RASSF4 controls SOCE and ER–PM junctions through regulation of PI(4,5)P2

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RASSF4 is involved in tumorigenesis and regulation of the Hippo pathway. In this study, we identify new functional roles of RASSF4. First, we discovered that RASSF4 regulates store-operated Ca2+ entry (SOCE), a fundamental Ca2+ signaling mechanism, by affecting the translocation of the endoplasmic reticulum (ER) Ca2+ sensor stromal interaction molecule 1 (STIM1) to ER–plasma membrane (PM) junctions. It was further revealed that RASSF4 regulates the formation of ER–PM junctions and the ER–PM tethering function of extended synaptotagmins E-Syt2 and E-Syt3. Moreover, steady-state PM phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] levels, important for localization of STIM1 and E-Syts at ER–PM junctions, were reduced in RASSF4-knockdown cells. Furthermore, we demonstrated that RASSF4 interacts with and regulates the activity of adenosine diphosphate ribosylation factor 6 (ARF6), a small G protein and upstream regulator of type I phosphatidylinositol phosphate kinases (PIP5Ks) and PM PI(4,5)P2 levels. Overall, our study suggests that RASSF4 controls SOCE and ER–PM junctions through ARF6-dependent regulation of PM PI(4,5)P2 levels, pivotal for a variety of physiological processes.

Introduction

Phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2) is an inositol-containing phospholipid enriched at the inner leaflet of the plasma membrane (PM; Balla, 2013). PI(4,5)P2 governs many cellular processes including membrane traffic, ion transport activities, and cytoskeleton–PM interactions. Aberrant metabolism of PI(4,5)P2 has been associated with cancer, ocularcerebrorenal syndrome of Lowe, and infectious diseases (Bunney and Katan, 2010; Balla, 2013; Staiano et al., 2015). Most PI(4,5)P2 is generated by type I phosphatidylinositol phosphate kinases (PIP5Ks), which phosphorylate the 5-position of the inositol ring of phosphatidylinositol 4-phosphate (PI[4]P; Roth, 2004). The activity and localization of PIP5Ks are regulated by multiple factors including ADP ribosylation factor 6 (ARF6), a small G protein that dynamically cycles between the inactive GDP-bound (ARF6-GDP) and the active GTP-bound (ARF6-GTP) states (van den Bout and Divecha, 2009). ARF6-GTP directly activates PIP5Ks to control the generation of PI(4,5)P2 (Honda et al., 1999; Brown et al., 2001).

In addition to its many functions in resting cells, PM PI(4,5)P2 is essential for Ca2+ signaling in receptor-stimulated cells (Chang and Liou, 2016). Receptor-activated PLC hydrolyzes PM PI(4,5)P2 to generate soluble inositol trisphosphate (IP3), triggering Ca2+ release from the ER. Depletion of ER Ca2+ activates Ca2+ influx across the PM, a process known as store-operated Ca2+ entry (SOCE), essential for a wide array of cellular functions (Prakriya and Lewis, 2015). Defective SOCE is associated with many human diseases, including severe combined immunodeficiency, cardiac hypertrophy, and cancer (Bergmeier et al., 2013).

The mediators of SOCE, stromal interaction molecule 1 (STIM1) and Orai1, were identified using RNA interference screens (Liou et al., 2005; Roos et al., 2005; Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006). STIM1 is a single-pass ER membrane protein that binds Ca2+ at steady state. ER Ca2+ depletion triggers conformational change of STIM1 to expose the Orai1-activating domain and the PM-targeting polybasic motif in its C-terminal cytosolic region (Liou et al., 2007; Walsh et al., 2010; Zhou et al., 2013; Maus et al., 2015). The exposed polybasic tail of STIM1 binds PI(4,5)P2 at the PM. As a result, STIM1 accumulates at ER–PM junctions, where the ER closely opposes the PM within a 30-nm gap distance (Wu et al., 2006; Liou et al., 2007; Henne et al., 2015). Localization at ER–PM junctions enables STIM1 interaction with the PM Ca2+ channel Orai1 and activation of SOCE (Luik et al., 2006).

Besides SOCE, PM PI(4,5)P2 is important for ER–PM junction formation. The best-characterized proteins mediating...
the formation of ER–PM junctions in mammalian cells are extended synaptotagmins E-Syt2 and E-Syt3 (Giordano et al., 2013). E-Syt2 and E-Syt3 are ER membrane proteins containing multiple C2 domains. Interaction of the very C-terminal C2 domain of E-Syt2 and E-Syt3 with PM PI(4,5)P₂ mediates ER tethering to the PM at ER–PM junctions.

Here, we identified RAS association domain family 4 (RASSF4) from a human siRNA screen for regulators of SOCE. Subsequently, we showed that RASSF4 functions as a novel regulator of PM PI(4,5)P₂, affecting not only SOCE, but also ER–PM junctions. RASSF4 belongs to the RASSF family of proteins containing a RAS association (RA) domain and a Salvador-RASSF-Hippo (SARAH) domain in the C terminus (Chan et al., 2013). The RA domain of RASSF4 is predicted to interact with small G proteins (Sherwood et al., 2010). The SARAH domain can mediate dimerization and protein–protein interactions (Scheel and Hofmann, 2003; Dittfeld et al., 2012; Makbul et al., 2013). RASSF4 associates with MST1, the mammalian orthologue of the Drosophila Hippo protein, and inhibits the Hippo pathway (Crose et al., 2014). Altered expression of RASSF4 is frequently associated with the progression of many cancer types, including nasopharyngeal, lung, head, and neck cancers, as well as alveolar rhabdomyosarcoma (Chow et al., 2004; Steinmann et al., 2009; Crose et al., 2014; Han et al., 2016).

By dissecting the mechanism underlying RASSF4-mediated control of PM PI(4,5)P₂ levels, we found that RASSF4 interacts with and regulates the activation of ARF6, an upstream regulator of PIP5Ks and PM PI(4,5)P₂. Overall, our study reveals novel functional roles of RASSF4 and provides new insights into the regulation of PI(4,5)P₂, Ca²⁺ signaling, and ER–PM junctions.

