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

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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 4-phosphate (PtdIns4P)) play critical roles 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 protein, Ena, has the ability to bind PtdIns4P and direct its insertion into the plasma membrane.

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Abbreviations used in this paper: 4-OHT, (Z)-4-hydroxytamoxifen; BP, band pass; IP, immunoprecipitation; iRFP, infrared RFP; KO, knockout; MβCD, methyl-β-cyclodextrin; MEF, mouse embryonic fibroblast; PI, phosphatidylinositol; PIK, PI kinase; PIPKI, type I PI monophosphate kinase; PtdIns(4,5)P2, PI 4,5-bisphosphate; PtdIns4P, PI 4-phosphate; RBO, rolling blackout; TIRF, total internal reflection fluorescence.

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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 localized not in the plasma membrane but, surprisingly, on intracellular vesicles. Proteins and lipids normally segregated in the plasma membrane are also found on these vesicles, emphasizing 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 membrane (Audhya and Emr, 2002), a GFP-tagged PI4KIIIα construct based on the published ORF (Nakagawa et al., 1996) displayed diffuse cytosolic localization with no discernable enrichment 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 untagged PI4KIIIα was slightly faster than the mobility of the endogenous protein, as assessed by Western blotting (Fig. 1 B, compare first and third lanes). We thus considered the possibility of an incorrect annotation and reexamined the reported translational 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), including an additional conserved Met residue (defined as M1). Western blot analysis demonstrated that exogenously expressed...
1005Control of PtdIns4P synthesis at the plasma membrane • Nakatsu et al.

the plasma membrane (Baird et al., 2008), our finding is consistent with a role for PI4KIII$\alpha$ at the plasma membrane. It was therefore of interest to determine how PI4KIII$\alpha$ is recruited to the plasma membrane.

PI4KIII$\alpha$ recruitment to the plasma membrane requires its interaction with EFR3 and TTC7

Immunoprecipitation (IP) of endogenous PI4KIII$\alpha$ 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 PI4KIII$\alpha$ 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 PI4KIII$\alpha$ recruitment, we expressed tagged versions of these proteins and performed knockdown experiments.

M1-PI4KIII$\alpha$ co-migrated with endogenous PI4KIII$\alpha$, in contrast to the smaller M1*-PI4KIII$\alpha$ (Fig. 1 B). Furthermore, determination of PI4KA 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 PI4KIII$\alpha$ and next investigated whether M1-PI4KIII$\alpha$ had a different subcellular localization than M1*-PI4KIII$\alpha$. The majority of GFP-M1-PI4KIII$\alpha$ fluorescence was still localized in the cytosol (Fig. 1 A, right). However, total internal reflection fluorescence (TIRF) microscopy revealed that GFP-M1-PI4KIII$\alpha$, but not GFP-M1*-PI4KIII$\alpha$, transiently visited the plasma membrane (Fig. 1 E and Video 1; also see Fig. 3). Although this dynamic behavior of PI4KIII$\alpha$ 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 PI4KIII$\alpha$ at the plasma membrane. It was therefore of interest to determine how PI4KIII$\alpha$ 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. 4A). Accordingly, a bioorthogonal metabolic labeling approach (Hang et al., 2011)

Individually, TTC7B was localized in the cytosol, whereas EFR3B was found constitutively at the plasma membrane (Fig. 2 C). Coexpression of GFP-M1-PI4KIIIα with EFR3B and TTC7B resulted in significant colocalization of all three proteins at the plasma membrane (Fig. 2 D). Control experiments demonstrated that although EFR3B could recruit TTC7B to the plasma membrane (Fig. 2 E), EFR3B alone was not sufficient to recruit GFP-M1-PI4KIIIα to this membrane (Fig. 2 F). We note that the stable association of a large fraction of the cellular GFP-M1-PI4KIIIα pool with the plasma membrane when it is cooverexpressed with TTC7B and EFR3B stands in contrast the puncta observed by TIRF microscopy in cells expressing only GFP-M1-PI4KIIIα (Fig. 1E). Thus, most likely, when M1-PI4KIIIα is overexpressed alone, its predominant localization in the cytosol as well as its dynamic behavior at the plasma membrane (Fig. 1 E) are explained by limiting amounts of endogenous EFR3B and TTC7B at the plasma membrane. Consistent with this possibility, siRNA-mediated knockdown of EFR3B (and its close homologue EFR3A) and/or TTC7B (and its close homologue TTC7A) resulted in a striking reduction of the dynamic GFP-M1-PI4KIIIα puncta at the plasma membrane (Fig. 3). Collectively, these experiments demonstrate

