PtdIns4P synthesis by PI4KIIIα at the plasma membrane and its impact on plasma membrane identity

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Plasma membrane phosphatidylinositol (PI) 4-phosphate (PtdIns4P) has critical functions via both direct interactions and metabolic conversion to PI 4,5-bisphosphate (PtdIns(4,5)P2) and other downstream metabolites. However, mechanisms that control this PtdIns4P pool in cells of higher eukaryotes remain elusive. PI4KIIIα, the enzyme thought to synthesize this PtdIns4P pool, is reported to localize in the ER, contrary to the plasma membrane localization of its yeast homologue, Stt4. In this paper, we show that PI4KIIIα was targeted to the plasma membrane as part of an evolutionarily conserved complex containing Efr3/rolling blackout, which we found was a palmitoylated peripheral membrane protein. PI4KIIIα knockout cells exhibited a profound reduction of plasma membrane PtdIns4P but surprisingly only a modest reduction of PtdIns(4,5)P2 because of robust up-regulation of PtdIns4P 5-kinases. In these cells, however, much of the PtdIns(4,5)P2 was localized intracellularly, rather than at the plasma membrane as in control cells, along with proteins typically restricted to this membrane, revealing a major contribution of PI4KIIIα to the definition of plasma membrane identity.

Introduction

Phosphoinositides are minor components of cellular membranes but play key regulatory roles in cell physiology (Di Paolo and De Camilli, 2006). Their phosphorylated head groups, which protrude from the cytosolic leaflet of membranes, bind with variable affinity and specificity to proteins located in or exposed to the cytosolic space (Lemmon, 2008). Through these interactions, the heterogeneous distribution of the seven phosphoinositides in different membranes helps to generate a code of membrane identity that plays a fundamental role in orchestrating the processes occurring on such membranes. Some phosphoinositides (in particular phosphatidylinositol [PI] 4,5-bisphosphate [PtdIns(4,5)P2]) also act as precursors of other intracellular messenger molecules.

Among phosphoinositides, PI 4-phosphate (PtdIns4P) plays numerous fundamental roles. It has critical functions in the Golgi complex, the endosomal system, and the plasma membrane, which are mediated by its direct interaction with PtdIns4P-binding proteins (D’Angelo et al., 2008). Additionally, phosphorylation of PtdIns4P at the 5 position by type I PI monophosphate kinases (PIPKIs) represents the major pathway for the synthesis of PtdIns(4,5)P2 (Doughman et al., 2003), which, in turn, is the precursor of other important signaling metabolites. PtdIns4P also has major regulatory actions on the localization and metabolism of other membrane lipids. For example, several oxysterol-binding protein–related proteins contain PtdIns4P binding sites that target them to PtdIns4P-containing membranes. One yeast oxysterol-binding

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two major yeast PI 4-kinases, Stt4 and Pik1, which mediate
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simple system, and also to some extent at the plasma mem-
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In view of these considerations, and of numerous findings
that link PtdIns4P, its downstream metabolites, and the lipids
indirectly controlled by PtdIns4P to human disease (Vicinanza
et al., 2008; McCrea and De Camilli, 2009), it is critically
important to understand the mechanisms controlling PtdIns4P
levels and metabolism on different membranes, in particular
the plasma membrane, whose PtdIns4P pool serves as the
precursor of the bulk of cellular PtdIns(4,5)P2. However,
formation on these mechanisms in metazoan cells is not as
advances as in yeast. The mammalian genome encodes four
PI 4-kinase isoforms. PI4KIIα and PI4KIIIβ (encoded by
PI4KA and PI4KB, respectively) are the homologues of the
two major yeast PI 4-kinases, Stt4 and Pik1, which mediate
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complex, the endosomal system, and also to some extent at the plasma membrane (Balla and Balla, 2006).

In yeast, Stt4 has been shown to be localized at plasma
membrane hotspots termed PI kinase (PIK) patches in a com-
plex with the accessory proteins Efr3 and Ypp1 (Baird et al.,
2008). Interestingly, considering that the bulk of the cortical ER
is tightly apposed to the plasma membrane in this organism,
Stt4 contains a so-called FFAT (two phenylalanines [FF] in an
acidic tract) motif that enables binding to the integral ER mem-
brane proteins Scs2/22. In fact, contact sites between the ER
and the plasma membrane serve as hubs controlling PtdIns4P
metabolism in this organism (Stefan et al., 2011).

Although a pharmacological and knockdown study has suggested that, as in the case of yeast Stt4, PI4KIIIα functions
in the generation of PtdIns4P at plasma membrane (Balla
and Balla, 2006), surprisingly little is known about this enzyme.
Based on immunofluorescence, a localization of PI4KIIIα in
the ER has been reported (Wong et al., 1997), but such a local-
ization remains highly debated, in spite of the partial conserva-
tion of its FFAT motif that would be expected to bind
VAP-A/B, the mammalian Scs2/22 homologues. The exoge-
 nous expression of tagged PI4KIIIα also failed to provide consistent results, and no evidence existed for its targeting
to the plasma membrane (Balla and Balla, 2006). In mam-
malian cells, ER–plasma membrane contacts are much less
extensive than in yeast, and the bulk of the ER is not local-
ized in close proximity to the plasma membrane. How the
mechanisms of PtdIns4P generation have adjusted to the dif-
ferent architecture of cells of higher eukaryotes is an import-
ant open question.

An issue of special interest is the link between the func-
tions of Stt4/PI4KIIIα and of Efr3 (Baird et al., 2008). Efr3 is
the yeast homologue of Drosophila melanogaster rolling black-
out (RBO), a protein expressed at high levels in the nervous
system of this organism whose mutation is responsible for a
temperature-sensitive paralysis (Huang et al., 2004). RBO was
proposed to be a transmembrane lipase, and its potential role in
lipid metabolism was supported by changes in phosphoinositide
and diacylglycerol levels in temperature-sensitive rbo mutants
at the restrictive temperature (Huang et al., 2004).

Additional interest in the localization and properties of
PI4KIIIα came from the recent identification by several groups
of this enzyme as a critical host factor for the hepatitis C virus
life cycle (Berger et al., 2009; Borawska et al., 2009; Tai et al.,
2009; Trotard et al., 2009; Alvisi et al., 2011), pointing to
PI4KIIIα as to a potential target for anti–hepatitis C virus ther-
apieties (Altan-Bonnet and Balla, 2012).