Results

Identification of RASSF4 as a positive regulator of SOCE

The key regulators of SOCE, STIM1 and STIM2, were identified from a screen for siRNAs inhibiting sustained Ca²⁺ signaling in HeLa cells stimulated with histamine and thapsigargin (TG) to deplete ER Ca²⁺ and activate SOCE (Liou et al., 2005). Another hit from this screen was RASSF4 (Fig. 1 A). Similar to siRNA targeting STIM1 (siSTIM1), siRASSF4 suppressed the sustained phase but not the initial peak of the Ca²⁺ response in stimulated HeLa cells (Fig. 1 B). To further characterize and validate the effect of RASSF4 on Ca²⁺ responses, two additional diced siRNA pools targeting the coding sequence of the N-terminal region (siRASSF4_N') and the C-terminal region (siRASSF4_C') of human RASSF4 protein (Fig. 1 A) were generated. We implemented a Ca²⁺ add-back experiment that enables separate monitoring of Ca²⁺ release from intracellular stores and the subsequent Ca²⁺ flux across the PM in HeLa cells treated with either siRASSF4_N' or siRASSF4_C'. Reduced Ca²⁺ flux across the PM was observed in cells treated with either siRASSF4_N' or siRASSF4_C', with no apparent effect on release of stored Ca²⁺ (Fig. 1 C). Moreover, expression of a RAS SF4 construct fused with YFP rescued the suppressed Ca²⁺ flux across the PM in cells treated with a diced siRNA pool targeting the 3' untranslated region of RASSF4 (Fig. S1 A). These data, derived using four different siRNAs targeting RASSF4, support the specificity of RASSF4 knockdown and a selective regulation of Ca²⁺ flux after store depletion by RASSF4.

We further tested the effect of RASSF4 knockdown on SOCE in primary human umbilical vein endothelial cells (HUVECs) and noncancerous retinal pigment epithelial 1 (RPE1) cells. Results similar to those in HeLa cells were obtained (Fig. 1, D and E; and Fig. S1, B and C), indicating that the effect of RASSF4 on SOCE is not confined to HeLa cells. The effect of RASSF4 on SOCE was supported by measuring the quenching of intracellular Fura-2 fluorescence by influx of Mn²⁺, a surrogate for Ca²⁺. Consistent with a role of RASSF4 in regulating Ca²⁺ influx, siRASSF4-treated cells exhibited suppressed Mn²⁺ quench rate triggered by TG (Fig. 1 F). Furthermore, we tested the effect of RASSF4 knockdown on the activation of nuclear factor of activated T cells (NFAT), a transcription factor activated by SOCE and essential for many physiological processes (Hogan et al., 2003), in TG-treated cells. The percentage of cells with nuclear translocation of NFAT, indicative of NFAT activation, was significantly reduced in siRASSF4-treated cells (Fig. 1 G). Together, these results demonstrate the importance of RASSF4 in the activation of SOCE and its downstream signaling events.

SOCE is activated by interaction of STIM1 and Orai1 at ER–PM junctions. Therefore, the effect of RASSF4 knockdown on SOCE could be caused by dysregulation of STIM1, Orai1, and/or ER–PM junctions. However, we found that the expression levels of STIM1 and Orai1 proteins were not affected in siRASSF4-treated cells (Fig. S1 D). In addition, knockdown of RASSF4 did not affect the expression of several other genes implicated in regulating SOCE (Fig. S1 E). Thus, we examined the function of Orai1 in siRASSF4-treated cells using a soluble fragment of the STIM1 C-terminus (STIM1-CT) that can activate Orai1 and mediate constitutive Ca²⁺ influx without localizing to ER–PM junctions (Sharma et al., 2013). Knockdown of RASSF4 did not affect Orai1 activation by STIM1-CT (Fig. 1 H). In contrast, knockdown of RASSF4 resulted in a reduction of SOCE in STIM1-overexpressing cells (Fig. 1 I), indicating that RASSF4 regulates STIM1 activation during SOCE. Therefore, the reduced SOCE observed in siRASSF4-treated cells is likely caused by impaired STIM1 activation and/or defects in ER–PM junctions.

RASSF4 regulates STIM1 localization at ER–PM junctions

To determine the effect of RASSF4 on STIM1 activation, confocal microscopy was applied to monitor the dynamic localization of STIM1 after ER Ca²⁺ depletion. TG-induced STIM1 accumulation at ER–PM junctions, which appears as puncta formation, was significantly suppressed in siRASSF4-treated cells (Fig. 2 A and Video 1). These observations were confirmed using total internal reflection fluorescence (TIRF) microscopy (TIRFM; Fig. 2 B), which allows for selective illumination of fluorescent probes within ~100 nm of the PM (Steyer and Almers, 2001). These results indicate a defect in STIM1 translocation to ER–PM junctions in RASSF4-knockdown cells. Conversely, RASSF4 overexpression enhanced STIM1 accumulation at ER–PM junctions induced by TG (Fig. 2 C). Together, these results demonstrate that RASSF4 regulates the activation of STIM1 during SOCE.

To further dissect the mechanism underlying RASSF4 regulation of STIM1 activation, we used a constitutively active STIM1-D76A construct that contains a point mutation disrupting the key Ca²⁺-binding residue in its luminal EF hand, adopts the activated conformation, and readily localizes at ER–PM junctions at steady state (Liou et al., 2005). The activated
Ca\(^{2+}\) influx caused by expressing STIM1-D76A was reduced in siRASSF4-treated cells (Fig. 3 A). Conversely, overexpression of RASSF4 enhanced Ca\(^{2+}\) influx induced by the expression of STIM1-D76A (Fig. 3 B). Consistent with these Ca\(^{2+}\) data, steady-state localization of STIM1-D76A at ER–PM junctions, measured by the density of STIM1-D76A puncta detected using TIRFM, was significantly decreased in siRASSF4-treated cells (Fig. 3 C). These results indicate that, even in the activated conformation, STIM1 fails to accumulate at ER–PM junctions to mediate Ca\(^{2+}\) influx. Moreover, overexpression of RASSF4 increased the density of steady-state STIM1-D76A puncta (Fig. 3 D), demonstrating that RASSF4 has a dominant effect over STIM1 and suggesting that RASSF4 regulates STIM1 localization at ER–PM junctions rather than its conformational change. Furthermore, time-lapse TIRFM imaging of stationary HeLa cells revealed that STIM1-D76A puncta were less stable in siRASSF4-treated cells than in control cells (Fig. 3 E). These results raise the possibility that both STIM1 activation and ER–PM junctions are defective in RASSF4-knockdown cells.

