Role of Two-Pore Channels in Embryonic Development and Cellular Differentiation

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Since the identification of nicotinic acid adenine dinucleotide phosphate (NAADP) and its putative target, the two-pore channel (TPC), the NAADP/TPC/ Ca^{2+} signaling pathway has been reported to play a role in a diverse range of functions in a variety of different cell types. TPCs have also been associated with a number of diseases, which arise when their activity is perturbed. In addition, TPCs have been shown to play key roles in various embryological processes and during the differentiation of a variety of cell types. Here, we review the role of NAADP/TPC/ Ca^{2+} signaling during early embryonic development and cellular differentiation. We pay particular attention to the role of TPC2 in the development and maturation of early neuromuscular activity in zebrafish, and during the differentiation of isolated osteoclasts, endothelial cells, and keratinocytes. Our aim is to emphasize the conserved features of TPC-mediated Ca^{2+} signaling in a number of selected examples.

It was the mid-1990s when nicotinic acid adenine dinucleotide phosphate (NAADP) was first demonstrated to activate Ca^{2+} stores that were distinct from those sensitive to inositol 1,4,5-trisphosphate (IP_3) or cyclic adenosine diphosphoribose ([cADPR]; Lee and Aarhus 1995). NAADP was reported to be highly potent, working at concentrations as low as ~10–20 nM to stimulate the release of Ca^{2+} from microsomes prepared from the eggs of the sea urchin, *Strongylocentrotus purpuratus*. This was in comparison to concentrations of ~75 nM cADPR and ~0.4 μM IP_3 required to elicit a similar Ca^{2+} response in the same cell preparation (Lee and Aarhus 1995). Furthermore, NAADP was also shown to induce rapid changes in intracellular Ca^{2+} in the intact eggs of another sea

urchin species, *Lytechinus pictus* (Lee and Aarhus 1995; Aarhus et al. 1996). Following these early reports using sea urchins, NAADP was shown to mobilize Ca^{2+} in the oocytes of a number of other marine eggs, including those of the starfish, *Asterina pectinifera*, and the ascidian *Phallusia mammillata* (Albrieux et al. 1998; Santella et al. 2000; Lim et al. 2001; Moccia et al. 2006). In addition, NAADP was demonstrated to mobilize Ca^{2+} in a number of mammalian cell types, such as in mouse pancreatic acinar cells, rat astrocytes, human Jurkat T-lymphocytes, and guinea pig atrial myocytes (Cancella et al. 1999; Berg et al. 2000; Singaravelu and Deitmer 2006; Collins et al. 2011). Although the list of species and cell types reported to mobilize Ca^{2+} in response to NAADP is not exhaustive,

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the diversity of the selection that do (i.e., from evolutionary ancient echinoderms [Zamora and Rahman 2014] to the relatively more modern mammals [Lee and Beck 2015]), indicate the possible ubiquitous nature of this intracellular messenger. With the identification of more cell types that were responsive to NAADP, the race was on to identify the intracellular stores involved in generating the Ca^{2+} signals as well as the molecular identity of the NAADP receptor. With regard to the former, lysosome-related acidic organelles were identified as being the source of the Ca^{2+} that was mobilized by NAADP (Churchill et al. 2002; Kinnear et al. 2004). In addition, in reports where Ca^{2+} imaging data were presented, NAADP was described as stimulating a “local elevation of Ca^{2+} ” or a “spatially restricted Ca^{2+} wave” in the eggs and cell types under investigation (Churchill et al. 2002; Kinnear et al. 2004; Calcraft et al. 2009). Furthermore, it was suggested that such localized Ca^{2+} signals from the lysosomes might stimulate or “trigger” longer-range Ca^{2+} signaling by Ca^{2+} -induced Ca^{2+} release (CICR) via ryanodine receptors (RyRs) or IP_3 receptors (IP_3 Rs) located in the membrane of the endo-/sarcoplasmic reticulum ([ER/SR]; Cancela et al. 1999; Kinnear et al. 2004). In addition, although there remains some controversy with regard to NAADP metabolism, target receptors/ion channels, and organelles involved (Guse and Diercks 2018), there is evidence to suggest that in certain cell types, NAADP stimulates the release of Ca^{2+} from the acidic organelles via two-pore channels (TPCs) (Calcraft et al. 2009; Galione et al. 2009; Brailoiu et al. 2010; Zhu et al. 2010; Ruas et al. 2015).

In animals, three members of this cation-selective ion channel family have been identified, namely, TPC1, TPC2, and TPC3. TPC1 and TPC3 are localized on endosomes, whereas TPC2 is localized on lysosomes (Calcraft et al. 2009). There is some debate with regard to the identity of the endogenous agonists of these channels, and whether they conduct Ca^{2+} or Na^+ or indeed both (Guse 2009, 2012; Wang et al. 2012; Cang et al. 2013). TPC1 is generally understood to be a voltage-regulated channel; however, there are diverging views about whether it is an NAADP-activated Ca^{2+} channel (Ry-

balchenko et al. 2012; Ogunbayo et al. 2015; Patel et al. 2017), or a phosphatidylinositol-3-5-bisphosphate ($\text{PI}(3,5)\text{P}_2$)-activated Na^+ channel (She et al. 2018). TPC2 is voltage insensitive and is reported to either conduct Ca^{2+} in response to NAADP, $\text{PI}(3,5)\text{P}_2$, Mg^{2+} and protein kinases such as P38 and JNK (Zhang et al. 2013; Jha et al. 2014; Capel et al. 2015) or, like TPC1, conduct Na^+ in response to $\text{PI}(3,5)\text{P}_2$. Similarly, TPC3 has been identified as a voltage-activated Na^+ channel (Cang et al. 2014; Kintzer and Stroud 2018), but it might also conduct Ca^{2+} in response to NAADP (Ogunbayo et al. 2015).

The genes of all three TPCs (*tpcns*) are reported to be expressed in most vertebrates although *tpcn3* is not expressed in primates (specifically humans and chimps) or rodents (Calcraft et al. 2009). In addition, *tpcns* are found to a greater or lesser degree in many invertebrates, including echinoderms, ascidians, and some insects (Calcraft et al. 2009). Because of the diversity of species expressing *tpcns*, it has been suggested that they might be part of an ancient gene family, and thus play a crucial role in regulating a diverse variety of cell functions (Calcraft et al. 2009). Indeed, it has been shown that TPCs play a role in modulating the excitability and stimulus-secretion coupling in pancreatic β cells (Arredouani et al. 2015); they are important for normal platelet function (Ambrosio et al. 2015), they regulate cell pigmentation (Ambrosio et al. 2016), are involved in β -adrenoceptor signaling in the heart (Capel et al. 2015), and play a role in the contraction of smooth muscle (Tugba Durlu-Kandilci et al. 2010).