Collectively, these findings highlight the importance of
addressing the still unclear and controversial subcellular local-
ization of PI4KIIIα and the function of the PtdIns4P pools regu-
lated by this enzyme. Here, we show recruitment of PI4KIIIα
to the plasma membrane and show that its targeting requires
presence in this membrane of EFR3/RBO, which we demon-
strate to be a palmitoylated peripheral scaffold protein, not a
transmembrane lipase. We also show that loss of PI4KIIIα leads
to a striking compensatory increase of PIPKIs that minimizes
a global decrease in cellular levels of PtdIns(4,5)P2, in spite of
the dramatic decrease of PtdIns4P. However, the bulk of
PtdIns(4,5)P2 produced by this compensatory response is local-
ized not in the plasma membrane but, surprisingly, on intra-
cellular vesicles. Proteins and lipids normally segregated in
the plasma membrane are also found on these vesicles, emphasis-
how targeting of PI4KIIIα to the plasma membrane is a
key upstream event in defining the proteomic and lipidomic
identity of this membrane.

Results

Full-length PI4KIIIα visits the plasma membrane dynamically
In contrast to yeast Stt4, which is localized at the plasma mem-
brane (Audhya and Emr, 2002), a GFP-tagged PI4KIIIα con-
struct based on the published ORF (Nakagawa et al., 1996)
displayed diffuse cytosolic localization with no discernable en-
richment on any cellular membrane (Fig. 1 A, left), as reported
previously (Balla and Balla, 2006). We noted, however, that the
mobility by SDS-PAGE of a construct corresponding to un-
tagged PI4KIIIα was slightly faster than the mobility of the en-
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mobility by SDS-PAGE of a construct corresponding to un-
tagged PI4KIIIα was slightly faster than the mobility of the en-
dogenous protein, as assessed by Western blotting (Fig. 1 B,
compare first and third lanes). We thus considered the possi-
bility of an incorrect annotation and reexamined the reported
translation start site of PI4KA (Nakagawa et al., 1996). An
alignment of vertebrate PI4KA homologues (Fig. 1 C) revealed
substantial conservation upstream of the reported translation
start site (hence defined as M1*; Nakagawa et al., 1996), in-
cluding an additional conserved Met residue (defined as M1).
Western blot analysis demonstrated that exogenously expressed
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the plasma membrane (Baird et al., 2008), our finding is consistent with a role for PI4KIIIα at the plasma membrane. It was therefore of interest to determine how PI4KIIIα is recruited to the plasma membrane. PI4KIIIα recruitment to the plasma membrane requires its interaction with EFR3 and TTC7

Immunoprecipitation (IP) of endogenous PI4KIIIα from mouse brain extracts, followed by mass spectrometry analysis, identified two interactors, EFR3B and TTC7B, that, interestingly, are the yeast homologues of Efr3 and Ypp1, the other constituents of yeast PIK patches (Fig. S1; Baird et al., 2008). The interaction of EFR3B and of TTC7B with PI4KIIIα was confirmed using IP followed by Western blotting with endogenous and exogenously expressed proteins (Fig. 2, A and B). To assess whether EFR3B and TTC7B have a role in PI4KIIIα recruitment, we expressed tagged versions of these proteins and performed knockdown experiments.

Figure 1. Full-length PI4KIIIα visits the plasma membrane dynamically. (A) Confocal imaging of HeLa cells transfected with GFP-M1*-PI4KIIIα (left) or GFP-M1-PI4KIIIα (right). (B) Anti-PI4KIIIα Western blot comparing electrophoretic mobility of endogenous (Endog.) PI4KIIIα and either exogenously expressed M1-PI4KIIIα or M1*-PI4KIIIα in lysates of HEK cells. Molecular masses are given in kilodaltons. (C) Alignment of the N-terminal region of vertebrate PI4KIIIα orthologues. The black shading denotes conservation of amino acid identity, and the gray shading denotes conservation of amino acid similarity. (D) Ribosome footprinting reveals M1 and T*, but not M1*, as likely PI4KIIIα translation start sites. The data shown were pooled from two biological replicate experiments. (E) TIRF microscopic analysis shows highly dynamic spots of GFP-M1-PI4KIIIα, but not of GFP-M1*-PI4KIIIα, at the plasma membrane, revealing very transient visits of the enzyme to this membrane. COS-7 cells were transfected with GFP-M1-PI4KIIIα (top row) or GFP-M1*-PI4KIIIα (bottom row) and imaged by TIRF microscopy, with a 1-s interval between each image acquisition. Bars: (A) 20 µm; (E) 2 µm.

M1-PI4KIIIα co-migrated with endogenous PI4KIIIα, in contrast to the smaller M1*-PI4KIIIα (Fig. 1 B). Furthermore, determination of PHKA translation initiation sites using ribosome footprinting after harringtonine treatment (Ingolia et al., 2011) suggested that M1, but not M1*, was a strong translation start site (Fig. 1 D). This analysis also revealed a potential non-AUG start site (ACG, encoding Thr and indicated as T* in Fig. 1 D) between M1 and M1*.

We considered the possibility that an incorrectly assigned start site may have affected previous studies of PI4KIIIα and next investigated whether M1-PI4KIIIα had a different subcellular localization than M1*-PI4KIIIα. The majority of GFP-M1-PI4KIIIα fluorescence was still localized in the cytosol (Fig. 1 A, right). However, total internal reflection fluorescence (TIRF) microscopy revealed that GFP-M1-PI4KIIIα, but not GFP-M1*-PI4KIIIα, transiently visited the plasma membrane (Fig. 1 E and Video 1; also see Fig. 3). Although this dynamic behavior of PI4KIIIα appeared to differ from that of its yeast homologue, Stt4, which forms stable PIK patches at the plasma membrane (Baird et al., 2008), our finding is consistent with a role for PI4KIIIα at the plasma membrane. It was therefore of interest to determine how PI4KIIIα is recruited to the plasma membrane.
that PI4KIIIα, like its yeast homologue, is at least partially targeted to the plasma membrane and requires both EFR3 and TTC7 for efficient recruitment to this membrane.