**RASSF4 regulates the formation and stability of ER–PM junctions**

Previous studies indicate that ER–PM junctions exist before the activation of SOCE (Wu et al., 2006), and STIM1 reversibly localizes to these loci depending on ER Ca\(^{2+}\) levels (Liou et al., 2005). We used electron microscopy (EM) to examine the effect of RASSF4 on the formation of ER–PM junctions in cells at steady state. ER–PM junctions were identified as tubules with...
ER-targeted HRP (HRP-ER) located within 50 nm of the PM (Wu et al., 2006). The percentage of PM length engaged in contact with the ER was calculated. Approximately 3.3% of the PM was occupied by the ER in control cells, compared with ∼1.6% in RASSF4-knockdown cells (Fig. 4 A). In contrast, overexpression of RASSF4 increased the PM occupancy of the ER to ∼4.8% (Fig. 4 B). These EM results reveal that RASSF4 regulates the formation of ER–PM junctions in cells at steady state. We validated the EM results using ER labeling in live cells imaged by time-lapse TIRFM to measure ER–PM junctions (Chang et al., 2013). The density of ER–PM junctions in siRASSF4-treated cells was reduced to ∼50% of that in control cells (Fig. 4 C). Conversely, overexpression of RASSF4 significantly increased the density of ER–PM junctions (Fig. 4 D). Together, these results show that RASSF4 regulates the formation of ER–PM junctions in cells at steady state.

Our group has previously developed a genetically encoded marker named MAPPER (membrane-attached peripheral ER) that selectively labels ER–PM junctions (Chang et al., 2013). Using MAPPER, similar reduction in the density of ER–PM junctions was observed in RASSF4-knockdown cells (Fig. 4 S2). Unlike the EM or the ER-labeling approach, MAPPER enables tracking of individual ER–PM junctions in real time and, thus, can be used to further determine the stability of single ER–PM junctions. By monitoring MAPPER-labeled ER–PM junctions in stationary HeLa cells, we observed that the percentage of stable ER–PM junctions was reduced in siRASSF4-treated cells (Fig. 4 E). Overall, the
results derived using three independent yet complimentary assays for ER–PM junctions demonstrate that RASSF4 regulates the formation as well as the stability of ER–PM junctions in cells at steady state.

The ER membrane proteins E-Syt2 and E-Syt3 contribute to the formation of steady-state ER–PM junctions by binding to PM PI(4,5)P2 (Giordano et al., 2013). We tested the dependence of RASSF4 on the ER–PM tethering function of E-Syt2 and E-Syt3 by examining GFP–E-Syt2 and GFP–E-Syt3 puncta using TIRFM. Knockdown of RASSF4 reduced the density of GFP–E-Syt2 and GFP–E-Syt3 puncta (Fig. 5, A and B), indicating a dominant effect of RASSF4 over E-Syt2 and E-Syt3 on the formation of ER–PM junctions. Furthermore, overexpression of RASSF4 had an additive effect on the formation of ER–PM junctions in cells overexpressing E-Syt2 or E-Syt3 (Fig. 5, C and D). Therefore, RASSF4 not only regulates SOCE mediated by STIM1 at ER–PM junctions in ER Ca2+-depleted cells, but also regulates the formation of ER–PM junctions mediated by E-Syt2 and E-Syt3 in cells at steady state.

**RASSF4 regulates PM PI(4,5)P2 levels in cells at steady state and after receptor stimulation**

Given the critical role of PM PI(4,5)P2 in governing the localization of STIM1, E-Syt2, and E-Syt3 at ER–PM junctions, we tested the effect of RASSF4 on PM PI(4,5)P2. First, we used a biosensor, PLCδ-PH (Stauffer et al., 1998), to detect steady-state PI(4,5)P2 levels at the PM using confocal microscopy. A significant reduction in the relative intensity of PLCδ-PH at the PM, indicative of reduced PI(4,5)P2, was observed in siRASSF4-treated cells (Fig. 6 A). Using another PI(4,5)P2 biosensor, Tubby-GFP (Quinn et al., 2008), similar results were obtained (Fig. S3 A). In addition, siRASSF4-treated cells exhibited a decrease in total PI(4,5)P2 levels directly measured...
using myo-[3H]inositol to label phosphoinositides (Fig. 6 B). Moreover, direct staining of PI(4,5)P$_2$ at the PM with an antibody, using a protocol established previously (Hammond et al., 2009), confirmed the reduction of PM PI(4,5)P$_2$ in RAS SF4-knockdown cells (Fig. 6 C). In contrast, overexpression of RAS SF4 resulted in an increase in steady-state PI(4,5)P$_2$ levels at the PM (Fig. 6 D). These results demonstrate that RASSF4 regulates PM PI(4,5)P$_2$ levels in resting cells.

In receptor-stimulated cells, PM PI(4,5)P$_2$ is hydrolyzed by PLC to generate IP$_3$ and diacylglycerol for activation of downstream signaling events. The levels of PM PI(4,5)P$_2$ are rapidly restored via a feedback pathway coupling PI(4,5)P$_2$ hydrolysis to its replenishment (Chang and Liou, 2016). The replenishment of PM PI(4,5)P$_2$ after receptor-stimulated hydrolysis was suppressed in RASSF4-knockdown cells (Fig. 6 E). Conversely, RASSF4 overexpression augmented the replenishment of PM PI(4,5)P$_2$ (Fig. 6 F). These results demonstrate that RASSF4 is not only important for maintaining PM PI(4,5)P$_2$ levels in cells at steady state, but also essential for replenishing PM PI(4,5)P$_2$ after its hydrolysis in receptor-stimulated cells.

**RASSF4 regulates the levels of PI(4)P and PIP5Ks at the PM**

Reduction in PM PI(4,5)P$_2$ in siRASSF4-treated cells might arise from insufficient precursors. The levels of PI(4)P, an immediate precursor of PI(4,5)P$_2$, at the PM were examined using a biosensor, N-PH-ORP5 (Chung et al., 2015), in cells at steady state. In contrast to the reduced PM PI(4,5)P$_2$ levels, an increase

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**Figure 4.** RASSF4 regulates the formation and stability of ER–PM junctions. (A and B, left) EM micrographs of HRP-KDEL–expressing HeLa cells treated with the indicated siRNA or plasmid for 2 d. Red arrows indicate ER–PM junctions. Bar, 2 µm. (Right) The percentage of PM length engaged in contact with the ER is shown. Means ± SEM of six to eight cells are plotted. N indicates the cell nucleus. (C and D) Densities of ER–PM junctions determined using a TIRF image series of YFP-KDEL–expressing HeLa cells that were treated with siRASSF4 or siControl (C) or transfected with RASSF4 or a control vector (D). More than 24 cells for each condition across three independent experiments were analyzed. Means ± SEM are plotted. (E) TIRF images of ER–PM junctions labeled by MAPPER in cells treated with siControl or siRASSF4. Red arrows indicate unstable junctions. Bar, 1 µm. The percentage of stable ER–PM junctions labeled by MAPPER was quantified. More than 500 puncta in six different cells for each condition were evaluated. Means ± SEM are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
RASSF4 regulates the localization of E-Syt2 and E-Syt3 at ER–PM junctions. (A) Localization of GFP–E-Syt2 monitored by TIRFM in siControl- or siRASSF4-treated HeLa cells. The density of GFP–E-Syt2 puncta in 20 cells across two independent experiments was quantified. Means ± SEM are shown, *P < 0.01. Bars, 2 µm.