Perhaps not surprisingly, evidence is accumulating to suggest that TPCs also play a role during embryonic development and cellular differentiation. We are especially interested in the role of TPC2-mediated Ca^{2+} signaling in the development and innervation of the skeletal muscle in zebrafish embryos (Kelu et al. 2015, 2017, 2018). This is because we had previously demonstrated that a series of distinct Ca^{2+} signals are generated in the developing slow skeletal muscle cells of intact zebrafish embryos, and that both IP_3 Rs and RyRs play a role in generating these signals (Cheung et al. 2011). In the same year, it

was reported that TPC2 is expressed in skeletal muscle tissue in mouse embryos and neonates, and that the differentiation of C2C12 myoblasts is stimulated by NAADP-induced Ca^{2+} release, and inhibited by the down-regulation of TPC2 (and TPC1; Aley et al. 2010a). In addition, it had previously been reported that NAADP-regulated localized Ca^{2+} signals from lysosomes initiates the global release of Ca^{2+} from RyRs in the SR, which stimulated the contraction of myocytes (Kinnear et al. 2004). These reports together with our own observations suggested that it would be important to investigate whether TPCs, and especially TPC2, might play some role in generating the Ca^{2+} signals we observed during the differentiation of slow skeletal muscle cells in zebrafish embryos.

In addition to skeletal muscle development, NAADP/TPC/ Ca^{2+} signaling is also reported to play a role in other aspects of development, including the acrosome reaction (and hence activation) of mouse spermatozoa (Arndt et al. 2014) and fertilization in sea star oocytes (Ramos et al. 2014), as well as in the differentiation of various cell types, including osteoclasts, keratinocytes, and endothelial cells (Notomi et al. 2012; Favia et al. 2014; Park et al. 2015). Thus, here we review the current knowledge regarding NAADP/TPC/ Ca^{2+} signaling during embryogenesis and tissue differentiation, and in doing so we hope to highlight the conserved features as well as the differences observed during the development of these various embryos and cell types.

TPC-MEDIATED Ca^{2+} SIGNALING DURING EARLY DEVELOPMENTAL AND DIFFERENTIATION EVENTS

As is quite often the case, new and important discoveries are made while working on a relatively simple nonmammalian species, and these are subsequently found to play a crucial role in the development and function of higher organisms: such was the case with the search for, and identification of, TPCs. This followed the pioneering work by Lee and colleagues who showed that NAADP could mobilize Ca^{2+} from an independent intracellular store other than the ER (i.e.,

from microsomes prepared from extracts derived from the sea urchins *S. purpuratus* [Lee and Aarhus 1995] and *L. pictus* [Lee and Aarhus 1995; Aarhus et al. 1996]). Similar results were then reported from ascidian and starfish oocytes (Albrieux et al. 1998; Santella et al. 2000), from plants (Navazio et al. 2000), and from higher eukaryotic calls (Cancela et al. 1999; Gambara et al. 2008; Aley et al. 2010a,b; Esposito et al. 2011), suggesting a highly conserved feature of this messenger molecule (Guse and Lee 2008), and presumably its endogenous receptor(s).

NAADP has been demonstrated to have distinct functions in a number of early developmental events, including during the acrosome reaction of sea urchin and mammalian sperm (Vasudevan et al. 2010; Arndt et al. 2014) and during fertilization in ascidian and starfish oocytes (Albrieux et al. 1998; Lim et al. 2001; Moccia et al. 2004). Thus, it is perhaps not surprising that TPCs have also been reported to play key roles in a variety of early developmental processes across a large number of cell types from a diverse range of organisms (e.g., from protozoans [Suárez-Cortés et al. 2017] to humans [Hockey et al. 2015; Ogunbayo et al. 2018]). These include the Ca^{2+} mobilization events that occur at fertilization of oocytes of the sea star *Patiria miniata*. Normally on fertilization in these oocytes, there is a rapid elevation of Ca^{2+} around the cortex (outermost region), called a “cortical flash,” which lasts for just a few seconds. This is followed by a longer-duration propagating wave of Ca^{2+} that crosses the oocyte starting at the location where the sperm makes contact (Ramos et al. 2014). When the expression of TPC1, TPC2, and TPC3 were individually perturbed via the introduction of morpholino oligonucleotides (MOs), there was little effect on the cortical flash or the subsequent propagating wave of Ca^{2+} . However, when they were concomitantly perturbed, these normally well-defined Ca^{2+} signals were altered; in some oocytes, the cortical flash occurred after the Ca^{2+} wave, whereas in others, Ca^{2+} waves were initiated from two locations. It was suggested that in these oocytes there is a cooperative activity among the three TPC isoforms (Ramos et al. 2014).

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The pharmacological inhibition of TPCs with *trans*-ned-19 (Naylor et al. 2009) has been shown to prevent the migration of invasive cancer cells and reduce lung metastasis of mammary mouse cancer cells (Nguyen et al. 2017). Here, the disruption of TPC1 and TPC2 function was reported to inhibit trafficking of β 1 integrin, resulting in its accumulation in early endosomes. As a result, invasive cancer cells were no longer able to form leading edges that are required for migration. As TPCs are expressed in the embryos of a number of different species (Brailoiu et al. 2009; Zong et al. 2009; Ramos et al. 2014), and key early developmental events, especially those associated with gastrulation, rely on cell migration events (Keller 2005), it is possible that TPCs might regulate early embryological processes such as these as well.

TPCs have also been shown to play an essential role in the development and completion of the complex life cycle of the malaria-carrying protozoan, *Plasmodium falciparum* (Suárez-Cortés et al. 2017). Treatment with *trans*-ned-19 prevented the progression of the asexual life cycle of this parasite by inhibiting the normal spontaneous Ca^{2+} oscillations that are generated, as well as blocking the transition of the parasite from the early to the late trophozoite stage, and the ability of the late trophozoite to develop to the multinucleated schizont stage (Suárez-Cortés et al. 2017).