**Figure 2.** PI4KIIIα, EFR3B, and TTC7B form a complex at the plasma membrane. (A) Western blot analysis of anti-EFR3B and control immunoprecipitates from mouse brain homogenates. (B) Western blot analysis of anti-GFP, anti-HA, and anti-FLAG immunoprecipitates from lysates of HeLa cells transfected with GFP-M1-PI4KIIIα, EFR3B-HA, and TTC7B-FLAG. (C) Confocal imaging of live HeLa cells transfected with TTC7B-GFP (top) or EFR3B-tdTomato (bottom). (D) Imaging analysis of HeLa cells triple transfected with GFP-M1-PI4KIIIα, TTC7B-mCherry, and EFR3B-HA. EFR3B-HA immunofluorescence was performed with an anti-HA antibody followed by an Alexa Fluor 647–conjugated anti–rat secondary antibody. (E and F) Imaging analysis of HeLa cells double transfected with EFR3B-HA and either TTC7B-mCherry (E) or GFP-M1-PI4KIIIα (F) after anti-HA immunofluorescence labeling. Insets show the region outlined with dotted lines at higher magnification. Bars: (full size images) 20 µm; (insets) 2 µm.
that also harbored a Cre recombinase-estrogen receptor fusion transgene (Badea et al., 2003). Addition of (Z)-4-hydroxytamoxifen (4-OHT) to primary cultures of mouse embryonic fibroblasts (MEFs) derived from these mice induced gene recombination, leading to complete disappearance of PI4KIIα within 10 d (Fig. 5 A) and a dramatic depletion of cellular PtdIns4P (to 25% of control cell levels; Figs. 5 B and S4).

The subcellular distribution of PtdIns4P in KO MEFs was probed via PtdIns4P biosensors that selectively bind to either the plasma membrane or the Golgi pool of this phosphoinositide (GFP-PH[PLC]δ [Balla et al., 2008] and GFP-GOLPH3 [Wood et al., 2009], respectively). These experiments revealed a selective loss of plasma membrane PtdIns4P in KO MEFs (Fig. 5, D–F). The morphology of the Golgi complex was not affected (Fig. 5, F and G), in contrast to the dramatic changes in Golgi complex structure and function observed in cells lacking PI4KIIβ 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-PI4KIIα but not GFP-M1*-PI4KIIα (Fig. 6, E–H; and Video 4), demonstrating that only GFP-M1-PI4KIIα is a fully functional protein.

Other PtdIns(4,5)P2-mediated processes occurring at the plasma membrane were clearly altered in PI4KIIα 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 PI4KIIα 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 PI4KIIα KO MEFs (Fig. S5 A). Additionally, most of these puncta were stable and did not turn over as in control MEFs, as visualized by TIRF microscopy (Fig. S5 B).

**Ectopic PtdIns(4,5)P2 synthesis and localization in PI4KIIα KO cells**

Expression of RFP-PH[PLC]δ, a biosensor for PtdIns(4,5)P2, revealed a robust reduction of PtdIns(4,5)P2 levels at the plasma membrane as expected (Fig. 7 A and B). Most surprisingly,
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\(_\delta\), no formation of intracellular PHPLC\(_\delta\)-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\(\alpha\) 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\(\alpha\) KO cells

Further characterization of the PtdIns(4,5)P\(_2\)-positive intracellular vesicles in PI4KIII\(\alpha\) 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\(\alpha\) KO cells. Although both PtdIns(4,5)P\(_2\), as detected by RFP-PH PLCI\(_\delta\), 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\(_\delta\) and for GFP-PIPKI\(\beta\) were detected intracellularly in KO cells (Fig. 7, A [inset] and C). Furthermore, PIPKIs (PIPKI\(\beta\) and PIPKI\(\gamma\)) 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\(\alpha\) 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 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.

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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 EFR3 and TTC7 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

Figure 6. Loss of PI4KIIIα causes perturbations of PtdIns4P- and PtdIns(4,5)P2-dependent processes at the plasma membrane. (A–H) Control (A and B) and PI4KIIIα KO MEFs (C–H) were transfected with STIM1-mRFP. For rescue experiments, KO MEFs were transfected with GFP-M1*-PI4KIIIα (E and F) or GFP-M1-PI4KIIIα (G and H). Plasma membrane–associated STIM1-mRFP fluorescence was imaged by TIRF microscopy before (−Tg) or 8 min after (+Tg) the addition of 1 µm thapsigargin (Tg). (I) F-actin in control and PI4KIIIα KO MEFs as revealed by staining with fluorescently labeled phalloidin. (J and K) Altered assembly of the endocytic clathrin machinery in control and PI4KIIIα KO MEFs as assessed by immunofluorescence labeling of AP-2 (J) and epsin 1 (K). Insets show a representative region at higher magnification. Bars: (A–H) 10 µm; (I–K, full size images) 20 µm; (J and K, insets) 5 µm.
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
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” very strongly up-regulate the expression of PIPKIs, which convert PtdIns4P to PtdIns(4,5)P2, so that PtdIns(4,5)P2 levels are much less drastically decreased than PtdIns4P levels. The intracellular signaling pathways underlying this compensatory change remain to be determined and represent an important question to be addressed by future work. Additionally, PtdIns(4,5)P2, which is normally primarily concentrated at the plasma membrane, was surprisingly mislocalized on intracellular structures in PI4KIIIα KO MEFs, leading to a partial relocation onto these vesicles of actin regulatory proteins and endocytic proteins normally restricted to the plasma membrane. Because binding to PtdIns4P contributes to the membrane targeting of PIPKIs (Kunz et al., 2002), PtdIns4P generated on endosomes by type II PI 4-kinases (Balla and Balla, 2006) may play a role in the ectopic localization of the PIPKIs and may function as a substrate for PtdIns(4,5)P2 generation. Thus, it remains possible that the very robust reduction in total PtdIns4P levels observed in PI4KIIIα KO MEFs may be caused not only by lack of PtdIns4P production at the plasma membrane but also by increased consumption of the PtdIns4P pool generated by type II PI 4-kinases.