EFR3 is a peripheral membrane protein whose N-terminal palmitoylation is essential for plasma membrane localization. To gain further understanding of the mechanisms that localize PI4KIIIα to the plasma membrane, we investigated the membrane targeting of EFR3, as this protein is a necessary factor for recruiting both TTC7 and PI4KIIIα to this membrane. At the molecular level, EFR3/RBO was proposed to function as an integral membrane lipase (Huang et al., 2004). Our bioinformatic analysis, however, predicted that none of the EFR3/RBO homologues from yeast to humans contains transmembrane regions and that the protein, instead, comprises multiple HEAT (Huntington, elongation factor 3, regulatory subunit A of protein phosphatase 2A, and target of rapamycin) repeats. Further sequence analysis identified a Cys-rich motif at the N terminus of EFR3 that is conserved in all metazoans and could potentially act as a palmitoylation site (Fig. 4 A). Accordingly, a bioorthogonal metabolic labeling approach (Hang et al., 2011)
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mutant was unable to recruit tagged TTC7B and M1-PI4KIII to the cytosol (Fig. 4 C), and the EFR3B palmitoylation-deficient mutant, palmitoylation-deficient mutants (C6S/C7S/C8S/C9S toylated at the N-terminal Cys-rich motif (Fig. 4 B). Importantly, palmitoylation-deficient mutants (C6S/C7S/C8S/C9S for EFR3A and C5S/C7S/C8S for EFR3B) were localized in the cytosol (Fig. 4 C), and the EFR3B palmitoylation-deficient mutant was unable to recruit tagged TTC7B and M1-PI4KIII to the plasma membrane (Fig. S2). These data suggest that EFR3 is a peripheral membrane protein whose posttranslational lipidation enables its localization to membranes and its function in PI4KIIIα-mediated PtdIns4P synthesis at the plasma membrane. Interestingly, the N-terminal Cys-rich motif is absent from yeast Efr3, in contrast to the dramatic changes in Golgi complex structure and function observed in cells lacking PI4KIIIβ or PI4KIIα, two PI 4-kinases known to synthesize PtdIns4P in this region (Wang et al., 2003; Balla et al., 2005, Balla and Balla, 2006). Additionally, no changes were observed in the levels of these other PI 4-kinases as well as of PI4KIIβ, a further confirmation of the nonredundant functions of the PI 4-kinase isoforms (Fig. 5 A).

Consistent with a decrease of plasma membrane PtdIns4P and thus of a predicted decrease also of plasma membrane PtdIns(4,5)P2, a physiological phenomenon known to require the presence of these lipids in this membrane, namely the formation of STIM1-dependent ER–plasma membrane contacts upon thapsigargin-induced depletion of intracellular Ca2+ stores (Korzeniowski et al., 2009; Walsh et al., 2010; Calloway et al., 2011), was impaired (Fig. 6, A–D; and Videos 2 and 3). Rescue experiments to confirm the specificity of this phenotype were successful using GFP-M1-PI4KIIIα but not GFP-M1*-PI4KIIIα (Fig. 6, E–H; and Video 4), demonstrating that only GFP-M1-PI4KIIIα is a fully functional protein.

Other PtdIns(4,5)P2-mediated processes occurring at the plasma membrane were clearly altered in PI4KIIIα KO MEFs. A drastic disruption of cortical actin, with a major loss of stress fibers, was observed, as assessed by phalloidin staining (Fig. 6 I). The organization of endocytic clathrin coat components was also affected. The normal fine puncta of immunoreactivity for the endocytic clathrin adaptors (and PtdIns(4,5)P2-binding proteins) AP-2 and epsin 1, which represent clathrin-coated pits selectively localized at the plasma membrane, were replaced in PI4KIIIα KO MEFs by more coarse puncta (Fig. 6, J and K), many of which were localized on intracellular structures (also see Fig. 8 A). Further direct assessment of plasma membrane clathrin organization confirmed the abnormal size and distribution of clathrin puncta in PI4KIIIα KO MEFs (Fig. 5 F and G), in contrast to the dramatic changes in Golgi complex structure and function observed in cells lacking PI4KIIIβ or PI4KIIα, two PI 4-kinases known to synthesize PtdIns4P in this region (Wang et al., 2003; Balla et al., 2005, Balla and Balla, 2006). Additionally, no changes were observed in the levels of these other PI 4-kinases as well as of PI4KIIβ, a further confirmation of the nonredundant functions of the PI 4-kinase isoforms (Fig. 5 A).

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To test whether the mere loss of plasma membrane PtdIns(4,5)P$_2$ in wild-type cells could cause a similar ectopic intracellular PtdIns(4,5)P$_2$ phenotype, we acutely depleted this lipid from the plasma membrane of HeLa cells using a recently developed optogenetic approach (Idevall-Hagren et al., 2012). Although light-mediated recruitment of an inositol 5-phosphatase to the plasma membrane resulted in a rapid and massive loss of PtdIns(4,5)P$_2$ in this membrane, as revealed by shedding of infrared RFP (iRFP)-PH PLC$_{δ}$, no formation of intracellular PHPLC$_{δ}$-positive vesicles was observed, even when the illumination was continually applied over a 1-h period (Fig. 7 G). To chronically prevent PtdIns(4,5)P$_2$ accumulation at the plasma membrane, we expressed an inositol 5-phosphatase construct with a C-terminal CAAX motif that ensures constitutive plasma membrane targeting (Milosevic et al., 2005). Even 16 h after transfection, no PtdIns(4,5)P$_2$-positive intracellular vesicles similar to those found in PI4KIII$_{α}$ KO MEFs were observed (Fig. 7 H), suggesting that more complex homeostatic mechanisms are involved in their formation.