TPC2-MEDIATED Ca^{2+} SIGNALING DURING SKELETAL MUSCLE DEVELOPMENT

The essential role of Ca^{2+} during mature muscle contraction has long been recognized (Ringer 1882). More recently, evidence has accumulated to suggest that intracellular Ca^{2+} regulation also plays a critical role in muscle differentiation and development (see reviews by Webb and Miller 2011; Tu et al. 2016). The initial research focused on the study and characterization of the role of ER/SR-resident Ca^{2+} channels (i.e., RyRs and IP₃Rs) during myogenesis. For example, Ferrari et al. (1996) demonstrated that cultured *Xenopus* myocytes exhibit spontaneous RyR-mediated Ca^{2+} transients during early differen-

tiation, and when these signals are inhibited, then myofibrillogenesis is disrupted (Ferrari et al. 1998). In addition, Powell et al. (2003) reported the presence of IP₃R-mediated nuclear Ca^{2+} transients in rodent myotubes, and demonstrated that these Ca^{2+} signals are involved in the regulation of gene transcription in muscle cells. Using live zebrafish embryos, Brennan et al. (2005) and Cheung et al. (2011) visualized and characterized a distinct pattern of Ca^{2+} signaling in the developing myotome. They suggested that these Ca^{2+} signals are mediated by RyRs and acetylcholine (Brennan et al. 2005) and/or by RyRs and IP₃Rs (Cheung et al. 2011), and are crucial for myotomal patterning and myofibrillogenesis (Brennan et al. 2005; Cheung et al. 2011).

It was only relatively recently that lysosomal Ca^{2+} release was implicated in the regulation of myogenesis. The requirement of TPC2/ Ca^{2+} signaling during skeletal muscle differentiation was first demonstrated in vitro using the C2C12 mouse myoblast cell line and primary murine myoblasts (Aley et al. 2010a). It was shown that in undifferentiated C2C12 cells, Ca^{2+} was released upon stimulation by NAADP-AM (a cell-permeant form of NAADP, an agonist of TPCs) and ATP (an agonist of IP₃Rs), but not ryanodine (an agonist of RyRs when used at a low, stimulatory concentration; Aley et al. 2010a). This demonstrated the existence of the machinery required for NAADP signaling in the skeletal muscle precursor cells. It was also shown that the differentiation of C2C12 cells was promoted by incubation with NAADP-AM, as indicated by the increase in both *myogenin* (a myogenic regulatory factor) and *skNAC* (a skeletal and heart muscle-specific transcription factor) transcripts, as well as an increase in the number of nuclei present in cells expressing the myosin heavy chain (a late terminal myogenic differentiation marker; Aley et al. 2010a). In addition, treatment with bafilomycin A1 (an inhibitor of lysosomal H⁺-ATPase, which depletes acidic Ca^{2+} stores) and *trans*-ned-19 both repressed *myogenin* and *skNAC* expression in C2C12 cells and primary murine myoblasts (Aley et al. 2010a). These treatments also decreased the number of nuclei present in myosin

heavy chain-positive cells (Aley et al. 2010a). It is important to note that treatment with xestospongins C (an IP₃R inhibitor), or dantrolene (a RyR inhibitor), did not affect the expression of *myogenin* and *skNAC* or the number of nuclei in myosin heavy chain-positive cells (Aley et al. 2010a), implicating the specific requirement of NAADP signaling during myogenesis in vitro.

The expression profile of the transcripts of various Ca²⁺ channels (i.e., *ip₃r1-3*, *ryr1-3*, and *tpcn1-2*) was also established in C2C12 cells and primary murine myoblasts (Aley et al. 2010a). It should be noted here, that somewhat similar to TPCs, there are multiple isoforms of both the IP₃R and RyR. These are called IP₃R1 to 3, and (in mammals) RyR1 to 3, and they are encoded by the *ip₃r1-3* and *ryr1-3* genes, respectively. These ER-based Ca²⁺ channels were first described in the mid-1980s (Berridge and Irvine 1984; Fleischer et al. 1985; Berridge 1987), and recent advances in our understanding of both are reviewed by Santulli et al. (2017). Using RT-PCR, *ip₃r2*, and *ryr3* were shown to be expressed in undifferentiated C2C12 cells, whereas *ip₃r1-3*, *ryr1*, and *ryr3* were shown to be expressed in these cells only once they had undergone differentiation (Aley et al. 2010a). In addition, using northern blotting the relative level of expression of *tpcn1-2* was characterized during the differentiation of C2C12 cells. The results showed that the expression of *tpcn2* preceded the induction of differentiation, but it decreased gradually after the initiation of differentiation. In contrast, *tpcn1* was expressed at a constant level before, during, and after the differentiation process (Aley et al. 2010a). Importantly, this same temporal pattern of expression of *tpcn1* and *tpcn2* was recapitulated in primary murine myoblasts that were obtained during embryonic and postnatal development. Thus, *tpcn2* was strongly down-regulated during development, whereas *tpcn1* was expressed constantly throughout all of the developmental stages examined (Aley et al. 2010a). It was suggested that TPC2 signaling might be required mainly for differentiation of the skeletal muscle cells rather than for their function once differentiation is complete (Aley et al. 2010a). To test the requirement for the various TPC isoforms in

the differentiation process, the expression of *tpcn1* and *tpcn2* was knocked down by transfecting C2C12 cells with siRNAs against each isoform (Aley et al. 2010a). The results showed that both differentiation and the subsequent cell–cell fusion events were repressed after TPC2-knockdown, whereas the differentiation process alone was inhibited after TPC1-knockdown (Aley et al. 2010a). Together, these data support the suggestion for the requirement of NAADP/TPC/Ca²⁺ signaling in skeletal muscle differentiation, and they suggest that TPC1 and TPC2 have different roles in skeletal muscle differentiation and function, such that TPC2 (but not TPC1) has a specific role in the cell fusion events that occur during differentiation, whereas TPC1 (but not TPC2) is expressed in mature muscle where it might act as a trigger to release Ca²⁺ from the SR via RyR1 during contraction (Aley et al. 2010a).