(B) Localization of GFP–E-Syt3 monitored by TIRFM in siControl- or siRASSF4-treated HeLa cells. GFP–E-Syt3 puncta density in 20 cells from two independent experiments was quantified. Means ± SEM are shown, *P < 0.01. Bars, 2 µm.

(C) Localization of GFP–E-Syt2 monitored by TIRFM in HeLa cells cotransfected with an empty vector or a vector for expression of RAS SF4. GFP–E-Syt2 puncta density in 20 cells across two independent experiments was quantified. Means ± SEM are shown, **P < 0.01. Bars, 2 µm.

(D) Localization of GFP–E-Syt3 monitored by TIRFM in siControl- or siRASSF4-treated HeLa cells. The density of GFP–E-Syt3 puncta in 20 cells from two independent experiments was quantified. Means ± SEM are shown, **P < 0.01. Bars, 2 µm.

in PM PI(4)P was detected in siRASSF4-treated cells compared with control cells (Fig. 7 A). Similar results were obtained using PH-Osh2×2 (Roy and Levine, 2004; Balla et al., 2008), another biosensor for PI(4)P (Fig. S3 B). Increase in PM PI(4)P levels in siRASSF4-treated cells was further confirmed by immunostaining using an anti-PI(4)P antibody (Fig. 7 B). The increased PI(4)P at the PM suggests that PI(4,5)P2 generation from PI(4)P by PIP5Ks is suppressed in RASSF4-knockdown cells.

PIP5K1B and PIP5K1C are two isoforms of PIP5Ks localized at the PM and linked to the regulation of PI(4,5)P2 levels and Ca2+ signaling in HeLa cells (Padrón et al., 2003; Wang et al., 2004). Using confocal microscopy, it was revealed that PM localization of both PIP5K1B and PIP5K1C was suppressed in siRASSF4-treated cells (Fig. 7 C and Fig. S3 C). These results suggest that altered localization of PIP5Ks might be the cause of defective PM PI(4,5)P2 levels, ER–PM junctions, and SOCE in RASSF4-knockdown cells. To test this hypothesis, we applied a previously established rapamycin-inducible approach (Suh et al., 2006) in which a FK506-binding protein (FKBP)–fused PIP5K1C (FKBP-PIP5K) is acutely recruited to the PM-localized FKBP-rapamycin binding (FRB) protein (Lyn-FRB) via heterodimerization to increase PM PI(4,5)P2 levels (Fig. 7 D). A kinase-dead mutant, FKBP-PIP5Ki, was used as a negative control. Addition of rapamycin induced a rapid translocation of FKBP-PIP5K or the control FKBP-PIP5Ki to the PM in cells cotransfected with Lyn-FRB (Fig. S4 A). Consistent with previous findings (Suh et al., 2006), acute recruitment of PIP5K to the PM increased PM PI(4,5)P2 levels in siControl-treated cells (Fig. 7 E, black bar vs. red bar). Notably, the acute recruitment of PIP5K to the PM was sufficient to overcome the deficiency of RASSF4 and restored PM PI(4,5)P2 levels in siRASSF4-treated cells (Fig. 7 E, gray bar vs. green bar). In addition, reduction in ER–PM junctions, measured using either ER labeling or MAPPER, was restored in siRASSF4-treated cells by induced targeting of PIP5K to the PM (Fig. 7 F and Fig. S4 B). Moreover, the impaired TG-triggered STIM1 translocation to ER–PM junctions, as determined by STIM1 puncta density, and SOCE were restored in siRASSF4-treated cells after induced targeting of PIP5K (Fig. S4 C and Fig. 7 G). Thus, the various defects in RASSF4-knockdown cells can be rescued by targeting PIP5K to the PM to increase PM PI(4,5)P2. Together, these results suggest that RASSF4 regulates PIP5Ks to control PI(4,5)P2 levels at the PM.

RASSF4 interacts with ARF6 and regulates its localization and activation

The small G protein ARF6 can directly activate PIP5Ks and regulate PM localization of PIP5Ks as well as PI(4,5)P2 production (Honda et al., 1999). ARF6 is partially localized at the PM (Donaldson and Honda, 2005). In siRASSF4-treated cells, a dramatic accumulation of ARF6 in large internal structures with reduced localization at the PM was observed (Fig. 8 A). This altered distribution of ARF6 is reminiscent of the internal structures formed by prolonged expression of ARF6-T27N, an ARF6 mutant that remains in the inactive GDP-bound state (Peters et al., 1995). This observation suggests a defect of ARF6 activation in siRASSF4-treated cells. Consistent with this notion, a reduction of ARF6 in the active ARF6-GTP state was observed in siRASSF4-treated cells compared with control cells (Fig. 8 B), indicating that RASSF4 regulates the localization and activation of ARF6.

To adddress the mechanism by which RASSF4 regulates ARF6 activation, we examined the subcellular localization of RASSF4-YFP using confocal microscopy and found that RASSF4 is diffusely distributed in the cytoplasm of HeLa cells (Fig. S5). As RASSF4 was predicted to bind small G proteins (Sherwood et al., 2010), it is plausible that RASSF4 binds ARF6 and regulates its activation. Indeed, RASSF4 was detected in the immunoprecipitation of ARF6 (Fig. 8 C). Reciprocally, ARF6 was detected in the immunoprecipitation of RASSF4 (Fig. 8 D), indicating that RASSF4 interacts with ARF6.

ARF6 dynamically switches between GTP-bound and GDP-bound states to perform its functions (Honda et al., 1999; Wong and Isberg, 2003). Several ARF6 mutants mimicking...
different nucleotide-binding states, such as ARF6-T27N (GDP bound), ARF6-Q67L (GTP bound), and ARF6-N122I (nucleotide free), were used to determine the state in which ARF6 interacts with RAS SF4. A preferential association of RAS SF4 with ARF6-T27N and ARF6-N122I, but not ARF6-Q67L, was detected (Fig. 8 D). These results support the specificity of RAS SF4–ARF6 interaction and suggest that RAS SF4 regulates ARF6 activation by interacting with ARF6 in its GDP-bound and nucleotide-free states.