Loss of plasma membrane identity in PI4KIII$_{α}$ KO cells

Further characterization of the PtdIns(4,5)P$_2$-positive intracellular vesicles in PI4KIII$_{α}$ KO MEFs revealed that they were positive for many proteins typically restricted to the plasma membrane. For example, they were decorated by AP-2–, epsin-, and dynamin-positive puncta (Fig. 8 A). Dynamin was also localized however, global levels of cellular PtdIns(4,5)P$_2$, as assessed biochemically, were only moderately reduced in KO MEFs (to 80% of control cell levels; Fig. 5 C). A potential explanation for this modest change, which contrasts with the much stronger reduction in global PtdIns4P levels (to 25% of control; Figs. 5 B and S4), in spite of the loss of PtdIns(4,5)P$_2$ at the plasma membrane, came from a further analysis of the localization of PtdIns(4,5)P$_2$ and of the expression levels and localization of PIPKIs (the enzymes that generate PtdIns(4,5)P$_2$ from PtdIns4P; Doughman et al., 2003) in PI4KIII$_{α}$ KO cells. Although both PtdIns(4,5)P$_2$, as detected by RFP-PH PLC$_{δ}$, and exogenously expressed PIPKIs are typically segregated at the plasma membrane (Fairn et al., 2009), numerous vesicles that were highly heterogeneous in size and positive for RFP-PHPLC$_{δ}$ and for GFP-PIPKI$_{β}$ were detected intracellularly in KO cells (Fig. 7, A [inset] and C). Furthermore, PIPKIs (PIPKI$_{β}$ and PIPKII) were found to be massively up-regulated (Fig. 7 D), and numerous actin comets were observed in the cytoplasm (Fig. 7 E and Video 5), a known effect of the ectopic accumulation of PtdIns(4,5)P$_2$ on intracellular organelles (Rozelle et al., 2000). Accordingly, PtdIns(4,5)P$_2$-positive vesicles were detected at the tip of comets (Fig. 7 F). Thus, loss of PI4KIII$_{α}$ in MEFs leads to initiation of homeostatic mechanisms by which cells attempt to restore PtdIns(4,5)P$_2$ levels through a more efficient conversion of PtdIns4P to PtdIns(4,5)P$_2$ in KO MEFs, but much of the PtdIns(4,5)P$_2$ generated under such conditions is ectopically localized.
in this membrane, and studies in both yeast and higher eukaryotes had implicated Stt4/PI4KIIIα in its generation (Audhya et al., 2000; Balla et al., 2005). However, the localization in cells of higher eukaryotes of this enzyme, which is expected to be of fundamental importance in cell physiology because its product is an essential precursor of key regulatory messenger molecules, had remained highly controversial (Balla and Balla, 2006). The prevailing view was that it was predominantly localized in the ER (e.g., Wong et al., 1997; Balla and Balla, 2006; Altan-Bonnet and Balla, 2012; Bianco et al., 2012), and no evidence has been reported for its targeting to the plasma membrane.

Because of lack of suitable antibodies for the detection of the endogenous protein by immunocytochemistry (despite repeated attempts), the subcellular targeting of PI4KIIIα can only be explored by the expression of tagged PI4KIIIα. Here, we show that the failure of a previous study to reveal an association of PI4KIIIα with the plasma membrane is explained by the incorrect identification of the N-terminal sequence of this enzyme (Balla and Balla, 2006). We demonstrate that EFR3/RBO and TTC7, the mammalian homologues of yeast Efr3 and Ypp1, play a key role in the recruitment of PI4KIIIα to the plasma membrane. As in yeast, EFR3 is constitutively localized at the plasma membrane, but it is not capable of recruiting PI4KIIIα in the absence of Ypp1/TTC7. TTC7 is a soluble protein that requires interaction with EFR3 for membrane recruitment and likely acts as a bridge between PI4KIIIα and EFR3.

Discussion

This study provides answers to long-standing questions concerning the generation of PtdIns4P at the plasma membrane. It has long been known that an important pool of PtdIns4P exists on actin comet tails (Video 6), as previously shown in cells overexpressing PIPKI (Lee and De Camilli, 2002). In contrast, the PtdIns(4,5)P2-positive vesicles did not colocalize with early or late endosomal markers (EEA1, Rab5, Rab9, and the PtdIns3P biosensor FYVE<sup>ΔN</sup>) or a Golgi/PtdIns4P marker (PH<sup>E380D</sup>; Fig. 8 A). Additionally, integral and peripheral membrane proteins normally selectively segregated at the plasma membrane, including fluorescently tagged M1 muscarinic receptor (M1R-YFP; Wess et al., 2007) and myristoylated/palmitoylated Lck membrane anchor (L<sub>10</sub>-GFP; Fig. 7 H; Rodgers, 2002), were found at these intracellular sites (Fig. 8 B). Likewise, wild-type EFR3B partially relocalized to the intracellular structures positive for PtdIns(4,5)P<sub>2</sub> (Fig. 8 B), suggesting a reciprocal requirement of both EFR3 and PI4KIIIα for correct targeting of the PI4KIIIα–EFR3–TTC7 complex to the plasma membrane. Additionally, plasma membrane levels of cholesterol, a lipid normally concentrated in this membrane, were ~20% lower in PI4KIIIα KO MEFs relative to controls, whereas intracellular levels were increased (Fig. 8 C). Thus, PI4KIIIα appears to be a critical determinant of the lipidomic and proteomic identity of the plasma membrane.

![Figure 5. PI4KIIIα and PtdIns4P production.](image)
In yeast, in which fluorescently tagged proteins were expressed under the regulation of the endogenous promoter (and thus at physiological levels), Stt4 was shown to be stably associated with the plasma membrane at “PIK patches.” In our experiments, the recruitment of transfected GFP-tagged PI4KIIIα to the plasma membrane was highly dynamic unless its binding partners EFR3B and TTC7B were also overexpressed, possibly reflecting its stoichiometric excess relative to EFR3 and TTC7 when expressed alone. Whether endogenous PI4KIIIα, EFR3, and TTC7 form stable patches in mammalian cells remains an open question.

A large fraction of the ER is constitutively closely apposed to the plasma membrane in yeast, and these contact sites play an important role in PtdIns4P metabolism, as the ER-localized PtdIns4P phosphatase Sac1 is thought to act in trans to dephosphorylate PtdIns4P in the plasma membrane (Stefan et al., 2011). Although the relationship between yeast PIK patches and ER–plasma membrane contact sites remains unclear (Baird et al., 2008), it is of interest that Stt4 contains an amino acid sequence matching the so-called FFAT motif, which enables binding of cytosolic proteins to the ER-resident proteins Scs2/Scs22. This motif is partially conserved in PI4KIIIα and thus could mediate the association of a small PI4KIIIα pool with the ER via the mammalian Scs2/Scs22 homologue VAP-B (Kaiser et al., 2005), although contacts between the cortical ER and the plasma membrane are less abundant and smaller in mammalian cells compared with in yeast. No evidence for a major association of PI4KIIIα with the ER was obtained in our study.