More recently, the role of TPC2 in mediating myogenesis in vivo was demonstrated (Kelu et al. 2015, 2017). Earlier work characterized the endogenous pattern of Ca²⁺ signaling in the developing myotome in zebrafish embryos (Brennan et al. 2005; Cheung et al. 2011). Using a transgenic line of zebrafish that expresses the bioluminescent Ca²⁺ reporter, aequorin, specifically in skeletal muscle cells, the muscle-generated Ca²⁺ signals were visualized (Cheung et al. 2011). Two distinct phases of Ca²⁺ signaling were discovered during the development of the slow muscle cells (the first myofibers to develop; Devoto et al. 1996). These were called signaling period 1 (SP1), which occurs from ~17.5 to ~19.5 hours post-fertilization (hpf), and signaling period 2 (SP2), which occurs after ~23.5 hpf (Fig. 1Aa; Cheung et al. 2011). Further pharmacological interventions suggested that the SP1 Ca²⁺ signals were mainly regulated by IP₃R (as they were attenuated by 2-APB: an IP₃R antagonist), whereas the SP2 Ca²⁺ signals were mainly regulated by RyR (as they were attenuated by an inhibitory concentration of ryanodine; Cheung et al. 2011). To investigate the role of TPC2-mediated Ca²⁺ signaling during slow muscle cell development in zebrafish embryos, Kelu et al. (2015) utilized antisense MO technology (see reviews by Blum et al. 2015; Stainier et al.

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2017) to knock down the expression of TPC2. Using the same aequorin transgenic line of zebrafish described by Cheung et al. (2011), Kelu et al. (2015) showed that the usual pattern of Ca^{2+} signaling in the developing myotome (between ~17 and ~30 hpf) was significantly attenuated after *TPCN2*-MO injection (Fig. 1Ac). In addition, when a *tpcn2* messenger RNA (mRNA) (that was not recognized by the *TPCN2*-MO) was injected into the morphants (MO-treated embryos), they demonstrated a partial rescue of the Ca^{2+} signaling signature (Fig. 1Ad); this supports the specificity of action

of the *TPCN2*-MO (Kelu et al. 2015). In these experiments, a *p53*-MO was always coinjected with the *TPCN2*-MO (Fig. 1Ab; Kelu et al. 2015). The coinjection of *p53*-MO with the desired MO has been suggested to alleviate potential MO off-targeting effects that have previously been reported (Robu et al. 2007; Bedell et al. 2011). In a subsequent study, a negligible level of apoptosis was seen in the neural and somite regions (using the TUNEL assay) after the coinjection of the *TPCN2*-MO with *p53*-MO (Kelu et al. 2017); this helped to confirm the efficacy of *p53* attenuation in the *TPC2* morphants. As a

Figure 1. Role of TPC2 in the differentiation of the slow muscle cells in zebrafish embryos. (A) Effect of morpholino oligonucleotide (MO)-based knockdown (without and with messenger RNA [mRNA] rescue) or pharmacological inhibition of TPC2 on the muscle-generated Ca^{2+} signals from ~17 to ~30 hours post-fertilization (hpf). These histograms show the mean $\pm$ SEM frequency of Ca^{2+} signals generated every 30 min in the trunk musculature in α -actin-aeq transgenic embryos that were (Aa) untreated, (Ab–Ad) injected at the 1- to 4-cell stage with (Ab) *p53*-MO, (Ac) *TPCN2*-MO plus *p53*-MO, or (Ad) *TPCN2*-MO plus *p53*-MO and *tpcn2* mRNA at the amounts shown; or (Ae–Ag) treated after the SP1 Ca^{2+} signals were first observed with (Ae) dimethylsulfoxide (DMSO), (Af) bafilomycin A1, or (Ag) *trans*-ned-19 (at the concentrations shown). Calcium signaling periods 1 and 2 (SP1 and SP2), and the signaling quiet period ([QP]; Cheung et al. 2011) are shown. (B) Effect of MO-based knockdown ($\pm$ mRNA rescue), CRISPR/Cas9-knockout, or pharmacological inhibition of TPC2 on the organization of the trunk musculature and the formation of sarcomeres. Embryos were (Ba) untreated or (Bb–Bd) injected with (Bb) *p53*-MO, (Bc) *TPCN2*-MO plus *p53*-MO, or (Bd) *TPCN2*-MO plus *p53*-MO, and *tpcn2* mRNA at the amounts shown in (A). In addition, (Be) shows a representative homozygous *tpcn2 dhkz1a* mutant, and (Bf–Bh) in some experiments embryos were treated with (Bf) DMSO, (Bg) bafilomycin A1, or (Bh) *trans*-ned-19 at the concentrations shown. All embryos were fixed at ~25 hpf and dual-labeled with phalloidin and the F59 antibody to visualize F-actin (in red) and myosin heavy chain (in green) in the trunk musculature, respectively. The panels show a series of optical sections projected as single images at (Bai–Bhi) low and (Baii–Bhii) higher magnification when the red and green channels are merged; overlapping regions are shown in yellow. The higher magnification images of the slow muscle cell myofibers reveal the presence (Baii, Bbii, Beii, Bfii) or absence (Bcii, Bgii, Bhii) of the sarcomeric banding pattern of the F-actin and myosin heavy chain. Morphants that were coinjected with *tpcn2* mRNA (Bdii) showed the appearance of some banding (white arrowheads) but not clear sarcomeres. Ant. and Pos. in (Bai) are anterior and posterior, respectively. (C) Localization of TPC2 in the slow muscle cells of (Cai) untreated embryos and in those injected with (Caii) *p53*-MO, (Caiii) *TPCN2*-MO plus *p53*-MO, or (Caiiv) *TPCN2*-MO plus *p53*-MO, and *tpcn2* mRNA (at the amounts shown in (A)) between the 1–4-cell stage and then fixed at 24 hpf prior to immunolabeling. The yellow dashed lines indicate the location of line scan analyses, which were performed along individual myofibers, after which (Cai*–Caiiv*) line graphs were plotted to show the change in the pattern of TPC2 expression with the different treatments. (Cb) Visualization of TPC2 and RyR by dual-immunolabeling and dual-color stimulated emission depletion microscopy (STED) superresolution imaging. This is a representative muscle cell that was dissociated from the trunk of a zebrafish embryo at ~48 hpf, plated onto a coverslip for 1 h, and then fixed and dual-immunolabeled with the 2137A anti-TPC2 and 34C anti-RyR primary antibodies. The region bounded by the yellow square in (Cbi) is shown at higher magnification in (Cbii). The white lines in (Cbii) indicate the presence of distinct gaps between the RyR and TPC2 clusters when observed via STED imaging. (Cc) Expression of TPC2 in a slow muscle cell at ~24 hpf in relation to (Cci–Cciii) that of the myosin heavy chain, and (Cciv–Ccvi) the nucleus. The former images were acquired from intact embryos fixed at ~24 hpf, whereas the latter images were acquired from primary cultured cells prepared from embryos at ~16 hpf and then cultured for ~24 h. Scale bars, 50 μm (Bai–Bhi); 2 μm (Baii–Bhii); 10 μm (Cai–Caiiv); 2 μm (Cbi); 200 nm (Cbii); 5 μm (Cc). (A, Ca, and Cc from Kelu et al. 2015; adapted, with permission, from UPV/EHU Press; B and Cb from Kelu et al. 2017; adapted, with permission, from Elsevier.)

