Constitutive recycling of the store-operated Ca\(^{2+}\) channel Orai1 and its internalization during meiosis

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The egg’s competency to activate at fertilization and transition to embryogenesis is dependent on its ability to generate a fertilization-specific Ca\(^{2+}\) transient. To endow the egg with this capacity, Ca\(^{2+}\) signals remodel during oocyte maturation, including inactivation of the primary Ca\(^{2+}\) influx pathway store-operated Ca\(^{2+}\) entry (SOCE). SOCE inactivation is coupled to internalization of the SOCE channel, Orai1. In this study, we show that Orai1 internalizes during meiosis through a caveolin (Cav)- and dynamin-dependent endocytic pathway. Cav binds to Orai1, and we map a Cav consensus–binding site in the Orai1 N terminus, which is required for Orai1 internalization. Furthermore, at rest, Orai1 actively recycles between an endosomal compartment and the cell membrane through a Rho-dependent endocytic pathway. A significant percentage of total Orai1 is intracellular at steady state. Store depletion completely shifts endosomal Orai1 to the cell membrane. These results define vesicular trafficking mechanisms in the oocyte that control Orai1 subcellular localization at steady state, during meiosis, and after store depletion.

Introduction

Vertebrate oocytes are arrested in prophase of meiosis I in an interphase-like stage of the cell cycle with an intact germlinal vesicle (nucleus; Masui and Clarke, 1979). Before such oocytes become fertilization competent, they undergo a cellular differentiation pathway known as oocyte maturation, during which they complete meiosis I and arrest at metaphase of meiosis II. We refer to these mature oocytes as eggs. Oocyte maturation encompasses remodeling of Ca\(^{2+}\) signaling pathways to endow the egg with the capacity to activate at fertilization and undergo the egg to embryo transition (Machaca, 2007). The fertilization-specific Ca\(^{2+}\) signal provides a digital cipher that encodes critical cellular events at fertilization in a sequential fashion, including the block to polyspermy and completion of meiosis. As part of Ca\(^{2+}\) signaling remodeling, the primary Ca\(^{2+}\) influx pathway, store-operated Ca\(^{2+}\) entry (SOCE), inactivates (Machaca and Haun, 2000; Machaca and Haun, 2002). SOCE inhibition could contribute to shaping the dynamics of the fertilization-specific Ca\(^{2+}\) transient and may represent a safety mechanism to prevent premature egg activation (Machaca and Haun, 2002; Ullah et al., 2007). SOCE also inactivates during mitosis of mammalian cells (Preston et al., 1991; Tani et al., 2007).

SOCE is activated by the level of Ca\(^{2+}\) in intracellular stores, primarily the ER. Lowering free ER Ca\(^{2+}\) below a certain threshold activates Ca\(^{2+}\) influx at the cell membrane through SOCE. RNAi screens identified the critical molecular determinants of SOCE. The stromal interaction molecule 1 (STIM1), an ER transmembrane protein with luminal EF-hands, was identified as the ER Ca\(^{2+}\) sensor (Liou et al., 2005; Roos et al., 2005), and Orai1 (also known as CRACM1) as the Ca\(^{2+}\) release-activated current channel (Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006). The Orai1 protein spans the membrane four times with intracellular N and C termini, has no sequence homology to other known channels, and is essential for Ca\(^{2+}\) release-activated current (Prakriya et al., 2006; Vig et al., 2006; Yeromin et al., 2006). A mutation in Orai1 (R91W) abrogates Ca\(^{2+}\) influx in T cells and causes severe combined immunodeficiency disorder (Feske et al., 2006). Depletion of Ca\(^{2+}\) stores releases Ca\(^{2+}\) from the STIM1 luminal EF-hand, resulting in a conformational change that leads to clustering of STIM1 into large puncta in a cortical ER domain that localizes 10–20 nm below the cell membrane (Wu et al., 2006; Liou et al., 2007; Stathopoulos et al., 2008; Orci et al., 2009). Clustered STIM1

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recruits Orai1 into coincident puncta and gates the channel leading to SOCE (Luij et al., 2006; Park et al., 2009; Yuan et al., 2009). STIM1 has also been shown to couple to and gate transient receptor potential canonical channels to induce SOCE (Huang et al., 2006; Yuan et al., 2007).