Importantly, our results shed new light on EFR3/RBO. They demonstrate that EFR3 is not a transmembrane lipase as originally proposed by a study in Drosophila (Huang et al., 2004). First, improved structural predictions do not support the presence of transmembrane regions or of a lipase-type fold. Instead, our bioinformatic analysis predicts that EFR3 is a cytosolic protein consisting entirely of α-helical HEAT repeats.

Second, the protein is palmitoylated at a conserved Cys-rich motif at its N terminus, and mutation of the Cys residues in this motif abolishes the plasma membrane targeting of EFR3. Interestingly, the palmitoylation motif is conserved in all metazoan EFR3 homologues but is absent from yeast Efr3, demonstrating a conservation of function in spite of potentially divergent membrane-targeting mechanisms. In view of this revised function of EFR3/RBO, it will be of interest to reassess and further...
provide conclusive evidence for a role of PI4KIIIα as a key contributor to plasma membrane PtdIns4P, as previously suggested by pharmacological and knockdown studies (Balla et al., 2005, 2008). Interestingly, in the absence of PI4KIIIα, cells investigate the mechanisms of the temperature-sensitive paralytic phenotype in Drosophila rbo mutants.

The association of PI4KIIIα with the plasma membrane that we show here and the results of our gene KO experiments provide conclusive evidence for a role of PI4KIIIα as a key contributor to plasma membrane PtdIns4P, as previously suggested by pharmacological and knockdown studies (Balla et al., 2005, 2008). Interestingly, in the absence of PI4KIIIα, cells...
Importantly and strikingly, the selective localization of specific intrinsic membrane proteins of the plasma membrane is disrupted by lack of PI4KIIIα. In PI4KIIIα KO MEFs, proteins normally segregated primarily in this membrane, including the M1 muscarinic receptor, a prototypical transmembrane protein (Wess et al., 2007), and the myristoylated/palmitoylated N-terminal anchor of Lck (Rodgers, 2002), were also found and enriched on PtdIns(4,5)P2-positive intracellular structures, which adopted a plasma membrane-like identity. Even EFR3, whose plasma membrane targeting is mediated by palmitoylation and thus at least partially via interactions with plasma membrane lipids (potentially cholesterol-enriched membrane rafts; Foster et al., 2003), localizes to such intracellular sites in PI4KIIIα KO MEFs, indicating that the unique protein composition of the plasma membrane relative to other membranes is perturbed. Effects produced by the deficiency of plasma membrane PtdIns4P on other lipids in this membrane, notably cholesterol, may contribute to these changes. Thus, impairing the production of PtdIns4P at the plasma membrane drastically affects the proteomic and lipidomic composition of the plasma membrane, pointing to PI4KIIIα as a key upstream factor in determining the “identity”

![Figure 8.](image_url) Loss of plasma membrane identity in PI4KIIIα KO MEFs. Proteins typically restricted to the plasma membrane are found on intracellular PtdIns(4,5)P2-positive vesicles, which are negative for a variety of endosomal and Golgi markers. (A) PtdIns(4,5)P2-positive intracellular vesicles, visualized with GFP- or RFP-PHPLCδ, colocalize with endocytic clathrin machinery components (endogenous AP-2, epsin 1, and dynamin 2–GFP) but not with markers of endosomes (endogenous EEA1, GFP-Rab5, and GFP-Rab9), the endosomal phosphoinositide PtdIns3P (GFP-2×FYVE), and Golgi-localized PtdIns4P (GFP-PHOSBP). For clarity, PHPLCδ is false colored green, and the second marker is false colored red in all images. (B) Mislocalization of plasma membrane proteins in cells lacking PI4KIIIα. PI4KIIIα KO MEFs were transfected with M1R-YFP, L10-GFP, or EFR3B-GFP and imaged by confocal microscopy. Note the accumulation of all markers in intracellular vesicles highlighted in the inset images. (C) Measurement of total and plasma membrane fraction of free cholesterol in control and PI4KIIIα KO MEFs. Total free cholesterol levels were measured by HPLC, after lipid extraction, and normalized to total cellular phospholipid levels. *, P < 0.0001 (n = 12 for control and KO). Plasma membrane free cholesterol was measured by treatment of cells with 10 mM MβCD for 1 min followed by quantification of extracted cholesterol using an enzyme-coupled assay and normalization to total protein content. Error bars indicate standard deviations. *, P < 0.02 (n = 6 for control and KO). Bars: (A and B, insets) 5 µm; (B, full size images) 20 µm.
of this membrane. We note that an independent study published just after our manuscript was submitted also reported that generation of PtdIns4P at the plasma membrane is a critical determinant of the identity of this membrane and that loss of plasma membrane PtdIns4P is not matched by a corresponding reduction in PtdIns4(5)P levels (some discrepancies between the two studies may depend upon the different experimental models: Hammond et al., 2012).

In conclusion, our study demonstrates that, in contrast to previous studies, at least a pool of PI4KIIIα is targeted to the plasma membrane. Furthermore, we provide mechanistic insight concerning the recruitment of PI4KIIIα to this membrane and show that the PtdIns4P pool produced by this enzyme plays essential roles not only as a precursor to a variety of key regulatory metabolites but also, either directly or via its downstream metabolites, as a critical determinant of plasma membrane homeostasis and identity.