Our data suggest that ARF6, regulated by RAS SF4, is involved in the regulation of SOCE. Consistent with this hypothesis, expression of the ARF6-N122I mutant, which has a dominant-negative effect on PM targeting of PIP5K1B (Honda et al., 1999), reduced SOCE in a fashion similar to siRAS SF4 treatment (Fig. 8 E). Based on our findings, we propose that RAS SF4 acts upstream of ARF6 to regulate the localization and activation of PIP5Ks and PI(4,5)P2 production (Fig. 8 F). By affecting the PM PI(4,5)P2 level, RASSF4 controls SOCE, ER–PM junctions, and other PI(4,5)P2-dependent biological processes.

Discussion

In this study, we report that RASSF4 is a novel regulator of SOCE and ER–PM junctions. The expression level of RASSF4 controls the number and stability of ER–PM junctions in resting cells and the extent of SOCE in receptor-stimulated cells. By affecting steady-state PM PI(4,5)P2 levels, RASSF4 regulates targeting of ER membrane proteins E-Syt2, E-Syt3, and STIM1 to ER–PM junctions. RASSF4 interacts with and regulates the localization and activation of ARF6, which can directly activate PIP5Ks and control the levels of PI(4,5)P2. This study not only reveals new functions of RASSF4, but also provides insights into molecular mechanisms underlying these functions.

RASSF4 is 1 of 10 RAS SF proteins implicated in tumorigenesis; yet, a role of RAS SF proteins in SOCE had not been demonstrated previously. Diced pools of siRNAs designed to target each of the 10 RAS SF proteins were included in the siRNA screen for SOCE regulators in HeLa cells (Liou et al., 2005). Only the siRNA pool targeting RASSF4_N' had a significant effect on sustained Ca2+ responses. Among the 10 family members, RASSF1–RASSF6 contain the homologous RA and SARAH domains at the C terminus with divergent N terminal regions (Chan et al., 2013). Notably, in cells treated with siRASSF4_N', targeting the coding sequence of the distinct N-terminal region of RASSF4 protein, SOCE was suppressed to the same extent as that in cells treated with siRASSF4_C' (Fig. 1 C). Furthermore, similar suppression of SOCE was observed in siRASSF4-treated noncancerous HUVECs and RPE-1 cells (Fig. 1, D and E). These findings indicate that among RASSF proteins, RASSF4 is the major regulator of SOCE in these cells.

SOCE is mediated by STIM1 interaction with Orai1 at ER–PM junctions. Several additional SOCE regulators have been identified using siRNA screens or biochemical approaches since the discovery of STIM1 and Orai1. Many of these regulators, including CRACR2A, SARAF, Junctate, α-SNAP,
STIM1, TEMEM110, and Junctophilin-4, modulate SOCE by binding to STIM1 and/or Orai1 (Srikanth et al., 2010, 2012; Palty et al., 2012; Miao et al., 2013; Jing et al., 2015; Quintana et al., 2015; Woo et al., 2016). Instead, RASSF4 regulates SOCE by affecting steady-state PM PI(4,5)P2 levels. Our finding supports an important role of PM PI(4,5)P2 in STIM1 targeting to ER–PM junctions and is consistent with the model in which STIM1 translocates to ER–PM junctions by binding to PM PI(4,5)P2, and subsequently recruits Orai1 to ER–PM junctions after ER Ca2+ depletion (Liou et al., 2007; Prakriya and Lewis, 2015). In addition, we demonstrate that PM PI(4,5)P2 is crucial for the formation and stability of steady-state ER–PM junctions using several assays for quantitation and tracking of ER–PM junctions. It is possible that some ER–PM junctions do not survive long enough to allow productive STIM1 interaction with Orai1 for SOCE activation in RASSF4-knockdown cells. Therefore, defective STIM1 targeting to ER–PM junctions in RASSF4-knockdown cells might be caused by insufficient PM PI(4,5)P2 and unstable ER–PM junctions.

PM PI(4,5)P2 is required for the ER–PM tethering function of E-Syts, and triple knockdown of ESYT1, ESYT2, and ESYT3 results in a reduction in ER–PM junctions without disrupting the activation of SOCE in HeLa cells (Giordano et al., 2013; Idevall-Hagren et al., 2015). In contrast to E-Syts, regulatory of SOCE and ER–PM junctions by RASSF4 • Chen et al. 2019
ple-knockdown cells, both SOCE and ER–PM junctions were disrupted in RAS SF4-knockdown cells. Our data reveal that, by controlling steady-state PM PI(4,5)P2 levels, RAS SF4 has a dominant effect over the activation of STIM1 to regulate SOCE and the function of E-Syts to form ER–PM junctions. As PM PI(4,5)P2 controls the localization and functions of numerous proteins in addition to STIM1 and E-Syts (Balla, 2013), it is expected that RAS SF4 impacts a broad array of physiological processes, such as cell motility, vesicular trafficking, and cytoskeleton reorganization.

Aside from Ca2+ signaling, ER–PM junctions are important for nonvesicular lipid transport between the ER and the PM (Henne et al., 2015). The formation and stability of ER–PM junctions may affect the functions of lipid transfer proteins such as Nir2 and Nir3 that exchange phosphatidylinositol and phosphatidic acid at ER–PM junctions to support receptor-induced signaling and maintain PM PI(4,5)P2 levels (Chang et al., 2013; Kim et al., 2013, 2015; Chang and Liou, 2015; Yadav et al., 2015). Consistently, the replenishment of PM PI(4,5)P2 after receptor-induced hydrolysis was suppressed in RAS SF4-knockdown cells and was promoted in RAS SF4-overexpressing cells (Fig. 6, E and F). Thus, the defective replenishment of PM PI(4,5)P2 in RAS SF4-knockdown cells may result from decreased ER–PM junctions and reduced production of PI(4,5)P2 from PI(4)P.

The accumulation of PM PI(4)P in RASSF4-knockdown cells may result from disrupted conversion of PI(4)P to PI(4,5)P2 caused by defects in ARF6 activation and localization of PIP5Ks. Indeed, acute recruitment of an active PIP5K to the PM rescued the defects in PM PI(4,5)P2 levels, SOCE, and ER–PM
junctions in RASSF4-knockdown cells (Fig. 7). In addition, reduction in ER–PM junctions in RASSF4-knockdown cells may contribute to the increase in PM PI(4,5)P2 by limiting transport of PI(4)P from the PM to the ER by oxysterol-binding protein–related protein 5 (ORP5) and ORP8 (Chung et al., 2015; Moser von Filseck et al., 2015) or by preventing Sac1-mediated dephosphorylation of PI(4)P to generate phosphatidylinositol at ER–PM junctions (Stefan et al., 2011; Dickson et al., 2016).