As with other ion channels, SOCE current amplitude depends in part on the levels of the channel protein in the target membrane. Indeed, defects in ion channel trafficking lead to serious clinical consequences. For example, a mutation in CFTR (ΔF508) produces a functional Cl− channel that never reaches the plasma membrane, thus hampering Cl− transport and leading to cystic fibrosis (Skach, 2000). Defects in ether-a-go-go (hERG) channel trafficking lead to long QT syndrome, a cardiac disorder that increases arrhythmias and the risk of sudden cardiac death (Perrin et al., 2008). Members of the transient receptor potential canonical channel family are also regulated by vesicular insertion into the cell membrane after agonist stimulation (Bezzerides et al., 2004; Singh et al., 2004). In addition, TRPC1 function depends on its localization to lipid raft domains (Lockwich et al., 2000), and caveolin (Cav) has been implicated in TRPC1 targeting to the plasma membrane and its association with STIM1 (Brazer et al., 2003; Pani et al., 2009).

A role for vesicular trafficking in SOCE was initially proposed in the context of the coupling mechanism between store depletion and SOCE activation based on the role of GTP in the process (Fasolato et al., 1993; Somasundaram et al., 1995). It was suggested that a secretion-like mechanism mediates translocation of the SOCE channel from an intracellular vesicular pool to the cell membrane, where it mediates Ca2+ influx. As with other early models of the SOCE coupling mechanism, this proposal was controversial. Broad action drugs that block vesicular transport, such as brefeldin A and primaquine, produce variable effects on SOCE dependent on the study (Somasundaram et al., 1995; Gregory and Barritt, 1996; Yao et al., 1999). A potential role for vesicular transport in SOCE was also implied through the effect of interventions that altered the cortical cytoskeleton (Patterson et al., 1999; Rosado et al., 2000). More specific approaches targeted at inhibiting different components of the SNARE machinery required for vesicular fusion also produced conflicting results (Yao et al., 1999; Alderton et al., 2000; Bakowski et al., 2003; Scott et al., 2003). It is difficult to reconcile these early findings with our current knowledge of the SOCE activation mechanism centered around STIM1–Orai1 modulation and interaction. Although significant knowledge has accumulated regarding STIM1 subcellular distribution dynamics (Lewis, 2007; Fahrner et al., 2009; Lee et al., 2010), little is known about the trafficking of Orai1. We have recently shown that Orai1 is gradually internalized during Xenopus laevis oocyte meiosis without any associate decrease in total Orai1 protein levels, arguing that it is not targeted for degradation (Yu et al., 2009). Furthermore, Orai1 recycles at rest, and its cell membrane enrichment requires a functional exocytic machinery because Orai1 distribution shifts intracellularly in cells expressing a dominant-negative SNAP-25 that blocks exocytosis (El Jouni et al., 2007; Yu et al., 2009). Herein, we define the endocytic mechanisms controlling both Orai1 internalization during meiosis and its recycling at the cell membrane at rest. We show that Orai1 constitutively recycles between the recycling endosome and the cell membrane through a Rho- and Rab5-dependent and largely dynamin-independent endocytic pathway. During meiosis, Orai1 trafficking shifts its dependence, leading to internalization to a late endosome compartment through a Cav-, Rab5-, and dynamin-dependent endocytic pathway. Interestingly, store depletion leads to a complete shift of the subcellular distribution of Orai1 away from the endosomal compartment into the cell membrane, showing that dynamic recycling of Orai1 in the oocyte is important to regulate channel density at the cell membrane. We further map Orai1 domains required for internalization and show a dependency on both the N- and C-terminal cytoplasmic domains for targeting Orai1 for internalization. We map a Cav-binding site in the Orai1 N terminus, which is required for internalization. These results define the regulation Orai1 trafficking at rest, after store depletion, and during M phase.