Materials and methods

Plasmids

The sources of cDNAs were as follows: STIM1-mRFP obtained from B. Baird (Cornell University, Ithaca, NY); GFP-PIK3CA obtained from T. Balla (National Institutes of Health, Bethesda, MD); GFP-GOLPH3 obtained from C. Burd (Yale University, New Haven, CT); CH<sup>h+z</sup>-mCherry obtained from W. Bement (University of Wisconsin-Madison, Madison, WI); GFP-Ph<sup>Pho</sup> obtained from A. De Matteis (Telethon Institute of Genetics and Medicine, Naples, Italy); RFP; GFP<sup>Pho</sup> obtained from S. Grinstein (University of Toronto, Toronto, Ontario, Canada); M1-RFP obtained from B. Hille (University of Washington, Seattle, WA); clathrin light chain-GFP obtained from J. Keen (Thomas Jefferson University, Philadelphia, PA), dynamin (University of Washington, Seattle, WA), C8<sup>z</sup>-GFP obtained from M. McNiven (Mayo Clinic, Rochester, MN), GFP-PIPK1<sup>L10-GFP</sup>, GFP-PIPK1<sup>5Kazusa DNA Research Institute</sup> using the following primer pairs: M1*<sup>5</sup>-GCTCGAGGCCACCATGTACG-<sup>3</sup> and 5<sup>3</sup>-GCACACTCAGTGCGTCAAACTTTCATC-<sup>5</sup>. EFR3B was amplified from mouse brain cDNA using the following primers: 5<sup>5</sup>-GCTCGAGGCCACCATGTACG-<sup>3</sup> and 5<sup>3</sup>-GCACACTCAGTGCGTCAAACTTTCATC-<sup>5</sup>. The PCR amplicon was digested with XhoI and EcoRI and cloned into the pEGFP-N1 vector (Takara Bio Inc.) using XhoI and SalI restriction sites to obtain constructs. Mouse EFR3A (GenBank accession no. BC007482) was amplified by PCR from the appropriate original reagents (Thermo Fisher Scientific), and splicing no. NM_058004.3) expanded the predicted ORF to include the sequence in between M1* and M1. EFR3A was subcloned into the appropriate original EFR3A-containing vector and cloned into the pEGFP-C2 vector (Takara Bio Inc.) for GFP-M1*-PI4KIII<sup>α</sup>. We note that, during the course of our study, an independent study published an alternative kit (QuickChange II XL; Agilent Technologies). Human TTC7B and M1-PI4KIII<sup>α</sup> constructs were generated by amplification from the appropriate original constructs. Mouse EFR3A (GenBank accession no. NM_058004.1 (GenBank access number; Kazusa DNA Research Institute) using the following primer pairs: M1*<sup>5</sup>-GCTCGAGGCCACCATGTACG-<sup>3</sup> and 5<sup>3</sup>-GCACACTCAGTGCGTCAAACTTTCATC-<sup>5</sup>. The PCR amplicon was digested with XhoI and EcoRI and cloned into the appropriate pEGFP<sup>C1</sup> vector (Takara Bio Inc.) for GFP-M1*-PI4KIII<sup>α</sup>. We note that, during the course of our study, an NCBI revision of human PI4KA (available from GenBank under accession no. NM_058004.3) expanded the predicted ORF to include the sequence in between M1* and M1. EFR3B was amplified from mouse brain cDNA using the following primers: 5<sup>5</sup>-GCTCGAGGCCACCATGTACG-<sup>3</sup> and 5<sup>3</sup>-GCACACTCAGTGCGTCAAACTTTCATC-<sup>5</sup>. The PCR amplicon was digested with XhoI and EcoRI and cloned into the pEGFP<sup>N1</sup> vector (Takara Bio Inc.). EFR3B was subsequently subcloned into the pDTomato-N1 vector (Takara Bio Inc.) using XhoI and EcoRI restriction sites to generate the EFR3B-tDTomato construct and into the pDCA3.0 vector (Invitrogen) using C-terminal FLAG (DYKDDDKK) or HA (YPYDVPDYA) tags incorporated into the antisense primers, using EcoRI and NotI restriction sites, to generate the EFR3B-FLAG construct. The C<sub>2</sub>,S<sub>2</sub> mutants of EFR3B-FLAG and EFR3B-tDTomato were generated using a mutagenesis kit (QuickChange II XL; Agilent Technologies). Human TTC7B (GenBank accession no. BC148529) was obtained from Thermo Fisher Scientific and cloned into the pEGFP<sup>N1</sup> vector using Xhol and Sall restriction sites. TCC7B was subsequently subcloned into the pmCherry-N1 vector (Takara Bio Inc.) using a single digestion with Xhol (proper orientation was assessed using a test digestion with BamHI) and into the pCDNA3.0 vector (Invitrogen) with a C-terminal FLAG (DYKDDDKK) tag incorporated into the antisense primers, using HindIII and XbaI restriction sites, to generate the TCC7B-FLAG construct.

Transfection and other reagents

MEFs were electroporated using the Amaxa Nucleofector method (Lonza), and Hela and COS-7 cells were transfected with plasmids using transfection reagent (FuGENE HD; Promega) and with siRNA duplexes (obtained from Integrated DNA Technologies) using Lipofectamine RNAiMAX (Invitrogen).

Antibodies

Antibodies against mouse PI4KIIIα and EFR3B were generated by immunization of rabbits with the following rabbits: PI4KIIIα, GST fusion to residues 1,021–1,207 (based on translation initiation at M1), and EFR3B, a keyhole limpet hemocyanin conjugate of the peptide Ac-TDREDLRKKSKGE-TISLQVCNH (Cocalico Biologicals, Inc.). Sera were affinity purified using the antigenic peptide immobilized on SulfoLink resin following the manufacturer’s instructions (Thermo Fisher Scientific). A full list of antibodies used in this study appears in Table S1. All Western blots were developed by chemiluminescence using the SuperSignal West Pico, Femto, or Dura reagents (Thermo Fisher Scientific) unless otherwise noted. Sources of all antibodies used in this study are provided in Table S1.

Ribosome profiling to determine PI4KIIIα translation start sites

HEK 293 cells were treated for 90 s with 2 µg/ml harringtonine (LKT Laboratories) to specifically immobilize initiating ribosomes while permitting runoff elongation. Cells were washed once in PBS and harvested in lysis buffer consisting of ribosome buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 100 µg/ml cycloheximide) with 0.5% Triton X-100. Cells were incubated for 10 min on ice and triturated, and the lysate was clarified by centrifugation. The clarified lysate was treated with 2.5 µl/µl RNase I (Ambion) for 45 min at room temperature, and ribosomes were harvested by ultracentrifugation (265,000 g for 4 h) through a 1.1 M sucrose cushion. RNA was extracted directly from ribosomal pellets and used to generate a deep sequencing library as previously described (Ingolia et al., 2011). Footprint sequencing libraries were analyzed on a sequencing system (HiSeq 2000; Illumina), and sequences were mapped to the human genome (hg19) using the TopHat short read aligner. Ribosome peptidyl site positions were identified as the location 12 nt (for 28- and 29S footprints) or 13 nt (for 30- and 31S footprints) downstream of the 5′ end of the footprint (Ingolia et al., 2011).