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complementary approach, embryos were treated with the lysosome/TPC inhibitors (i.e., bafilomycin A1 or *trans*-ned-19), and both were shown to result in a similar disruption to the muscle-generated Ca^{2+} signaling (Fig. 1Ae–Ag; Kelu et al. 2015). Together, the molecular knockdown and pharmacological inhibition approaches provided strong evidence for the involvement of lysosome/TPC2/ Ca^{2+} signaling during slow muscle cell development in vivo.

To further characterize the role of TPC2 during slow muscle myogenesis, two major muscle proteins, F-actin and the myosin heavy chain, were labeled after the molecular knockdown (using *TPCN2*-MOs), pharmacological inhibition (using bafilomycin A1 and *trans*-ned-19), or genetic knockout (using CRISPR/Cas9-mediated mutagenesis) of TPC2 (Fig. 1B; Kelu et al. 2017). Strikingly, a similar pattern of muscle deformity was induced using the three different approaches, such that the slow muscle fibers became more flexuous, the somites became U-shaped, and the sarcomeric banding became disrupted (Kelu et al. 2017). Importantly, the number of slow muscle cells that were formed also decreased significantly after TPC2 intervention (Kelu et al. 2017). Together, these data suggest that in zebrafish, TPC2-mediated Ca^{2+} release from the lysosome is essential for the formation and differentiation of slow muscle cells, the overall patterning of the skeletal myotome, and the organization of the sarcomeres with respect to their essential contractile microfilaments.

To study the expression and localization of TPC2 during slow muscle development, a zebrafish-specific TPC2 antibody was custom made and used to label endogenous TPC2 at 2-hour intervals from ~16 to ~24 hpf via whole mount immunohistochemistry (Kelu et al. 2015). These stages were chosen because of the robust development of the slow muscle cells reported to occur within this time window (Devoto et al. 1996). To examine the expression of TPC2 in the slow muscle cells, in this series of experiments the slow muscle cells were dual-immunolabeled with the TPC2 antibody and a myosin heavy chain antibody (Kelu et al. 2015). At ~16 hpf, TPC2 expression in the slow muscle cells was almost undetectable; at ~18 hpf, TPC2 ex-

pression increased but remained relatively homogenous along the length of the slow muscle cells; at ~20 hpf, TPC2 expression increased further, and started to assume a banding pattern as visualized by line-scan analysis. By ~22 and ~24 hpf, a robust and clear TPC2 banding was observed in the slow myofibrils; this observation was supported by the appearance of clear peaks of TPC2 labeling in the line-scan analysis (Fig. 1Ca; Kelu et al. 2015). The specificity of the custom-made zebrafish TPC2 antibody was also validated using the TPC2 morphants, where a clear attenuation of the TPC2 labeling was seen in the slow muscle cells. In addition, the normal localization pattern was rescued to some extent when the embryos were coinjected with the *TPCN2*-MO and *tpcn2* mRNA (Fig. 1Ca; Kelu et al. 2015). Together, these data suggested that TPC2 is expressed in the right place (i.e., in the slow muscle cells) and at the right time (i.e., from ~16 to 24 hpf) to generate the essential Ca^{2+} signaling that is required for regulating the development of the slow muscle cells.

It has been suggested that Ca^{2+} released from the lysosome is highly localized, and as such, it might be able to trigger further Ca^{2+} release from the ER/SR via CICR when the acidic stores and ER/SR are in close proximity (Gallione et al. 2009; Gallione 2015). Using STED superresolution microscopy, Kelu et al. (2017) showed that in immunolabeled primary cultured muscle cells, TPC2 was in close proximity to the RyR and that labeling for both was localized in the sarcomeric I-band region (Fig. 1Cb) as confirmed when TPC2 was colabeled with the myosin heavy chain (Fig. 1Cc). As subsequent quantification indicated that the separation between TPC2 and the RyR was between 57 and 82 nm (Kelu et al. 2017), it was suggested that such a nanometer distance lies within a finite range for functional interactions between the two Ca^{2+} release channels to be possible (Morgan et al. 2013; Fameli et al. 2014; Penny et al. 2015). Unfortunately, as the currently available TPC2 and IP_3R antibodies are both raised in the same host species (i.e., rabbit), dual-immunolabeling of the TPC2 and IP_3R was not conducted. Nonetheless, the results from the dual-immunolabeling and superresolution imaging of

the RyR and IP₃R suggest that all three of the Ca²⁺ release channels are localized to the sarcomeric I-bands, and that TPC2 might be even more closely opposed to the IP₃R than it is to the RyR (Kelu et al. 2017). The juxtapositioning of TPC2 with the IP₃R and RyR therefore supports the proposed trigger hypothesis of TPC2 function (Galione et al. 2009; Galione 2015), and indicates that the subsequent recruitment of the SR to generate globalized Ca²⁺ signaling via CICR might be essential for myogenesis. This hypothesis was further tested by Kelu et al. (2017), who demonstrated that in the TPC2 morphants, stimulation of the IP₃R or RyR could partially rescue the development of (and the generation of Ca²⁺ signals in) the slow muscle cells, with stimulation of the IP₃R leading to a greater extent of rescue than stimulation of the RyR (Kelu et al. 2017). This partial rescue observed might be explained if there is a pool of TPC2 that works independently of IP₃R or RyR in these cells. Indeed, it has been shown that in addition to being associated with the sarcomeric I-bands, TPC2 (and LAMP1; a lysosomal marker) is also localized in the perinuclear region of slow muscle cells (Fig. 1Cc; Kelu et al. 2015). IP₃R and RyR are also expressed in the perinuclear and nuclear regions of these cells but the expression pattern of all these channels (TPC2, IP₃R, and RyR) is more diffuse than it is in the vicinity of the myofibrils, so whether they are coupled or work independently is not yet known. However, it has been reported that TPCs can function independently of the ER/SR Ca²⁺ channels (Masgrau et al. 2003), and it has been suggested that the elementary Ca²⁺ signals generated by these channels might directly gate Ca²⁺-activated cation channels on the plasma membrane (Calcra et al. 2009). This concept of distinct pools of TPC2 in cells (where one pool is coupled to the ER, while the other functions independently), has also been indicated in rat cortical neurons as the simultaneous inhibition of IP₃R and RyR reduced, but did not completely block, the generation of NAADP-mediated Ca²⁺ signals (Brailoiu et al. 2005).