A role of RASSF proteins in regulating the levels of phosphoinositides had not been shown previously. Our data support a model in which RASSF4 regulates ARF6 activation, which directly activates PIP5Ks to affect PM PI(4,5)P2 levels (Fig. 8 F). Several small G proteins, including ARF6, RhoA, and Rac1, have been reported to regulate the activity and localization of PIP5Ks (van den Bout and Dicevea, 2009). Nevertheless, the activation of PIP5K by RhoA and Rac1 are likely to be indirect (Funakoshi et al., 2011). RASSF4 preferentially binds ARF6 in its GDP-bound and nucleotide-free states, suggesting that RASSF4 is involved in the nucleotide exchange process from ARF6-GDP to ARF6-GTP (Cherfils and Chardin, 1999). However, RASSF4 is not related to any known guanine nucleotide exchange factors of ARF6. Thus, it is of interest to investigate the mechanism by which RASSF4 affects the activation process of ARF6 and the upstream signaling pathways that regulate RASSF4.

Involvement of RASSF4 in cancer progression has been demonstrated by numerous studies (Chow et al., 2004; Crose et al., 2014; Han et al., 2016). In addition, RASSF4 has been shown to bind MST1 of the Hippo pathway (Crose et al., 2014). Here, we discover that RASSF4 regulates PM PI(4,5)P2 via the ARF6/PIP5K pathway. Notably, RASSF4, ARF6, PIP5K, and the Hippo pathway are all linked to cancer development (van den Bout and Dicevea, 2009; Harvey et al., 2013; Hashimoto et al., 2016; Yoo et al., 2016). Therefore, our new findings on RASSF4 open a field in studying the interactions among ARF6, phosphoinositide metabolism, Ca2+ signaling, ER–PM contact sites, and the Hippo pathway. These studies may advance our understanding of tumorigenesis associated with these pathways and help design new therapeutic strategies for treating human cancers.

**Materials and methods**

**Reagents and antibodies**

TG, pluronic F-127, and Fura-2 AM were purchased from Invitrogen. All chemicals for extracellular buffer (ECB: 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 20 mM Heps, 10 mM glucose, and 1.5 mM CaCl2, pH 7.41) penicillin and streptomycin solutions, rapamycin, histamine, and EGTA were obtained from Sigma-Aldrich. siRNAs used in this study were generated as described previously (Liou et al., 2005). Primers used for siRNA generation are listed in Table S1. Monoclonal anti-PI(4,5)P2 antibody (clone 2C11) and anti-PI(4)P antibody were obtained from Echelon Bioscience. Anti-RASSF4 antibody (C18182) was obtained from Assay Biotech. Anti-STIM1 antibody (4916) was obtained from Cell Signaling Technology. Anti-Orai1 antibody (PA1-74181) and anti-GAPDH antibody (MAS-15738) were obtained from Thermo Scientific. Anti-GFP antibody (ab290) was purchased from Abcam. Anti-mCherry antibody (632496) was purchased from Clontech. Protein standard (161-0373) and DNA ladder (SM1153) were purchased from Bio-Rad and Thermo Scientific, respectively.

**Cell culture and transfection**

HeLa cells purchased from American Type Culture Collection were cultured in minimum essential medium supplemented with 10% fetal bovine serum (HyClone) and 1x penicillin and streptomycin solution. 50 ng of DNA plasmids and 25 nM siRNAs were cotransfected into HeLa cells with TransIT-LT1 reagent (Mirus Bio) for DNA plasmids and TransIT-TKO reagent (Mirus Bio) for siRNAs. For experiments with NEAT-YFP, STIM1-CT, STIM1-D76A, MAPPER, PIP5K, or ARF6, sequential transfection of siRNA and DNA plasmids was performed using TransIT-TKO reagent for siRNAs and TransIT-X2 reagent (Mirus Bio) for DNA plasmids. HUVECs were obtained from T. Meyer (Stanford University, Stanford, CA) and cultured using the EGM-2 BulletKit (CC-3162; Lonza). Transfection of DNA plasmids and siRNAs into HUV ECs was done as described previously (Tsai and Meyer, 2012) using Lipofectamine 2000 (Invitrogen) for DNA plasmids and RNAiMAX (Invitrogen) for siRNAs. RPE-1 cells purchased from American Type Culture Collection were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone) and 1x penicillin and streptomycin solution. siRNAs were transfected into RPE-1 cells with RNAiMAX (Invitrogen).

**DNA constructs**

Construction of mCherry-STIM1, mCherry-STIM1-D76A, YFP-KDEL (Lys-Asp-Glu-Leu; the ER marker), and MAPPER was described previously (Liou et al., 2005; Chang et al., 2013). RASSF4-YFP was constructed by replacing the Orai1 region of Orai1-YFP (Varnai et al., 2007) with the full-length coding region of human RASSF4 (NCBI RefSeq accession no. NM_032023.3). mCherry-STIM1-CT was generated as previously described (Sharma et al., 2013). GFP-E-Syt3 and ARF6-mCherry were constructed using PCR and a human cDNA library. PIP5K1B-HA and PIP5K1C-HA were provided by H. Yin (University of Texas Southwestern Medical Center, Dallas, TX). YFP-PIP5K1B and YFP-PIP5K1C were constructed by inserting a PCR fragment of full-length PIP5K1B or PIP5K1C into a YFP-C1 vector. GFP-FKBP-PIP5K was constructed by replacing the CFP region of CFP-FKBP-PIP5K with GFP. Mutants of ARF6 were generated using a QuikChange site-directed mutagenesis kit (Agilent Technologies). All constructs were verified by sequencing. Oligonucleotides used in this study are listed in Table S1. The GFP–PLCδ–PH (Staubf et al., 1998), CFP–FKBP–PIPK (Suh et al., 2006), CFP–FKBP–PIPKi (Suh et al., 2006), and Lyn–FRB (Inoue et al., 2005) DNA constructs were provided by T. Meyer (Stanford University, Stanford, CA). The GFP–PH–Osh2x2 (Balla et al., 2008) and Orai1–YFP (Varnai et al., 2007) DNA plasmids were provided by T. Balla (National Institutes of Health, Bethesda, MD). The N–PH–ORP5–GFP (Chung et al., 2015) and GFP–E–Syt2S (Giordano et al., 2013) DNA plasmids were gifts from P. De Camilli (Yale University, New Haven, CT). The HRP–ER3 (Wu et al., 2006) DNA plasmid was provided by R. Lewis (Stanford University, Stanford, CA). The Tubby–GFP (Quinn et al., 2008) DNA plasmid was a gift from A. Tinker (University College London, London, England, UK).

