RASSF4: Regulator of plasma membrane PI(4,5)P_2

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Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is a negatively charged phospholipid that plays a major role in recruiting and regulating proteins at the plasma membrane–cytosol interface. In this issue, Chen et al. (2017. *J. Cell Biol.* https://doi.org/10.1083/jcb.201606047) demonstrate that RAS association domain family 4 (RASSF4) positively influences PI(4,5)P_2 synthesis through ARF6-dependent regulation of PIP5K.

Phosphoinositides (PIs) are a family of dynamic signaling phospholipids found in the cytoplasmic leaflet of various subcellular organelles. PI levels are tightly regulated spatially and temporally by the action of numerous lipid kinases and phosphatases that add or cleave phosphate groups at specific positions on their inositol ring. The precise localization of each of these enzymes, coupled with the activities of lipid transfer proteins (Chang et al., 2013; Kim et al., 2015), ensures maintenance of the heterogeneous distribution of PIs despite continuous membrane flow from one compartment to another. The signature PI of the plasma membrane (PM) is phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2). There, it orchestrates a host of essential biological events, including membrane budding and fusion, actin cytoskeleton dynamics, second messenger signaling cascades, and transport of ions across membranes, including store-operated calcium (Ca^{2+}) entry (SOCE; Balla, 2013; Hille et al., 2015; Prakriya and Lewis, 2015). SOCE is the process through which Ca^{2+} ions move across the PM (into the cytoplasm) in response to depletion of ER Ca^{2+} stores. Orai channels in the PM and Stim1 proteins in the ER membrane interact together to choreograph this event. In brief, during periods of ER Ca^{2+} depletion, Stim1 dimers oligomerize and translocate to ER–PM junctions, where they interact and trap freely diffusing Orai Ca^{2+} channels to form high-order puncta. The consequence of these interactions is the flux of Ca^{2+} into the cytoplasm, refilling of the ER via Ca^{2+}-ATPase pumps, and activation of downstream transcriptional pathways. Thus, mechanisms that control this pathway, or control the localization and/or abundance of PM PI(4,5)P_2, are of great interest to cell biologists and neuroscientists.

RAS association domain family (RASSF) proteins are a family of 10 mammalian proteins, known as RASSF1 to RASSF10. RASSF proteins are devoid of any known enzymatic activity and most likely function as adapter proteins or scaffolds for larger multiprotein complexes. To this end, RASSF proteins carry several characteristic domains. The first six RASSF proteins, termed C-terminal or classical RASSF proteins, possess both a C-terminal RAS association (RA) domain and a Salvador-RASSF-Hippo (SARAH) domain, with RASSF1 and 5a having an additional C1 domain. RASSF7 through 10, termed N-terminal RASSF proteins, have the RA domain but do not have a SARAH domain. RA domains are thought to interact with various RAS GTPases, whereas the C-terminal SARAH domains of RASSF1–6 have been shown to facilitate dimerization between other SARAH domain–containing proteins, including the family of proapoptotic kinases known as the mammalian sterile 20–like kinases (Rawat and Chernoff, 2015).

Initially identified from an siRNA screen looking for the molecular components of SOCE (Liou et al., 2005), Chen et al. now report a novel role for the ubiquitously expressed RASSF4 (Fig. 1 A) as a positive regulator of PM PI(4,5)P_2 synthesis. The mechanism through which RASSF4 exerts these effects appears to be mediated via Arf6-dependent activation of PIP5K to control the levels of PM PI(4,5)P_2 (Fig. 1 B, yellow box). Supporting this hypothesis, the authors nicely show that knocking down RASSF4 expression decreases the ability of Arf6 and PIP5K1B to localize to the PM, resulting in an ∼30% reduction in PM PI(4,5)P_2 levels. This decrease in PM PI(4,5)P_2 is sufficient to reduce the number and stability of ER–PM junctions in resting cells and reduce the magnitude and kinetics of receptor-stimulated SOCE. Overexpression of RASSF4 has the inverse effect, resulting in increased PM PI(4,5)P_2, with equivalent increases in SOCE and ER–PM junctions. Thus, expressional changes in RASSF4 correlate with PM PI(4,5)P_2 abundance to control SOCE and ER–PM junctions.