Results
Expression of GFP-Orai1 with the ER marker Cherry-KDEL in oocytes shows a steady-state enrichment of Orai1 at the cell membrane, with a fraction of the protein pool localizing to an intracellular endocytic compartment (Fig. 1 A, oocyte). Orai1 distribution is shifted during meiosis, where in the egg, Orai1 is enriched in an endosomal compartment and is practically absent from the cell membrane (Fig. 1 A, egg). To confirm internalization of Orai1 during meiosis, we sought a protein that maintains its cell membrane localization during meiosis. The Ca2+-activated Cl− current is essential for the membrane depolarization observed at fertilization, which mediates the fast block to polyspermy (Machaca et al., 2001), and its amplitude is enhanced in eggs (Machaca and Haun, 2000), showing that the channel maintains its cell membrane localization. Recently, the Ca2+-activated Cl− channel was cloned from both Xenopus and rodents (Schroeder et al., 2008; Yang et al., 2008). We therefore synthesized the Xenopus TMEM16A gene and tagged it with mCherry to follow its trafficking during meiosis. TMEM16A is enriched at the cell membrane in both oocytes and eggs and, as such, is not internalized during meiosis (Fig. 1 B). Coexpression of TMEM16A with Orai1 shows colocalization of both proteins at the cell membrane in oocytes, whereas in eggs, Orai1 is enriched in endosomes and is absent from the cell membrane marked with TMEM16A (Fig. 1 B).

Identity of the Orai1-positive endosomal compartments
To identify the endocytic compartments to which Orai1 traffics in both oocytes and eggs, we expressed with GFP-Orai1 a set of tagged Rab markers that define different endocytic compartments (Fig. 1 C). Rab proteins constitute a large family of small GTPases that are central to multiple aspects of vesicular trafficking, including targeting of vesicles to the appropriate compartment and recruitment of effector proteins (Stenmark, 2009). Rab4 and Rab5 were used to visualize early endosomes, Rab11 for the recycling endosome, Rab9 and Rab7 for the late endosome (Stenmark, 2009), and LysoTracker to visualize lysosomes.
We used various approaches to define endocytic pathways through which Orai1 internalizes. Transferrin was used as a marker for clathrin-dependent endocytosis, and cholera toxin B (CTB), which binds the ganglioside GM1 (Parton, 1994), as a marker for raft-dependent endocytosis (Fig. 2A). Orai1 colocalizes efficiently with CTB but not transferrin, arguing that Orai1 is endocytosed through a raft-dependent and clathrin-independent pathway (Fig. 2A). CTB in different cell types can also be internalized through clathrin-dependent and caveolae- and clathrin-independent pathways (Torgersen et al., 2001; Kirkham and Parton, 2005). Therefore, the colocalization of Orai1 with CTB has to be interpreted carefully.

To expand on these results, we interfered with the function of dynamin, a GTPase required for pinching endocytic vesicles during both clathrin- and Cav-dependent endocytosis (Sever, 2002). A dominant-negative dynamin mutant (K44A) abrogates Orai1 internalization, resulting in a decrease in the number and size of Orai1-positive endosomes in eggs and a steady-state enrichment of Orai1 at the cell membrane (Fig. 2B, Dyn K44A).

Another important endocytic pathway in Xenopus oocytes is the Rho-dependent constitutive endocytosis (Schmalzing et al., 1995). This pathway can be inhibited using the Clostridium botulinum C3 exoenzyme, which ADP-ribosylates RhoA, -B, and -C (Schmalzing et al., 1995). C3 exoenzyme only mildly inhibits Orai1 internalization, despite resulting in dramatic vesiculation at the cell membrane (Fig. 2B, C3 exoenz), which is consistent with the increased membrane surface area previously described (El Jouni et al., 2007). The large vesiculations after C3 exoenzyme treatment are cell membrane invaginations, as confirmed by coexpression of TMEM16A (Fig. S1C).
Orai1 during meiosis (Fig. 3 B). As previously reported, MβCD treatment enhanced the rate of oocyte maturation (Sadler and Jacobs, 2004), showing functionality of the drug. These data argue that Orai1 internalization does not require cholesterol. There is precedent for stable functional raft domains at the cell membrane that are cholesterol independent yet contain Cav-1 (Hansen et al., 2001). Given the effect of Rab5 on Orai1-positive endosomes, we were interested in its potential role in mediating Orai1 internalization. A dominant-negative Rab5 mutant (S34N) effectively blocks Orai1 internalization (Fig. 2 C), revealing a requirement for Rab5 in Orai1 internalization. Rab5 has been implicated in cargo sorting and vesicle tethering and fusion (Stenmark, 2009), and, thus, its requirement for Orai1 internalization could be at the level of Orai1 trafficking.