**Ca2+ measurements**

Cells were loaded with 0.5 µM Fura-2 AM, a Ca2+ indicator, in ECB containing 0.05% pluronic F-127 and 0.1% BSA for 30 min at room temperature. Then, Fura-2–loaded cells were washed twice with ECB containing 0.1% BSA and incubated in ECB for another 15–30 min before the experiments. Single-cell Fura-2 images were taken with a Plan Fluor 4×/0.15 objective on a microscope (Ti-E; Nikon) with a camera (HQ2; Photometrics). Intracellular Ca2+ levels were reported as the ratio of the fluorescence intensity at 510 nm excited at 340 nm over that excited at 380 nm (F340/F380). For Ca2+ add-back experiments, 100 µM histamine, 1 µM TG, and 2 mM EGTA were simultaneously added to cells to stimulate ER Ca2+ release while inhibiting ER Ca2+ release.
refill and chelating extracellular Ca\(^{2+}\). After the ER Ca\(^{2+}\) store was depleted, 2 mM Ca\(^{2+}\) was added back to the ECB, enabling Ca\(^{2+}\) influx. For monitoring constitutive Ca\(^{2+}\) influx mediated by either STIM1-CT or STIM1-D76A, 2 mM EGTA was used to remove extracellular Ca\(^{2+}\), and 2 mM Ca\(^{2+}\) was subsequently added back to reinitiate Ca\(^{2+}\) influx.

**Mn\(^{2+}\) quench assays**

Mn\(^{2+}\) influx–induced Fura-2 quenching was used to measure divalent cation influx, using Mn\(^{2+}\) as a surrogate for Ca\(^{2+}\) (Liu et al., 2005). Cells were loaded with 0.5 µM Fura-2 AM in ECB containing 0.05% sodium cacodylate buffer (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 20 min at room temperature. The HRP signals in fixed cells were amplified with a TSA Biotin system (PerkinElmer) and the ABC kit (Vector Laboratories) for 30 min each before the first reaction with 1 mg/ml DAB (Sigma-Aldrich) in Tris-buffered saline for 15 min and a subsequent reaction with DAB with 0.01% H\(_2\)O\(_2\) for 30 min. DAB reaction was stopped by three sequential rinses with 0.1 M sodium cacodylate buffer. After postfixation with 1% OsO\(_4\) and en bloc staining with 1% uranyl acetate, cells were further processed as previously described (Ahmari et al., 2000). After embedding in Embed 812 resin (Electron Microscopy Sciences), glass coverslips were removed. Cells were located using light microscopy and trimmed out. 60–70-nm thin sections were cut, mounted on formvar-coated grids, and viewed by a transmission electron microscope (Tecnai G2 spirit; FEI) equipped with a LaB6 source using a voltage of 120 kV.

**Analyses of PM occupancy by ER–PM junctions using EM**

To measure lengths of HRP-ER–labeled ER tubules in close contact with the PM, micrographs with cells in which the nucleus was visible were selected. The total length of the PM and the length of HRP-positive ER segments making close contact with the PM (<50 nm) were measured. The percentage of PM occupancy by the ER was calculated as the sum of length of ER making contact with the PM divided by the length of the PM.

**Quantitation of the density of ER–PM junctions using EM**

To determine the density of ER–PM junctions in a cell using EM labeling, TIRFM images of live cells expressing YFP-KDEL were taken every 15 s for 2.5 min. A time series of YFP-KDEL TIRFM images was summed, background subtracted, and subjected to Fourier transformation with a high-pass filter to create binary-like images that helped identify ER–PM junctions. The transformed images were subjected to threshold analysis to obtain the total number of ER–PM junctions. PM areas detected by a fluorescent protein–tagged PM marker (mCherry-KRAS-tail) were measured to obtain the density of ER–PM junctions (Fivaz and Meyer, 2005; Chang et al., 2013).

**Live-cell confocal and TIRFM imaging**

Cells were plated on 8-well Lab-Tek chambered cover glass (Nunc) at a low density the day before transfection. Transfected cells were washed with ECB and imaged in ECB at room temperature with a CFI Apo TIRF 60x/1.49 or a CFI Apo TIRF 100x/1.49 objective on a spinning-disc confocal and TIRFM system built around a Ti-E Perfect Focus microscope (Nikon) with an HQ2 camera and an EM camera (c9100-13; Hamamatsu) controlled by Micro-Manager software (Edelstein et al., 2010). For mCherry-STIM1 translocation experiments, cells with low to moderate expression levels of mCherry-STIM1 were imaged near the adhesion surface using spinning-disc confocal microscopy or TIRFM. For TIRFM imaging experiments, a PM marker was cotransfected into cells to monitor the focus and cell movements.