TIRF microscopy

TIRF microscopy was performed at 37°C using an objective-type inverted microscope (IX-70; Olympus) fitted with a 60×, NA 1.45 TIRF microscopy lens (Olympus) and controlled by i>3 software (Andor Technology). Laser lines (488 and 568 nm) from argon and argon/krypton lasers (CVI Melles Griot) were coupled to the TIRF microscopy condenser through a single optical fiber. The calculated evanescent field depth was 100 nm. Cells were typically imaged without binning and with 0.2–0.5-s exposures and detected with a back-illuminated electron-multiplying charge-coupled device camera (512 × 512 pixels; 16 bit; iXon 887; Andor Technology).

Spinning-disc confocal microscopy

All imaging experiments with the exception of TIRF microscopy were performed on a spinning-disc confocal microscope, using the UltraVIEW VoX system (PerkinElmer) including an inverted microscope (Ti-E Eclipse; Nikon) equipped with Perfect Focus, temperature-controlled stage, 14-bit electron-multiplying charge-coupled device camera (C9100-50; Hamamatsu Photonics), and spinning-disc confocal scan head (CSU-X1; Yokogawa Corporation). TIRF microscopy was performed at 37°C using a 60×/1.45 N.A. objective (Olympus) and a 1-mW diode laser. Images were acquired through a 60 or 100× objective (1.4 NA, CFI Plan Apochromat VC). Green fluorescence was excited with a 488-nm/50-mW diode laser (Coherent) and collected by a band pass (BP) 527/55-nm filter. Red fluorescence was excited with a 561-nm/50-mW diode laser (Coherent) and collected by a BP 615/70-nm filter. Near-infrared fluorescence was excited with a 640-nm/50-mW diode laser (CVI Melles Griot) and collected by a BP 705/90-nm filter. Multicolor images were acquired sequentially.
IP experiments
For mouse brain IP experiments, mouse brain homogenates were generated by ~50 pulses in a Dounce homogenizer of ~20 frozen mouse brains thawed into IP lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, and 1% NP-40, pH 7.4, supplemented with an EDTA-free inhibitor cocktail tablet [Complete; Roche]). The suspension was centrifuged at 16,000 g for 30 min. The supernatant was subjected to IP as described in this paragraph. For IP experiments from transfected Hela cells, Hela cells were harvested 24 h after transfection with the appropriate combinations of GFP-M1-P14KIII, TCCB-FLAG, and EFR3B-HA, resuspended in IP lysis buffer, sonicated briefly, and centrifuged for 10 min at 16,000 g. The supernatant was immunoprecipitated in IP lysis buffer by addition of the appropriate primary antibody (or no primary antibody as a negative control) and rocking for 1 h at 4°C followed by the addition of 20 µl protein G–conjugated Sepharose (GE Healthcare) and an additional 1-h incubation at 4°C. The resin was then isolated by centrifugation at 1,000 g, rinsed three times with IP lysis buffer, and analyzed either by SDS-PAGE or Western blotting. In the case of the P14KIII IP, candidate bands were excised from the Coomassie-stained gel, and protein identification by liquid chromatography-mass spectrometry was performed at the Keck Research Facility at Yale University.

Biochemical analysis of EFR3 palmitoylation
Hela cells were transfected with the appropriate EFR3-FLAG construct using FuGENE HD, and palmitoylated proteins were labeled using the protocol of Collier et al. (2011). 5 h after transfection, the media were exchanged for media made with dialyzed serum that was supplemented with 10 µM alk-1 (17-octadecenoyl acid; Cayman Chemicals), and the cells were incubated for an additional 18 h. The cells were then rinsed with PBS, lysed, with M2 anti-FLAG–conjugated agarose (Sigma-Aldrich), and rinses were performed using alk-1 IP buffer (150 mM NaCl, 50 mM triethanolamine, supplemented with an EDTA-free protease inhibitor cocktail tablet [Complete]). After the rinses, the supernatant was aspirated, and biotin-azide (a gift from M. Boyce and C. Bertozzi, University of California, Berkeley, Berkeley, CA) was conjugated to the resin-bound EFR3-FLAG using copper-catalyzed click chemistry. Where indicated, an aliquot of this solution was incubated with 2.5% neutral hydroxylamine (NH2OH). The samples were then boiled and analyzed by Western blotting, detecting with an infrared imaging system (Odyssey; LI-COR Biosciences).

Generation of P14KIIIKO conditional KO mice
The P14KA conditional targeting strategy flanked exons 48–52 with loxP sites and inserted a neomycin resistance gene flanked by flipase recognition target sites into intron 47 (Fig. S3). The targeting vector (Gene Dynamics) was electroporated into hybrid C57BL/6J-129S1/Sv mouse embryonic stem cells (Yale Cancer Center Animal Genomics Shared Resource), and positive clones were identified by Southern blotting (Gene Dynamics; Fig. S3). Targeted embryonic stem cells were injected into blastocysts of C57BL/6J mice and transferred to the uteri of pseudopregnant recipients. Chimeric male offspring were subsequently crossed to an enhanced flipase recombinase-expressing deleter strain (B6.Cg-Tg[ACTFLPe9205Dym]; The Jackson Laboratory) to remove the neomycin resistance gene. Obtained conditional mutant mice were then crossed with either a β-actin-Cre mouse (B6.129-Gt(ROSA)26Sortm1(cre/ERT)Nat/J; The Jackson Laboratory) to generate mutant mice to delete the floxed allele, 5’ TGTGCCATTGTGCCTG-3’; KO allele, 5’ CTGATAAACATGTACACGCACTAA-3’; and N-Tg(ACTB-cre)2Mrt/J; The Jackson Laboratory) to delete the gene and analyze in homozygous condition. Obtained mutant mice were then crossed with either a C/D/N Isotopes Inc. in brief, total cellular lipid extracts were dissolved in a methyl-2,6-di-tert-butylnaphthalene/methanol (1:1) layer with 1:1 methanol/2 mM oxalic acid, and evaporation of the solvent. Analysis was performed as described previously in this paragraph, and the resultant glycerol-inositol phosphate peaks were analyzed by an HPLC system ( Dionex; Thermo Fisher Scientific) with a conductivity detector (Nakatsu et al., 2010).