This shows that Rho-dependent constitutive endocytosis is not the major route directing Orai1 removal from the cell membrane. This is compatible with the fact that the majority of Orai1 is removed from the cell membrane during meiosis, arguing that Orai1 is specifically targeted for endocytosis.

We also modulated ARF6-dependent endocytic pathway by injecting constitutively active (Q67L) or dominant-negative (T22N) ARF6 mutants (Fig. 3 A). Neither treatment affects Orai1 internalization during meiosis or Orai1 recycling in oocytes (Fig. 3 A), arguing that ARF6-dependent endocytosis is not involved in Orai1 trafficking.

Trafficking of Orai1 with CTB argues for potential involvement of lipid rafts, which typically entails cholesterol-rich domains that can be disrupted by methyl-β-cyclodextrin (MβCD), a cholesterol-chelating drug (Ilangumaran and Hoessli, 1998). Treating oocytes with MβCD did not affect the level of internalization of Orai1 during meiosis (Fig. 3 B). As previously reported, MβCD treatment enhanced the rate of oocyte maturation (Sadler and Jacobs, 2004), showing functionality of the drug. These data argue that Orai1 internalization does not require cholesterol. There is precedent for stable functional raft domains at the cell membrane that are cholesterol independent yet contain Cav-1 (Hansen et al., 2001).

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Domains required for Orai1 trafficking

The complete internalization of Orai1 during meiosis argues that Orai1 is specifically targeted for endocytosis. To determine multiple steps along the endocytic pathway. In contrast to Rab5, two different dominant-negative Rab4 mutants (N121I and S22N) had no effect on Orai1 internalization during meiosis (Fig. 3 C).

Orai1-recycling endocytic pathway

Using similar approaches, we dissected the endocytic pathway that underlies Orai1 recycling in oocytes. The dynamin dominant-negative mutant exhibits only a modest inhibitory effect on Orai1 recycling (Fig. 4 A) and a corresponding modest decrease in the number of recycling endosomes (Fig. S1 D). In contrast, C3 exoenzyme effectively blocks Orai1 recycling, leading to loss of Orai1 from the endocytic compartment visualized by Rab5 coexpression (Fig. 4 B). Furthermore, dominant-negative Rab5 (S34N) effectively blocks Orai1 recycling (Fig. 4 C), just as it blocked internalization during meiosis. This shows that in the interphase-like oocyte, Orai1 recycles primarily through a Rho-dependent, dynamin-independent endocytic pathway. During meiosis, Orai1 internalization switches to a dynamin-dependent, cholesterol-independent endocytic pathway.

