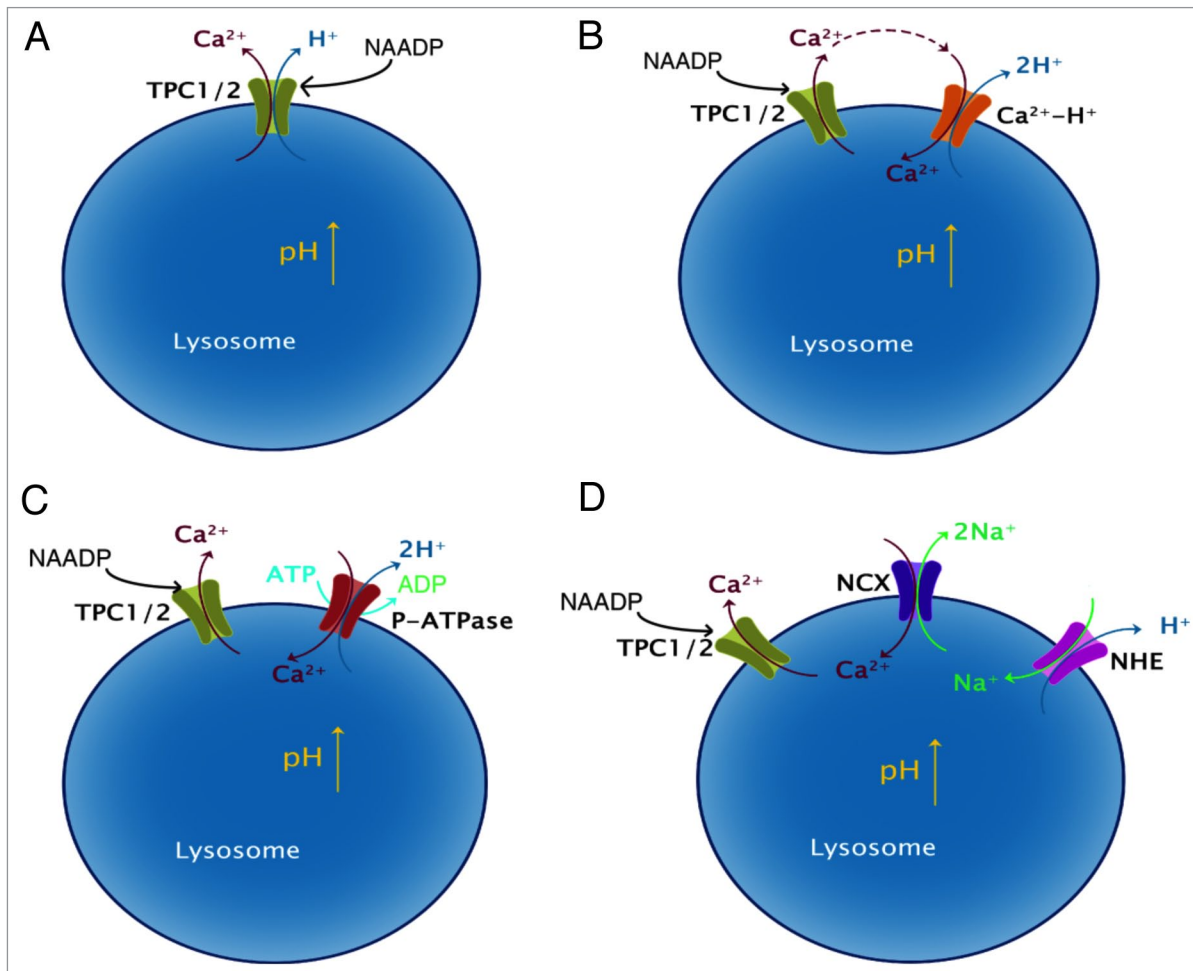
early neural differentiation of ES cells facilitates the fusion between autophagosome and lysosome, thereby enabling faster energy recycling to be utilized for target differentiation. On the other hand, TPC2 overexpression blocks the fusion to prevent energy recycling and inhibits neural differentiation. Taken together, our results established a physiological function of TPC2-mediated autophagy inhibition during early neural differentiation of ES cells.<sup>8</sup>

It has previously been reported that activation of NAADP/TPC2 signaling increased LC3-II levels,<sup>34,35</sup> and downregulation of TPC2 by presenilin decreased it.<sup>36</sup> Although these reports concluded that the increased LC3-II results from induction of autophagy by NAADP/TPC2 signaling, we clearly demonstrated that the increased LC3-II is actually due to the inhibition of autophagosomal-lysosomal fusion, not autophagy induction, by NAADP/TPC2 signaling (Fig. 2).<sup>7</sup> Because of its diversified physiological roles, the efforts to generate NAADP analogs chemically or from the corresponding NADP analogs using ADP-ribosyl cyclase are already underway. Since NAADP antagonists accelerate autophagosomal-lysosomal fusion whereas NAADP agonists inhibit it, the development of potent and cell permeable NAADP agonists or antagonists in the near future should provide a novel approach to specifically manipulate autophagy.