Chen et al. (2017) also provide insight into the mechanism through which RASSF4 may regulate the activation of Arf6 to control PM PI(4,5)P_2. Using several ARF6 mutants, which mimic different nucleotide-binding states, they find that RASSF4 preferentially binds the inactive, GDP-bound form of Arf6 (Arf6-GDP). These data imply that RASSF4 may act as a scaffold for the nucleotide exchange process of Arf6. Supporting this view, knockdown of RASSF4 reduces the total amount of Arf6-GTP. How does RASSF4 bind Arf6-GDP? Could it be through the RA domain, or are there additional proteins coordinating these activities in a macromolecular complex? Future experiments are needed to fully uncover the mechanisms surrounding these RASSF4–Arf6 interactions.

Could the expression of RASSF4 activate a parallel pathway that synergistically contributes to increase PM PI(4,5)P_2 (Fig. 1 B, blue box)? In this hypothetical scenario, RASSF4-mediated activation of Arf6-GTP would not only directly activate PIP5K but also activate PLD (Cockcroft et al., 1994). Activated PLD catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid. Phosphatidic acid could then directly function as a cofactor for the activation of PIP5K or recruit the PI transfer

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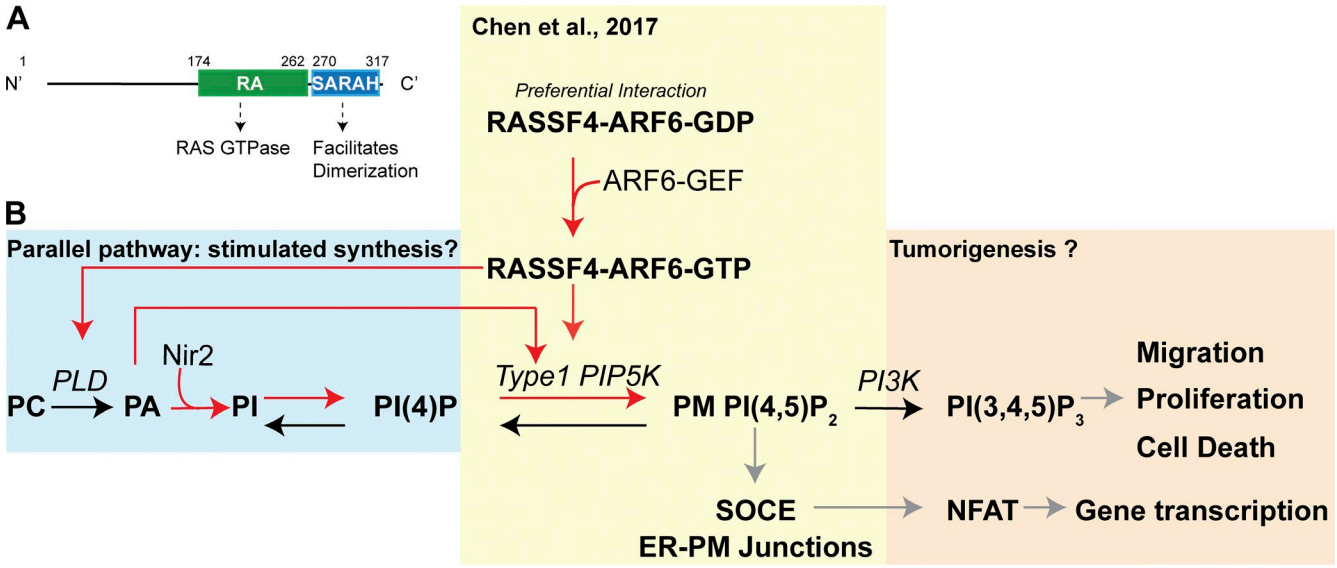