Cav is required for Orai1 internalization but not its recycling

We have previously shown that Cav colocalizes with Orai1 in endosomes (Yu et al., 2009). To address the functional role, if any, for Cav in Orai1 internalization, we expressed either wild-type Xenopus Cav or a dominant-negative Cav mutant (P168L). The Cav (P168L) mutant corresponds to the P132L in human Cav (Fig. S2 A), a mutation that is frequently detected in breast cancers and seems to contribute to the generation of the metastatic phenotype (Bonuccelli et al., 2009). In contrast to the cell membrane association of wild-type Cav in both oocytes and eggs (Fig. S2 B), Cav-P168L is intracellular and localizes to both the ER and Golgi, as shown by its distribution and by coexpression of GalNAc-GFP (Fig. S2 C). The equivalent human mutation mislocalizes to the Golgi (Bonuccelli et al., 2009). Expression of Cav-P168L limits Orai1 trafficking to the cell membrane, and traps it in the ER (Fig. S2 D), thus precluding testing the role of Cav in Orai1 internalization. We circumvented this limitation by first expressing Orai1 and allowing it enrich at the cell membrane, then expressing Cav-P168L (Fig. 5 B). Under these conditions, Cav-P168L effectively inhibits Orai1 internalization, as illustrated by the reduced number of endosomes and maintenance of cell membrane enrichment of Orai1, but does not affect Orai1 recycling in the oocyte (Fig. 5 B). Overexpression of wild-type Cav with the P168L mutant rescues Orai1 internalization inhibition (Fig. 6), arguing that P168L acts by sequestering endogenous Cav. These results argue that Cav is required for Orai1 internalization during meiosis but not for its recycling in the oocyte.
which Orai1 domains are involved in this targeting, we generated different deletion mutants of Orai1 focusing primarily on the cytoplasmic N and C termini. Deletion of the entire N-terminal cytoplasmic domain (ΔN90-Orai1-CFP) decreases Orai1 trafficking to the cell membrane, trapping it primarily in the ER (Fig. 7 B and Fig. S3 A). Deleting the first 70 residues of Orai1 (ΔN70-Orai1-CFP) results in normal Orai1 trafficking to the cell membrane in oocytes; however, this mutant is not internalized during meiosis (Fig. 7 C and Fig. S3 D). This argues that sequence motifs within the first 70 residues of Orai1 are required for targeting for internalization during meiosis. Given the role of Cav in Orai1 internalization, we tested whether expression of Cav with ΔN70-Orai1 rescues its internalization defect; however, this was not the case (Fig. S3 D). In addition, ΔN70-Orai1 recycles normally in oocytes, showing that this domain is not required for Orai1 recycling.

Deletion of the last 12 residues of Orai1 (GFP-Orai1ΔC289) has no effect on Orai1 trafficking (Fig. 7 D and Fig. S3 C). In contrast, deletion of the entire cytoplasmic C-terminal domain (Orai1ΔC267) inhibits Orai1 internalization without affecting trafficking to the cell membrane or recycling in oocytes (Fig. 7 E and Fig. S3 B). Interestingly, in this case, co-expression of Cav but not Rab5 effectively rescues Orai1-ΔC267 internalization (Fig. 7 E and Fig. S3 E).

The Orai1 cytoplasmic C-terminal domain is predicted to fold into a coiled-coil motif that is important for STIM1–Orai1 interaction (Zhang et al., 2006; Muik et al., 2008). To test whether Orai1 internalization requires this coiled-coil domain, we generated the L273S mutant, which is predicted to disrupt the coiled-coil and the L282S mutant, which is predicted to stabilize the coiled coil (Muik et al., 2008). Orai1-L282S traffics normally in oocytes and eggs (Fig. 7 F and Fig. S4 A), whereas the L273S mutant fails to reach the cell membrane and is retained in the ER (Fig. 7 G and Fig. S4 B). These data suggest that proper folding of the Orai1 C-terminal coiled-coil domain is important for Orai1 targeting to the cell membrane. However, because the biogenesis of Orai1-ΔC267 is unaffected, this argues that Orai1-L273S is retained in the ER because it does not fold properly.
Orai1 contains an additional consensus Cav-binding site between residues 250 and 258 (FIVFYHVF), which is predicted to be part of TM4 of Orai1 (Hogan et al., 2010). Because Cav inserts into the bilayer through a hairpin, this region of Orai1 could potentially interact with Cav. However, an Orai1 mutant with the first two aromatic residues mutated (F250,253A) traffics normally in oocytes and is internalized during maturation (Fig. S5 B), showing that this site is not required for Orai1 internalization.

Immunoprecipitation of wild-type Orai1 pulls down Cav, showing that Orai1 and Cav interact in vivo (Fig. 8 D). In contrast, significantly less Cav coprecipitates with Orai1-Y52,W55A, which is consistent with the inhibition of internalization of this mutant. Similar results were obtained whether Orai1 was co-expressed with human or *Xenopus* Cav (Fig. 8 D). Human Cav colocalizes with Orai1 in both oocytes and eggs (Fig. S5 A). The specificity of the anti-GFP immunoprecipitation is illustrated by the lack of Cav pull-down when the immunoprecipitation was performed using a nonspecific antigen, the inositol 1,4,5-trisphosphate receptor (Fig. 8 E).