Acute depletion of PtdIns(4,5)P2 using an optogenetic approach
PtdIns(4,5)P2 was depleted from Hela cells essentially as recently described (Idevall-Hagren et al., 2012). In brief, Hela cells were transfected with CIBN-CAAX, mCherry-CRY2-5Ptase OCRL, and iRFP-PH PLC (Idevall-Hagren et al., 2012). In brief, HeLa cells were transfected with FuGENE HD, and palmitoylated proteins were labeled using the protocol of Collier et al. (2011). 5 h after transfection, the media were exchanged for media made with dialyzed serum that was supplemented with 10 µM alk-1 (17-octadecenoyl acid; Cayman Chemicals), and the cells were incubated for an additional 18 h. The cells were then rinsed with PBS, lysed, with M2 anti-FLAG–conjugated agarose (Sigma-Aldrich), and rinses were performed using alk-1 IP buffer (150 mM NaCl, 50 mM triethanolamine, supplemented with an EDTA-free protease inhibitor cocktail tablet [Complete]). After the rinses, the supernatant was aspirated, and biotin-azide (a gift from M. Boyce and C. Bertozzi, University of California, Berkeley, Berkeley, CA) was conjugated to the resin-bound EFR3-FLAG using copper-catalyzed click chemistry. Where indicated, an aliquot of this solution was incubated with 2.5% neutral hydroxylamine (NH2OH). The samples were then boiled and analyzed by Western blotting, detecting with an infrared imaging system (Odyssey; LI-COR Biosciences).

Chronic depletion of PtdIns(4,5)P2
Chronic depletion of PtdIns(4,5)P2 was achieved by transfection of Hela cells with a constitutively membrane-anchored 5-phosphatase (CAAX-tagged catalytic domain of synaptojanin 1; Milosevic et al., 2005), IRFP-PH(1-110) to monitor PtdIns(4,5)P2, and the myristoylated/palmitoylated anchor L10-GFP to monitor the plasma membrane. Images were acquired 16 h after transfection by confocal microscopy.

Cholesterol measurements
Total free cholesterol was analyzed as described previously (Shui et al., 2011). Only HPLC-grade solvents were used, and the deuterated cholesterol standard (cholesterol-d4; 125/127-d4 in D2O) was obtained from C/D/N Isotopes Inc. In brief, total cellular lipid extracts were dissolved in a 1:1 mixture of chloroform/methanol, spiked with 2.5 µg/ml cholesterol-d4, and analyzed using an HPLC system (1100 Series; Agilent Technologies) coupled to a mass spectrometer (3200 QTRAP; Applied Biosystems). The multiple reaction monitoring transitions for endogenous cholesterol (369.4/161.0) and cholesterol-d4 (375.4/161.0) were monitored in positive ionization mode. Total cholesterol levels were first normalized to the cholesterol-d4 standard and finally presented relative to the measured phospholipid concentration to account for sample-to-sample variation. The values shown in Fig. 8 C represent the mean free cholesterol shown as a percentage of control cell levels. Error bars represent the standard deviation (n = 12 each for control and KO). Plasma membrane cholesterol levels were quantified by brief exposure of cells to 25 µM 1-cyclohexyl-cyclodextrin (MCD) in serum-free medium for 20 min. Mass spectrometric analysis of the amount of extracted cholesterol using enzyme-coupled assay, normalizing to total protein content determined after cell lysis (Zidovetzki and Levitan, 2007). In brief, control and P14KIII KO MEFs were rinsed three times with PBS and incubated with 10 mM MβCD (in PBS) at room temperature for 1 min. The solution was then collected, and the concentration of free cholesterol was measured using the enzymereacted assay kit (Amplex Red; Invitrogen) according to the manufacturer’s instructions.
with fluorescence detection on a fluorescence microplate reader (Infinity M1000; Tecan). The cells were lysed in lysis buffer [150 mM NaCl, 20 mM Tris, 1% Triton X-100, and 1 mM EDTA, pH 7.4, supplemented with an EDTA-free protease inhibitor cocktail tablet [cOmplete]], and protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific). The relative cholesterol concentration for each sample was normalized to protein concentration (n = 6 each for control and KO).

Image analysis
Image analysis was performed using Volocity and ImageJ (National Institutes of Health). For the quantification of GFP-PI3Kα and GFP-PI3Kβ, a ratio of plasma membrane to cytosolic signal (derived from a line scan) was computed for each cell and averaged (n = 29 cells for control; n = 30 cells for KO for GFP-PI3Kα and n = 22 cells for control; n = 23 cells for KO for GFP-PI3Kβ). For quantification of GFP-M1-PI4KIIα plasma membrane puncta (Fig. 3), samples were blinded, a random 100 × 100–pixel area was cropped, and puncta were counted manually (n = 4–6 cells per condition).

Statistical analysis
In all cases throughout the paper, statistical significance was calculated using an unpaired two-tailed Student’s t test with unequal variance, and error bars represent standard deviation.

Online supplemental material
Fig. S1 shows mass spectrometry data supporting the interaction of EF3B and TTC7B with PI4KIIα. Fig. S2 shows that palmitoylation of EF3B is required for recruitment of TTC7B and PI4KIIα to the plasma membrane. Fig. S3 depicts the strategy for generation of a PI4KIII KO mouse. Fig. S4 shows a time course of PtdInsP and PtdInsP 2 levels in PI4KIII KO MEFs. Video 1 shows dynamic behavior of GFP-M1-PI4KIII in this study. Video 1 shows induction of STIM1-mRFP–containing ER–plasma membrane contact sites upon thapsigargin stimulation. Video 2 shows localization of dynamin 2 on dynamic actin comet tails in PI4KIII KO MEFs. Video 6 shows ectopic actin nucleation and dynamic actin comet tails in PI4KIII KO MEFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201010605/DC1.

References