Figure 1. **RASSF4 domains and signaling pathways.** (A) Linear representation of RASSF4. (B) Experimentally validated (yellow box; Chen et al., 2017) and hypothetical (blue and orange boxes) RASSF4 signaling pathways. PC, phosphatidylcholine; PA, phosphatidic acid. Red arrows indicate potential stimulatory effects on PI(4,5)P_2 synthesis.

protein Nir2 to the PM (Kim et al., 2015). The VAPA/B-mediated recruitment of Nir2 to ER–PM junctions (Chang et al., 2013) would be expected to supply phosphatidylinositol to the PM for phosphorylation by PM-localized PI 4-kinases (Nakatsu et al., 2012) to generate PM phosphatidylinositol 4-phosphate (PI(4)P), the substrate for PIP5K. Indeed, depending on the kinetics of each arm of this bifurcating pathway, Arf6 could stimulate the parallel production or supply of PM PI, PI(4)P, and PI(4,5)P_2. Why would such a mechanism be beneficial for primary cells like neurons? Given the importance of PM PI(4,5)P_2 for regulating a plethora of signaling events, it could represent a mechanism that safeguards against net depletion of the lipid. Alternatively, for G_q-coupled receptor activation, it could represent (a) a mechanism for faster recovery of PI(4,5)P_2 or (b) a mechanism that ensures continuous IP_3-mediated Ca^{2+} signaling in the face of persistent PI(4,5)P_2 depletion. The concept of stimulated synthesis has been previously incorporated into kinetic models of receptor-mediated PI(4,5)P_2 metabolism. Interestingly, in those models, the rate of the PI 4-kinase and PI 5-kinases had to be elevated temporarily during G_q-coupled receptor activation to account for the sustained production of IP_3 (Dickson et al., 2013). Supporting these data, Chen et al. (2017) find that recovery of PI(4,5)P_2 after G_q receptor activation is faster with RASSF4 overexpression. Further experiments are certainly required to determine whether this hypothesis is correct, but given its upstream location and effects on Arf6 activity, RASSF4 is ideally positioned to be a key regulator of such a pathway.

Many of the RASSF proteins have been shown to play a role in tumor suppression (van der Weyden and Adams, 2007). There is conflicting evidence regarding the function of RASSF4, with some studies suggesting it shows tumor suppressor characteristics, and others suggesting that it functions in promoting cell proliferation (van der Weyden and Adams, 2007). In the context of the current study, up-regulation of RASSF4 is expected to increase NFAT (nuclear factor of activated T cells)-mediated transcription and also PM PI(4,5)P_2. Thus, RASSF4 could facilitate tumorigenesis via multiple mechanisms (Fig. 1 B, orange box). First, it could increase NFAT-mediated transcription through its positive effects on SOCE to drive expression of potential tumorigenic genes. Second, it could influence the production of PI(3,4,5)P_3 at the PM. In this second scenario, receptor-activated PI 3-kinase would have an increased substrate, potentially leading to augmented AKT activation and potentiation of its downstream effects, including apoptosis, cell migration, or proliferation. More work is required to fully elucidate these mechanisms.

Our knowledge of RASSF proteins and their roles in regulated cell biology is in its infancy. The original findings of Chen et al. (2017) highlight an important role for RASSF4 in regulating PM PI(4,5)P_2-dependent events. There remain many important unanswered questions; for example, how does RASSF4 exert effects at the PM when it has a cytosolic distribution? What is upstream of RASSF4, and how does this influence its ability to signal? Is RASSF4 part of a larger signaling complex? To conclude, the work of Chen et al. (2017) provides new insights into the regulation of PI(4,5)P_2, Ca^{2+} signaling, and ER–PM junctions. This study advances our understanding of tumorigenesis associated with these pathways and may inform and inspire the design of new therapeutic strategies for treating human cancers.

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