We further generated a GFP-tagged construct that contains the first 90 residues of Orai1, either as the wild-type construct (GFP–N-term–Orai1) or with residues Y52 and W55 mutated to Ala. Both constructs express equally well, as illustrated by the Western blot (Fig. 8 F). Immunoprecipitation of wild-type GFP–N-term–Orai1 efficiently pulls down Cav, whereas the N-term–Y52,W55A mutant was significantly less effective at pulling down Cav (Fig. 8 F). The fact that some Cav coprecipitates with Orai1-Y52,W55A either in the context of the full-length protein or the N-terminal domain argues that Cav can still interact with this mutant, albeit with drastically lower efficiency. Nonetheless,
this residual Cav association is not sufficient to mediate Orai1 internalization, even when Cav is overexpressed (Fig. 8 B).

**Store depletion shifts Orai1 distribution from endosomes to the cell membrane**
The fact that Orai1 recycles continuously between an endosomal compartment and the cell membrane raises the interesting question of the distribution of Orai1 after store depletion. Depleting Ca\(^{2+}\) stores in cells coexpressing STIM1 and Orai1 leads to coclustering of both proteins into large clusters referred to as puncta (Fig. 9 A). Importantly, the endosomal Orai1 pool is exhausted, arguing that recycling Orai1 is trapped by STIM1 puncta at the cell membrane, leading to depletion of the endosomal Orai1 pool. In 84% of the cells analyzed, no endosomal Orai1 was detected after store depletion (Fig. 9 A). We scored cells with any visible endosomal Orai1 as positive even if it was detected at significantly lower levels than in control cells. The presence of endosomal Orai1 after store depletion appears to correlate with lower levels of STIM1 expression.

Interestingly, rare events of intracellular STIM1–Orai1 puncta could be resolved in oocytes (Fig. 9 A, +TPEN, box). These puncta are detected deep below the membrane plane: in the example shown in Fig. 9 A, the highlighted punctum is 1.2 µm below the focal plane defined by STIM1–Orai1 puncta.
Although multiple membrane proteins are internalized during meiosis, others such as the \(\text{Ca}^{2+}\)-activated Cl\(^{-}\) channel, which is required for the block to polyspermy, remain enriched in the cell membrane (Fig. 1 B). This reveals specific sorting of cell membrane proteins during meiosis and argues that the subset of endocytosed proteins is specifically tagged to achieve effective internalization. This sorting is fundamental for egg activation and future embryonic development. In preparation for embryonic development, the egg internalizes both cell membrane proteins and lipids, leading to a dramatic decrease in cell membrane surface area (Kado et al., 1981; Machaca and Haun, 2000). A similar decrease in membrane surface area and modulation of vesicular trafficking has been observed during mitosis and shown to be important for M phase (Boucrot and Kirchhausen, 2007). The early rapid embryonic divisions lack the gap phases of the cell cycle required for accumulation of the molecular building blocks. Therefore, as the early blastomeres divide, they incorporate intracellularly stored vesicles rich in ionic transporters into their basolateral membrane with the apical membrane formed by the egg’s cell membrane, which contains a different transporter complement (Angres et al., 1991; Gawantka et al., 1992). This results in the formation of the first polarized epithelium during embryogenesis with the capacity to transport salts and water in a vectorial fashion, and as such, contributing to the formation of the fluid-filled blastocoel cavity (Müller and...
Hausen, 1995; Müller, 2001). Our experiments on the internalization of Orai1 during meiosis provide insights into the mechanisms involved in this critical development process. Orai1 is enriched at the cell membrane in oocytes and internalizes during meiosis into a late endosomal compartment that is Cav, Rab5, Rab7, and Rab9 positive. This targeted endocytosis process requires dynamin, Cav, and Rab5 and does not follow the clathrin-dependent endocytic pathway. Cav may function as a scaffolding platform to recruit the necessary molecular machinery required for Orai1 internalization, as there is evidence of a direct interaction between Cav and Rab5 (Hagiwara et al., 2009). Surprisingly, however, Orai1 internalization is independent of cholesterol, arguing that it is not through the classical lipid raft endocytic pathway. This is in contrast to the requirement for cholesterol for the internalization of the plasma membrane Ca$^{2+}$-ATPase during meiosis (El Jouni et al., 2008). This suggests that internalization of membrane proteins during meiosis does not follow the same endocytic pathway.