In zebrafish, it was previously proposed that the SP1 Ca²⁺ signals are required for slow mus-

cle cell differentiation by mediating excitation–transcription (ET) coupling, whereas the SP2 Ca²⁺ signals are required for slow muscle cell function by mediating excitation–contraction (EC) coupling (Cheung et al. 2011). As TPC2 and LAMP1 have a perinuclear localization in primary cultured muscle cells (Fig. 1Cc), it was suggested that the activation of TPC2 around the nucleus during SP1 might stimulate IP₃Rs and RyRs (which are also localized in the perinuclear/nuclear regions; Kelu et al. 2015) to modulate Ca²⁺-dependent transcription of muscle genes during ET coupling (Avila et al. 2001; Powell et al. 2001; Jaimovich and Carrasco 2002; Carrasco et al. 2003; Cárdenas et al. 2005; Stiber et al. 2005; Valdés et al. 2007). Indeed, the visualization of Ca²⁺ signals in the nucleus in zebrafish slow muscle cells was previously reported during the SP1 period (Cheung et al. 2011), where the duration of the elevated nuclear signal (>7.5 sec) far exceeded that of the cytoplasmic signal (~0.58 sec), thus placing it in the time window for stimulating gene expression suggested by Berridge et al. (2003).

Even though the NAADP/TPC2 pathway has been implicated in agonist-evoked contractions and/or EC coupling in both smooth muscle (Tugba Durlu-Kandilci et al. 2010; Aley et al. 2013) and cardiac muscle (Collins et al. 2011; Capel et al. 2015), relatively little is known about its role in mediating skeletal muscle cell contraction. It was shown that the spontaneous coiling behavior of zebrafish embryos (Saint-Amant and Drapeau 1998) after TPC2-knockdown was severely retarded from ~17 to ~28 hpf, but motility was partially rescued following injection of *tpcn2* mRNA (Kelu et al. 2017). In addition, when determining the touch-evoked response of embryos (Saint-Amant and Drapeau 1998), only ~3% of TPC2 morphants responded to touch, when compared with 100% of the untreated control embryos. Again, a partial rescue was induced by the *tpcn2* mRNA such that ~30% of the embryos responded to touch (Kelu et al. 2017). Nonetheless, further investigation is required to confirm whether the effects on the muscle function are secondary to the effects on the muscle development and motor/sensory neuron activity after TPC2 knockdown.

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In addition to the *in vitro* (Aley et al. 2010a) and *in vivo* (Kelu et al. 2015, 2017) evidence that is accumulating to indicate the essential requirement of NAADP/TPC2/ Ca^{2+} signaling in skeletal muscle development and function, the role of lysosomal Ca^{2+} release during muscle autophagy and aging, as well as during myopathy and muscle repair have recently been described (Cheng et al. 2014, 2015; Lin et al. 2015). Thus, more in-depth investigations into the role of the TPC2/ Ca^{2+} signaling pathway in muscle development and function might help to resolve the pathophysiology of skeletal muscle disorders.

TPC2-MEDIATED Ca^{2+} SIGNALING DURING NEURONAL DIFFERENTIATION

Ca^{2+} has long been known to regulate various aspects of neuronal development and function (reviewed by Simons 1988; Berridge 1998; Gruol et al. 2012; Kawamoto et al. 2012; Brini et al. 2014). Ca^{2+} entering the cell either via voltage-gated channels or via receptor-operated channels, which are regulated by ionotropic neurotransmitters, acts as a second messenger of various signal transduction pathways that regulate a diverse range of cellular processes, including neuronal excitability (Berridge 1998; Roussel et al. 2006; Lu et al. 2010), neuronal gene expression (Ghosh et al. 1994; West et al. 2001), neurotransmitter release (Harvey and MacIntosh 1940; Dodge and Rahamimoff 1967; Augustine et al. 1987; Südhof 2012), and synaptic plasticity (Lamont and Weber 2012), as well as many other events that contribute to the processing and storage of information that underlie learning and memory (Gibbs et al. 1979; Baker et al. 2013). Ca^{2+} signaling is also involved in various aspects of the development of the nervous system (Rosenberg and Spitzer 2011), playing a role in neuronal migration (Komuro and Rakic 1992; Tam et al. 2000), growth cone motility and guidance (Henley and Poo 2004; Bolsover 2005; Gasperini et al. 2017), neurite outgrowth and branching (Lankford and Letourneau 1989; Rønn et al. 2002), and synaptogenesis (Basarsky et al. 1994; Feng et al. 2002). Ca^{2+} influx from the extracellular milieu via voltage-gated Ca^{2+} channels, and/or the mobilization of Ca^{2+}

from the ER via activation of IP_3Rs or RyRs have long been implicated in the Ca^{2+} -regulated aspects of neuronal development and function (Bandtlow et al. 1993; Takei et al. 1998; Tam et al. 2000; Numakawa et al. 2003; Hertle and Yeckel 2007; Gasperini et al. 2017). It is not surprising, therefore, that NAADP-mediated release of cytosolic Ca^{2+} from acidic stores has also been demonstrated to play a role in the extension of neurites in neurons isolated from newborn rat cerebral cortex (Brailoiu et al. 2005), and induce neuronal differentiation of PC12 cells (Brailoiu et al. 2006). More recently, Zhang et al. (2013) demonstrated that TPC2 has opposing effects on different stages of neural differentiation of mouse embryonic stem cells (ESCs), such that it inhibits the early differentiation of ESCs to neural progenitors, but it is required for the later stages of neuronal differentiation.

Most recently, NAADP/TPC2-mediated Ca^{2+} signaling was demonstrated to play a role in the establishment of synchronized activity in the primary motor neurons (PMNs) of intact, normally developing zebrafish embryos (Kelu et al. 2018). The SAIGFF213A:GCaMP7a double transgenic line of fish was used in which the fluorescent Ca^{2+} indicator, GCaMP7a is expressed in the caudal PMNs (CaPs) (Muto and Kawakami 2011; Muto et al. 2011). In normally developing fish at 18 hpf, low frequency and long duration Ca^{2+} signals are generated, which appear to arise in an almost stochastic manner (Kelu et al. 2018). By ~24 hpf, however, the CaP-generated Ca^{2+} signals are ipsilaterally (i.e., along the same side of the trunk) correlated and contralaterally (i.e., on opposite sides of the trunk) anticorrelated (Muto and Kawakami 2011; Muto et al. 2011; Kelu et al. 2018); thus, Ca^{2+} signals are normally generated synchronously first along one side of the trunk and then along the other side (Fig. 2Aa, Ad). However, Kelu et al. (2018) showed that the MO-mediated knockdown (Fig. 2Ab), genetic knockout (via CRISPR/Cas9; Fig. 2Ae), or pharmacological inhibition (with bafilomycin A1 or *transferrin-19*; Fig. 2Af–Ah) of TPC2 resulted in a loss of this synchronized (i.e., both the correlation and anticorrelation) Ca^{2+} signaling in the CaPs. TPC2 knockdown, knockout, or inhibi-

Figure 2. (Legend on following page.)