**EM**

EM and HRP-ER cytochemistry were performed as previously described (Wu et al., 2006). After transfection of the HRP-ER plasmid with either siRNAs or DNA plasmids, HeLa cells were plated onto coverslips (MatTek Corporation) and fixed with 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 20 min at room temperature. The HRP signals in fixed cells were amplified with a TSA Biotin system (PerkinElmer) and the ABC kit (Vector Laboratories) for 30 min each before the first reaction with 1 mg/ml DAB (Sigma-Aldrich) in Tris-buffered saline for 15 min and a subsequent reaction with DAB with 0.01% H\(_2\)O\(_2\) for 30 min. DAB reaction was stopped by three sequential rinses with 0.1 M sodium cacodylate buffer. After postfixation with 1% OsO\(_4\) and en bloc staining with 1% uranyl acetate, cells were further processed as previously described (Ahmari et al., 2000). After embedding in Embed 812 resin (Electron Microscopy Sciences), glass coverslips were removed. Cells were located using light microscopy and trimmed out. 60–70-nm thin sections were cut, mounted on formvar-coated grids, and viewed by a transmission electron microscope (Tecnai G2 spirit; FEI) equipped with a LaB6 source using a voltage of 120 kV.
mCherry–PLCδ–PH, YFP-PIP5K1B, and YFP-PIP5K1C at the PM to that in the cytosol by line scan using confocal images as described previously (StaufFer et al., 1998). For direct measurements of PI(4)P and PI(4,5)P₂ using immunostaining, monoclonal anti-PI(4)P and anti-PI(4,5)P₂ antibody (clone 2C11), respectively, were used following a previously described protocol (Hammond et al., 2009). Cells plated on 8-well chamber cover glass were fixed with 4% formaldehyde and 0.2% glutaraldehyde in PBS for 15 min at room temperature. After fixation, cells were washed three times with PBS containing 50 mM NH₄Cl. Cells on cover glass were chilled on ice for at least 2 min. Then, cells were blocked and permeabilized for 45 min at 4°C with prechilled buffer A (20 mM Pipes, pH 6.8, 137 mM NaCl, and 2.7 mM KCl) containing 5% (vol/vol) normal goat serum (NGS), 50 mM NH₄Cl, and 0.5% saponin. Cells were rinsed twice with buffer A before incubation with primary antibodies (2.5 µg/ml for anti-PI(4,5)P₂ antibody and 8 µg/ml for anti-PI(4)P antibody) in buffer A with 5% NGS and 0.1% saponin for 1 h at 4°C. Cells were washed twice with buffer A before incubation with the secondary antibody (anti-mouse Alexa Fluor 488; 1:10,000 dilution) in buffer A with 5% NGS and 0.1% saponin at 4°C for 45 min. Cells were rinsed four times with buffer A, postfixed in 2% formaldehyde in PBS containing 50 mM NH₄Cl on ice for 10 min, and then incubated at room temperature for 5 min. Cells were rinsed once with distilled water and two more times with PBS. Cells were imaged using a spinning-disc confocal setup built around a Ti-E Perfect Focus microscope equipped with an EM camera (9100-13). For measurement of dynamic changes in PM PI(4,5)P₂ levels, PI(4,5)P₂ levels induced by histamine stimulation, HeLa cells transfected with histamine H₁ receptor (H₁R) and GFP–PLCδ–PH DNA plasmids were imaged using a TIRF imaging system built around a Ti-E Perfect Focus microscope equipped with an HQ2 camera. Changes in PM PI(4,5)P₂ levels were determined by the ratio of the intensity of GFP–PLCδ–PH measured at each time point to that measured before the addition of histamine.

**Analysis of myo-[³H]inositol–labeled lipids**
HeLa cells plated on 6-well plates were transfected with siRNAs and labeled with 20 µCi/ml myo-[³H]inositol (PerkinElmer) in isoinositol-free DMEM supplemented with 5% dialyzed fetal bovine serum for 24 h. The labeling was terminated by the addition of a 250-µl mixture of methanol and 1 M hydrochloric acid (1:1 vol/vol). Then, cells were scraped and transferred into tubes, and 125 µl of chloroform was added to extract lipids. The samples were vortexed and centrifuged at 14,000 rpm for 1 min, and then the phospholipids were separated by TLC in an n-propyl alcohol/H₂O/NH₄Cl (65:20:15) solvent system as described previously (Barylko et al., 2001). TLC plates were sprayed with autoradiography enhancer (KODAK) and then exposed to x-ray films. Radioactive PI(4,5)P₂ levels were quantified by densitometry analysis (Image J).

**ARF6 activation assay**
ARF6 activity was measured using an ARF6 Pull-Down Activation Assay Biochem kit (BK033-3; Cytoskeleton) according to the manufacturer’s instructions. In brief, ARF6-mCherry–transfected cells were lysed on ice for 10 min with cell lysis buffer (25 mM Tris, pH 7.2, 5 mM MgCl₂, 150 mM NaCl, 1% IGEPEAL, and 1x protease inhibitor cocktail). Lysates were clarified by centrifugation at 10,000 g for 20 min. Cell lysates were incubated with GGA3 beads for 1–2 h at 4°C. Then, the beads were washed three times with wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl₂, and 40 mM NaCl). Proteins bound to the beads were eluted into 2x sample buffer, separated by SDS-PAGE, and subjected to immunoblotting using anti-mCherry antibody and anti-ARF6 antibody. Chemiluminescence detection of immunoblotting was performed using SuperSignal West Dura Extended Duration substrate (34076; Thermo Scientific).

**Immunoprecipitation**
For immunoprecipitation of YFP-tagged or mCherry-tagged protein, transfected cells were collected and lysed in NP-40 lysis buffer (50 mM Tris, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, and 1x protease inhibitor cocktail) on ice for 20 min. Then, cell lysates were centrifuged at 4°C and 16,000 g for 10 min, and supernatants were incubated with Chromotek GFP-trap or RFP-trap magnetic beads (Allele Biotech) at 4°C for 16 h. Immunoprecipitated proteins were eluted with 2x sample buffer (Bio-Rad) after washing the beads five times with wash buffer (10 mM Tris, pH 7.5, and 150 mM NaCl) and analyzed by immunoblotting using antibodies against GFP or mCherry.

**Statistical analysis**
Unpaired Student’s t test was used for statistical analysis. A p-value < 0.05 was considered statistically significant.

**Online supplemental material**
Fig. S1 shows Ca²⁺ traces demonstrating that RASSF4-YFP expression rescued SOCE in siRASSF4-treated cells, and results, similar to those in HeLa cells, were obtained in RPE-1 cells. It also shows immunoblots and RT-PCR results demonstrating that expression levels of STIM1 and Orai1 proteins, as well as several other genes implicated in regulating SOCE, were not affected in siRASSF4-treated cells. Fig. S2 shows quantitation of MAPPER-labeled ER–PM junctions demonstrating reduction in ER–PM junctions in RASSF4-knockdown cells. Fig. S3 shows confocal images of HeLa cells transfected with Tubby-GFP, GFP-PH-Osh2x2, or YFP-PIP5K1C and treated with either siControl or siRASSF4 demonstrating that RASSF4 regulates PI(4,5)P₂, PI(4)P, and PIP5K levels. Fig. S4 shows confocal images of HeLa cells demonstrating rapamycin-induced PM targeting of FKBP-PIP5K and FKBP-PIP5K and shows quantitation of MAPPER-labeled ER–PM junctions and STIM1 puncta demonstrating reconstitution of ER–PM junctions and TG-triggered STIM1 translocation in RASSF4-knockdown cells by PM targeting of PIP5K. Fig. S5 shows confocal images of HeLa cells demonstrating the diffuse distribution of RASSF4–YFP in the cytoplasm. Video 1 is a time-lapse video demonstrating RASSF4 regulates mCherry-STIM1 translocation to ER–PM junctions during SOCE in live HeLa cells. Table S1 is available as an Excel file and shows oligonucleotides used in this study.

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The authors declare no competing financial interests. Author contributions: Y.-J. Chen designed and performed all experiments except those indicated below. C.-L. Chang performed the experiments and analyzed the data shown in Figs. 1 C and 2.
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