**Orai1 domains involved in Orai1 internalization**

We further mapped the domains within Orai1 that are required for its internalization and show that a consensus Cav-binding site in the Orai1 N terminus is essential for Orai1 endocytosis during meiosis (Fig. 8). In addition, we reveal a role for residues 267–289 in the Orai1 cytoplasmic C terminus to target Orai1 for internalization. However, this region is not essential for Orai1 internalization because Orai1–ΔC267 is internalized when coexpressed with Cav. The Orai1 C terminus is predicted to form a coiled coil required for interaction with STIM1 (Muik et al., 2008). We were unable to test whether the coiled coil by itself is required for endocytic targeting because a mutant predicted to disrupt the coiled-coil Orai1-L273S (Muik et al., 2008) did not effectively traffic to the cell membrane (Fig. 7 B). In contrast, others have shown that in mammalian cells, the L273 mutant traffics to the plasma membrane (Muik et al., 2008; Yuan et al., 2009), and in one case, the Orai1–ΔC deletion was reported to be trapped in the ER (Yuan et al., 2009). Furthermore, deletion of the N-terminal domain (Orai1–ΔN90) also disrupts Orai1 biogenesis in oocytes, although in this case, some membrane targeting is detectable (Fig. 7 B). In contrast, an Orai1 N-terminal deletion mutant has been reported to traffic normally to the plasma membrane; however, in this case, a YFP or CFP tag was placed on the N terminus (Muik et al., 2008; Park et al., 2009), whereas in our case, the CFP tag is placed on the C terminus of Orai1. This argues that the N-terminal tag may help Orai1 folding and, as such, enhance its trafficking to the cell membrane. Another study reported normal trafficking of N-terminally deleted Orai1 with a C-terminal tag (Li et al., 2007). These discrepancies in the trafficking of various Orai1 mutants may be because of different expression levels in the different studies that would drive enrichment at the cell membrane because the subcellular distribution of Orai1 is a homeostatic process that depends on the capacity of various trafficking compartments along the biogenesis and recycling pathways. Alternatively, these differences may argue for differential regulation of Orai1 trafficking in different cell types caused either by predominant active trafficking pathway and developmental stage of the cell or by accessory proteins that interact with Orai1. Indeed, the trafficking of Orai1 is quite distinct in the same cell, the *Xenopus* oocyte dependent on its developmental stage (oocyte or egg), as illustrated in this study.

**Orai1 constitutive recycling**

Orai1 is enriched at the cell membrane in oocytes. We estimate that ~80% of the total Orai1 protein pool localizes to the cell membrane in *Xenopus* oocytes, this under conditions of expression of exogenous Orai1, arguing that the Orai1 trafficking machinery has a large capacity. We can also detect Orai1 recycling in CHO cells that express a YFP-tagged Orai1 (unpublished data), arguing that Orai1 recycling is conserved among different species and cell types. The constitutive recycling of Orai1 maintains a significant percentage of the total Orai1 protein pool in the recycling endosome. Importantly, store depletion results in the translocation of endosomal Orai1 to the cell membrane, where it is stabilized in STIM1–Orai1 puncta. A recent study reported an increase in surface Orai1 after store depletion (Woodard et al., 2008). However, in this case, the Orai1 increase was dependent on a rise in cytoplasmic Ca$^{2+}$ levels, arguing that it is mechanistically different from the Orai1 translocation to the cell membrane we describe, because in our experiments, Ca$^{2+}$ stores were depleted with TPEN, which does not involve a rise in cytoplasmic Ca$^{2+}$ levels. Nonetheless, endosomal Orai1 represents a readily mobilizable pool of channels that could contribute to the SOCE amplitude and development kinetics. The extent of such a modulation would depend on the amount of Orai1 in the endosomal compartment relative to total cellular Orai1, on the rate of exocytosis of Orai1 from the recycling endosome, and on the levels of STIM1 relative to Orai1. Together, our experiments argue that Orai1 trafficking is an important homeostatic mechanism that regulates Orai1 levels at the cell membrane dynamically, dependent on physiological needs during cellular development.