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tion also resulted in a decrease in the frequency and amplitude of the CaP-generated Ca^{2+} signals and an increase in their duration. However, coinjection of embryos with *TPCN2*-MO and *tpcn2* mRNA resulted in the ipsilateral and contralateral pattern of Ca^{2+} signaling as well as the frequency, amplitude, and duration of the signals being (at least partially) rescued (Fig. 2Ac; Kelu et al. 2018). TPC2 and LAMP1 (lysosomal-associated membrane protein 1) were shown to be expressed by the CaPs both in the cell body and in clusters along the axon (Fig. 2Ba, Bb). In addition, IP₃Rs type I and II (but not IP₃R type III or any of the RyR subtypes) were shown to be localized in the CaPs (Fig. 2Bc–Bf). Following MO-mediated TPC2 knockdown, treatment of embryos with the IP₃R agonist, IP₃/BM (but not the RyR agonists, caffeine, or ryanodine at a low agonistic concentration), partially rescued the CaP-mediated Ca^{2+} signals (Kelu et al. 2018). It was therefore suggested that these data support the trigger hypothesis, where the localized release of Ca^{2+} via TPCs in acidic stores stimulates larger-scale Ca^{2+} release via Ca^{2+} channels in the ER (IP₃R or RyR) via CICR (Fig. 3; Kin-

near et al. 2004; Galione et al. 2009). Indeed, the acidic organelles and ER have previously been shown to be closely apposed in primary rat medullary neurons (Brailoiu et al. 2009), and NAADP-induced Ca^{2+} release has been demonstrated to be linked to the activation of the IP₃R in astrocytes isolated from newborn mice (Heidemann et al. 2005), as well as to the activation of IP₃R or RyR in isolated nuclei from the ganglia of adult *Aplysia californica* and *Arbacia punctata* (Bezin et al. 2008). The work by Kelu et al. (2018) provides the first evidence in an intact animal model that TPC2 is a key molecular component of the Ca^{2+} signaling pathway that coordinates the establishment of the motor neuronal circuitry during development.

TPC2-MEDIATED Ca^{2+} SIGNALING DURING OSTEOCLASTOGENESIS

Osteoclasts are giant multinucleated cells of the monocyte/macrophage family, which degrade and resorb bone (Udagawa et al. 1990). Working together with osteoblasts (which are responsible for synthesizing bone), osteoclasts regulate the

Figure 2. Role of two-pore channel 2 (TPC2) in the differentiation of the primary motor neurons in zebrafish embryos. (A) Effect of morpholino oligonucleotide (MO)-based knockdown (without and with messenger RNA [mRNA] rescue), CRISPR/Cas9 heterozygous knockout, or pharmacological inhibition of TPC2 on the spontaneous Ca^{2+} activity of the caudal primary motor neurons (CaPs) in SAIGFF213A;UAS:GCaMP7a embryos at ~24 hours post-fertilization (hpf). SAIGFF213A;UAS:GCaMP7a embryos were injected with (Aa) *p53*-MO; (Ab) *TPCN2*-MO plus *p53*-MO; or (Ac) *TPCN2*-MO plus *p53*-MO and the *tpcn2* mRNA at the amounts shown in Figure 1A. In addition (Ad), untreated SAIGFF213A;UAS:GCaMP7a embryos (termed wild-type *tpcn2* controls) were imaged and the adults were crossed with homozygous *tpcn2 dhkz1a* mutants to generate (Ae) double-transgenic heterozygous *tpcn2 dhkz1a* mutants. Furthermore (Af–Ah), some embryos were treated at ~17 hpf with (Af) DMSO; (Ag) bafilomycin A1; or (Ah) *trans*-ned-19 (at the concentrations shown). (A*a) All embryos were imaged in a dorsal orientation and (A*b, A*c) time-lapse fluorescence images were acquired to show the changes in GCaMP7a fluorescence in the CaPs at different time intervals in the various treatment groups. Regions of interest (ROIs) were placed on two selected CaP cell bodies on the left (L1 and L2) and right (R1 and R2) sides of the spinal cord. Ant. and Pos. are anterior and posterior, respectively. Scale bar, 50 μm . (Aa–Ah) Line graphs showing the $\Delta F/F_0$ against time (over a period of ~300 sec) in the ROIs selected for each embryo. (B) Expression and localization of TPC2, lysosomal-associated membrane protein 1 (LAMP1), IP₃Rs, and RyRs in presumptive CaPs isolated from SAIGFF213A;UAS:GFP embryos and cultured for 24 h. The trunk of these embryos, which express green fluorescent protein (GFP) in the CaPs, was dissected at ~18 hpf. The cells were dissociated and cultured for 24 h and then they were immunolabeled with antibodies for (Ba) TPC2, (Bb) LAMP1, (Bc–Be) IP₃R types I–III, respectively, or (Bf) RyR (all subtypes). (Bg) Secondary antibody control. The cells were costained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Each panel is a series of optical sections that have been projected as a single confocal image, and they show the red (individual antibody) and blue (nuclei) channels when merged. CB and AX are the cell body and axon, respectively. The yellow, white, and blue arrowheads indicate TPC2, LAMP1, and IP₃R type I clusters, respectively, localized along the axons. Scale bars, 10 μm . (From Kelu et al. 2018; adapted, with permission, from Elsevier.)