Ca<sup>2+</sup> has long been established as an essential ion for membrane fusion,<sup>37,38</sup> and a local Ca<sup>2+</sup> increase via extracellular Ca<sup>2+</sup> influx is essential for exocytosis of synaptic vesicles, in which synaptotagmin acts as Ca<sup>2+</sup> sensor.<sup>39</sup> Thus we originally expected that NAADP/TPC2-mediate Ca<sup>2+</sup> release should facilitate autophagosomal-lysosomal fusion. However, whether local Ca<sup>2+</sup> release from internal Ca<sup>2+</sup> stores is required for intracellular membrane fusion events has

actually not been determined. NAADP can induce Ca<sup>2+</sup> release from lysosomes, and we found that NAADP treatment actually inhibited autophagosomal-lysosomal fusion. These data argue that local Ca<sup>2+</sup> release from lysosomes does not facilitate autophagosomal-lysosomal fusion. On the other hand, BAPTA-AM, a Ca<sup>2+</sup> chelator, quickly blocked autophagosomal-lysosomal fusion, indicating that Ca<sup>2+</sup> itself is required for this process (Fig. 3). Therefore, these data suggest that the basal cytosolic Ca<sup>2+</sup> level, not a local Ca<sup>2+</sup> increase, is permissive for autophagosomal-lysosomal fusion, which is supported by the fact that membrane fusion can be reconstituted in an in vitro system that only contains the basal Ca<sup>2+</sup> concentration.<sup>40,41</sup> Notably, the accumulation of both LC3-II and p62 induced by BAPTA-AM was transient (Fig. 3), suggesting that Ca<sup>2+</sup> differentially regulates autophagy at different levels. Along this line, numerous studies indeed have already documented that intracellular Ca<sup>2+</sup> can differentially modulate autophagy within the context of time, space, Ca<sup>2+</sup> source, and cell status.<sup>31,42</sup>

Importantly, our findings added TPC2 to a handful of transporters, including V-ATPase and chloride channels, which can modulate lysosomal pH. V-ATPase, a multi-subunit protein complex, pumps protons into the lysosomal lumen against an electrochemical gradient at the expense of ATP hydrolysis to generate the acidic milieu in lysosomes.<sup>43</sup> The positive lysosomal membrane potential, created by the influx of protons, reciprocally, could prevent the V-ATPase from continuing to pump protons. Obviously, efflux of cations, or influx of anions, or both, is needed to maintain the balance of acidic pH and membrane potential inside lysosomes.<sup>44</sup> Along this line, Cl<sup>-</sup> influx has already been proposed to dissipate the restrictive electrical gradient for maintaining



**Figure 4.** Models of NAADP/TPC2/Ca<sup>2+</sup> signaling induced lysosomal alkalization in mammalian cells. (A) NAADP induces the release of Ca<sup>2+</sup> and H<sup>+</sup> from lysosomes via TPCs or an unknown channel. (B), (C), and (D) After Ca<sup>2+</sup> release from lysosomes via TPC2, Ca<sup>2+</sup> refilling via a putative Ca<sup>2+</sup>/H<sup>+</sup> counter-exchanger (B), or a P-type Ca<sup>2+</sup> pump (C), or the sequential coupling of several cation counter-exchangers, e.g., Ca<sup>2+</sup>/Na<sup>+</sup> and Na<sup>+</sup>/H<sup>+</sup> (D), could also lead to lysosomal alkalization.

acidic lysosomal pH.<sup>45,46</sup> Yet, whether Cl<sup>-</sup> is the main counter ion remains to be determined, let alone the identity of Cl<sup>-</sup> transporters in lysosomes.<sup>43</sup> Ca<sup>2+</sup>, whose concentration in lysosomes is high,<sup>47,48</sup> is another potential counter ion, yet the channels for Ca<sup>2+</sup> release or refilling in lysosomes also remain elusive. Interestingly, NAADP induced Ca<sup>2+</sup> release via TPC2 from lysosomes is accompanied by an increased lysosomal pH, indicating proton efflux with Ca<sup>2+</sup> release. These data suggested that Ca<sup>2+</sup> is not the counter ion for maintaining acidic pH in lysosomes.

How NAADP/TPC2-induced Ca<sup>2+</sup> release alkalizes lysosomal pH remains mysterious. One explanation is that Ca<sup>2+</sup> and protons are simultaneously released from lysosomes via TPC2, or TPC1, or other unidentified channels upon NAADP treatment (Fig. 4A). Yet, whether TPCs are proton permeable and the identity of other proton channels remain to be determined. On the other hand, lysosomal alkalization might be the result of proton efflux accompanied by Ca<sup>2+</sup> refilling after its release from lysosomes. A putative vacuolar Ca<sup>2+</sup>/H<sup>+</sup> counter-exchanger,<sup>49</sup> like the one in yeast or plant, can be activated to refill the lysosomal Ca<sup>2+</sup> pools at the expense of proton efflux upon Ca<sup>2+</sup> release from lysosomes via

TPC2 (Fig. 4B). Alternatively, an unidentified lysosomal P-type Ca<sup>2+</sup> ATPase,<sup>48</sup> similar to SERCA in ER, can also refill Ca<sup>2+</sup> in exchange of luminal protons at the expense of ATP hydrolysis (Fig. 4C). Another possible way for Ca<sup>2+</sup> to enter lysosomes is the coupling of several lysosomal proton-cation counter-transporters, such as the sequential action of Ca<sup>2+</sup>/Na<sup>+</sup> exchangers and Na<sup>+</sup>/H<sup>+</sup> exchangers (Fig. 4D). Unfortunately, except for the V-ATPase, ion channels or transporters responsible for the creation of the unique ionic environment inside lysosomes have not been identified.<sup>49</sup> We anticipate that an ongoing lysosomal proteomics study in the lab will help identify the respective transporters.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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