**Materials and methods**

**Oocyte isolation and cell culture**

*Xenopus laevis* oocytes were prepared and handled as previously described (Machaca and Haun, 2000). TRVb-1 cells, a CHO cell line lacking the endogenous transferrin receptor and stably expressing the human transferrin receptor (McGraw et al., 1987), were grown in bicarbonate-buffered Ham’s F-12 medium (Invitrogen) containing 5% FBS, 100 U/ml penicillin/streptomycin, and 200 μg/ml genetin. Cells were grown at 37°C in a humidified atmosphere of 5% CO$_2$. TRVb-1 cells were transfected with HA-extraspDS-Orai1-YFP plasmid DNA using Lipofectamine 2000 (Invitrogen) and plated onto coverslip-bottom dishes. Cells were used for experiments 48 h after transfection.

**Materials**

MjCD, cholesterol, and TPEN (N,N,N,N-Tetakis[2-pyridylmethyl]ethylenediamine) were obtained from Sigma-Aldrich. C3 exoenzyme was obtained from EMD. LysoTracker red DND99, Alexa Fluor 555–labeled CTB, and Alexa Fluor 633–labeled transferrin were obtained from Invitrogen.

**Molecular biology**

*Xenopus* expression plasmids pSGEM-GFP-Orai1, pSGEMmCherry-STIM1, and pSGEMmCherry-xCav1 were described previously (Yu et al., 2009).
Mammalian expression HA-extra pDS-Orai1-YFP was performed by R. Lewis (Stanford University, Palo Alto, CA). xTMM16α (GenBank/EMBL/DDB) accession no. NM_001135237 was synthesized by imoGenes. xTMM16α was C-terminally tagged with mCherry using PCR with primers 5'-CTCTCG-CAAGCTTACATTAGTATGGGCCAACGTCGAGC-3' and 5'-GAATGG- GTACCGCGCTAGCTACCCCACTCCGGGTGGAGTACG-3' and then inserted into XbaI-EcoRV pSGEM. To construct pSGEM-mCherry-Orai1, the NotI-BamHI PCR fragment was subcloned into NotI-BamHI site of pSGEM with the primer pairs 5'-AGCGGCGCGCTAGTGGAAGGGCGA-3' and 5'-GCTGACATTTAATCCCTTCTTTATGATCTGATC-3'. To make pSGEM-mCherry-Rab4b, mCherry were amplified using primers 5'-AGCGGCGCGCTAGTGGAAGGGCGA-3' and 5'-GCTGACATTTAATCCCTTCTTTATGATC-3'. Rab1b (5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGG- GCCGCACTGGCACTCGTCTGCTCTC-3') and Rab1b (5'-CTGATGCTCGAGT- TCTTGTACAGCTGGTCTAGAACGATACCATAGC-3' and 5'-GCTGACATTTAATCCCTTCTTTATGATC-3') were subcloned into XbaI-BamHI of pSGEM. Plasmids containing human Rab9b, Rab1b (Addgene), and human Cav1 (OR2) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab9b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Orai1-CFP, pSGEM-Orai1-C289, pSGEM-Orai1-C267, and pSGEM-Orai1-C264, PCR fragments using the following primers were subcloned into NotI-BamHI of pSGEM: 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. To construct pSGEM-P168L, pSGEM-Orai1-CFP was amplified using primers 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-GFP-N-termOrai1(Y52,W55A), the GFP–N terminus of human Orai1 was subcloned into NotI–Klenow–EcoRI of pSGEM. To construct pSGEM-GFP-N-termOrai1(Y52,W55A), the GFP–N terminus of human Orai1 was subcloned into NotI–Klenow–EcoRI of pSGEM. To construct pSGEM-GFP-N-termOrai1(Y52,W55A), the GFP–N terminus of human Orai1 was subcloned into NotI–Klenow–EcoRI of pSGEM. To construct pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. Rab4b and Rab4b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'. For the construction of pSGEM-Rab4b, Rab4b (Addgene) and Rab1b (Addgene) were subcloned into BamHI-XhoI of pSGEMmCherry by PCR using the following primers: Rab4b, 5'-CAAGACTCAACCTGGAAATATACATC-3' and 5'-GTTAACAGGGCGGCACTGGCACTCGTCTGCTCTC-3'.
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