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overall mass and structure of the skeleton. The major players and main mechanisms that control osteoclast differentiation and bone resorption have been described previously and are the subject of a number of excellent reviews (Chambers 2000; Teitelbaum 2000; Boyle et al. 2003; Asagiri and Takayanagi 2007; Teitelbaum 2007; Yavropoulou and Yovos 2008; Nakashima et al. 2012). In brief, it is known that osteoclasts differentiate under the control of two main cytokines, receptor activator of nuclear factor κ B ligand (RANKL) (Hsu et al. 1999; Wada et al. 2006), and macrophage colony-stimulating factor (Yoshida et al. 1990). Ca^{2+} signaling is known to play a key role in the differentiation of osteoclasts. Indeed, it has been shown that RANKL stimulates the generation of Ca^{2+} oscillations, which result in the calcineurin-mediated activation and nuclear localization of nuclear factor-activated T cells c1 (NFATc1), which in turn activates the gene transcription that regulates the terminal differentiation of these cells (Takayanagi et al. 2002). Following the Ca^{2+} oscillations, there is also an influx of Ca^{2+} on the basolateral side of osteoclasts, which is mediated by TRPV4, a Ca^{2+} -permeable channel of the transient receptor potential family. It has been suggested that the oscillations and influx of Ca^{2+} together help to maintain the intracellular Ca^{2+} levels to ensure that NFATc1-regulated gene transcription (and hence osteoclast differentiation) is maintained (Masuyama et al. 2008).

It was also recently reported that TPC2 plays a key role in the differentiation of osteoclasts (Notomi et al. 2012, 2017). TPC2 was shown to be expressed by mouse bone marrow cells and the mouse osteoclast precursor cell line, RAW 264.7. In TPC2-knockdown cells, RANKL-regulated processes were reported to be inhibited, including the generation of the Ca^{2+} signals, and the nuclear localization of NFATc1 (Notomi et al. 2012). Furthermore, the TPC2 function was reported to be dependent on the amount of Mg^{2+} , which (along with Ca^{2+}) is stored in bone (Swaminathan 2003). Thus, whereas in normal Mg^{2+} conditions, TPC2 promoted osteoclast differentiation; when the level of Mg^{2+} was low, TPC2 inhibited this process. In addition, TPC2 was demonstrated to affect these

changes by distinct mechanisms, such that it promoted and inhibited osteoclastogenesis via a Ca^{2+} -dependent mechanism and via $\text{PI}(3,5)\text{P}_2$ -dependent changes in Na^+ (rather than in Ca^{2+}), respectively (Notomi et al. 2017). This might be the first example of the dual action of TPC2 (i.e., demonstrating its ability to transport both Ca^{2+} and Na^+) in the same cell type (i.e., in RAW 264.7 cells) when exposed to different extracellular environments.

TPC-MEDIATED Ca^{2+} SIGNALING DURING THE DIFFERENTIATION OF ENDOTHELIAL AND EPITHELIAL CELLS

The formation of new blood vessels from pre-existing vessels (angiogenesis) is a key process during embryogenesis, and during the reproductive cycle of female mammals. Angiogenesis is also important during the repair and regeneration of damaged tissue, as well as in various diseases (Gupta and Zhang 2005). This process involves the proliferation, migration, and remodeling of endothelial cells from pre-existing blood vessels (Folkman and Shing 1992; Risau 1995), and it is regulated by vascular endothelial growth factor (VEGF) and its receptors, VEGFR1 and VEGFR2 (Michiels 2003). It has recently been shown both in vitro and in vivo that a VEGF/NAADP/TPC2/ Ca^{2+} signaling cascade plays a key role in the formation of blood vessels (Favia et al. 2014). For example, treatment of human umbilical vein endothelial cells with *trans*-ned-19 or *tpcn2* shRNA inhibited the usual VEGF-stimulated release of Ca^{2+} and formation of capillary-like tubes. Furthermore, in intact mice, the VEGF-induced formation of blood vessels was inhibited by *trans*-ned-19 in C57BL/6 (wild-type) animals and it failed to occur at all in *Tpcn2*^{-/-} mutants (Favia et al. 2014).

Although there is currently no direct evidence that TPCs play a role in the differentiation of epithelial cells, NAADP has been shown to stimulate an increase in Ca^{2+} and promote the differentiation of human epidermal keratinocyte cells (Park et al. 2015). The latter was demonstrated by the effect of NAADP treatment on the level of expression of several protein markers for early and late keratinocyte differentiation,

and the increase in activity of transglutaminases (Park et al. 2015), which are required for cross-linking proteins during terminal keratinocyte differentiation (Tharakan et al. 2010). The NAADP-stimulated Ca^{2+} signaling in these cells was shown to be inhibited by treatment with *trans*-ned-19, dipyridamole or bafilomycin, but was not affected by treatment with 8-bromo-cADPR (a cADPR inhibitor), xestospongine C (an IP_3R inhibitor), or ryanodine at a RyR inhibitory concentration (Park et al. 2015). These data therefore indicate that TPCs might also play a key role in the differentiation of this specialized epithelial cell type.

CONCLUDING REMARKS

Here, we have reviewed what has been described so far about the role of NAADP/TPC/ Ca^{2+} signaling during development and cellular differentiation. Tracking down the exact target or targets of NAADP, and identifying whether NAADP is the sole agonist of TPCs or not, as well as discovering whether TPCs conduct Na^+ rather than Ca^{2+} is a matter of great interest for researchers in the Ca^{2+} signaling field. It is perhaps not surprising that a cation channel family with an evolutionary lineage such as the TPCs might have more than one agonist as well as a number of agonist-binding strategies (Lin-Moshier et al. 2014; Rahman et al. 2015; Feijóo-Bandin et al. 2017). The fact that TPCs have a somewhat promiscuous relationship with regard to the agonists that induce their opening, and the cations that they subsequently gate, in addition to their ability to act as a Ca^{2+} release trigger (Galione et al. 2009; Patel et al. 2010; Davis et al. 2012; Morgan et al. 2013), makes them a very interesting and challenging component of the Ca^{2+} signaling toolkit (Berridge et al. 2000) to study and understand, especially when it comes to the regulation of developmental and cellular differentiation. Furthermore, when considering the size and relative mobility of the acidic organelles that present TPCs to the cytosolic environment, this provides additional tantalizing evidence to suggest that they might be major players in intracellular Ca^{2+} signaling pathways. Indeed, endosomes and lysosomes along with

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closely associated ER/SR have been described as being a regulatory hub at the intersection of intracellular Ca^{2+} signaling (Marchant and Patel 2015; Perera and Zoncu 2016; Kilpatrick et al. 2017). In addition, while it is becoming clear that in some circumstances NAADP does appear to play a significant role in the Ca^{2+} -mediated signaling pathways that orchestrate embryogenesis and differentiation, whether it does so via a TPC family member or some other target is (in many cases) still to be determined. Resolving these issues is essential for the role played by this pleiotropic channel family in key developmental events to be fully appreciated.

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