Figure 8. 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-PHPLCβ1, 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-Δrs), and Golgi-localized PtdIns4P (GFP-PHOSBP). For clarity, PHPLCβ1 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 PtdIns(4,5)P2 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 PI4KIIα is targeted to the plasma membrane. Furthermore, we provide mechanistic insight concerning the recruitment of PI4KIIα 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: STM1-mKFP obtained from B. Baird (Cornell University, Ithaca, NY), GFP-PH-domain obtained from T. Balla (National Institutes of Health, Bethesda, MD), GFP-GOLPH3 obtained from C. Burd (Yale University, New Haven, CT), CHIapo, mCherry obtained from W. Bement (University of Wisconsin-Madison, Madison, WI), GFP-PH<sup>1ph</sup> obtained from A. De Mattei (Telethon Institute of Genetics and Medicine, Naples, Italy), RFP-PH<sup>1ph</sup> obtained from S. Grinstein (University of Toronto, Toronto, Ontario, Canada), M1RFP obtained from B. Hille (University of Washington, Seattle, WA), chitin light chain-GFP obtained from J. Keen (Thomas Jefferson University, Philadelphia, PA), dynamin 2-GFP obtained from M. McNiven (Mayo Clinic, Rochester, MN), GFP-Rab9 obtained from S. Pfeffer (Stanford University, Stanford, CA), GFP-2xFYVE<sup>th</sup> obtained from H. Stenmark (Oslo University, Oslo, Norway), and L<sup>1</sup>-GFP, GFP-FIPIK1<sup>k</sup>87, IFRF-PH<sup>1ph</sup>, CIBN-CAAX, mCherry-CRY2-SPhage<sup>1ch</sup>, mFP-FP1-CAAX, GFP-RoB5, and GFP-PH<sup>8ph</sup> obtained from the De Camilli laboratory. M1*–PI4KIIα and M1–PI4KIIα were amplified by PCR from the following cDNA clones, respectively: NM_058004.1 (GenBank accession number; OriGene) and AB210002 (GenBank accession number; Kazusa DNA Research Institute) using the following primer pairs: M1*–PI4KIIα, 5′-GCACACTCAGGGGGATGTCATTCTGA-3′ and 5′-GCTCGAGGCCACCATGTACG-3′, 5′-GCACACTCAGGGGGATGTCATTCTGA-3′ and 5′-GTGTGTGTGGCTGC-3′, 5′-GTGTGTGTGGCTGC-3′ and 5′-GCTCGAGGCCACCATGTACG-3′, and 5′-GCTCGAGGCCACCATGTACG-3′ and 5′-GTGTGTGTGGCTGC-3′. We note that, during the course of our study, an antisense primer for GFP-M1*-PI4KIIα-containing vector and cloning into the pEGFP-C2 vector (Takara Bio Inc.) and cloning into the pEGFP-C1 vector (Takara Bio Inc.) were amplified by PCR from the human genome (hg19) using the TopHat short read aligner. Ribo- somes were harvested by ultracentrifugation (265,000 × g for 4 h) through a 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 pellet 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 iG 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 of America) controlled by Velocity software (PerkinElmer). All images were acquired through a 60 or 100× oil objective (1.4 NA, CFI Plan Apochro- mat 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.

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 PI4KIIα and EFR3B were generated by immunization of rabbits with the following constructs: PI4KIIα, 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-TDEDRLSKRKSIGE-2051Control of PtdIns4P synthesis at the plasma membrane • Nakatsu et al. on December 31, 2017 jcb.rupress.org Downloaded from
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-PI4KIII, TTC7B-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. Rocking. 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 PI4Klll 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 Ingham et al. (2011). 5 h after transfection, the media were exchanged for media made with dialyzed serum that was supplemented with 10 µM alk-16 (17-octadecynoic acid; Cayman Chemicals), and the cells were incubated for an additional 18 h. The cells were then rinsed with PBS, lysis, IP with M2 anti-FLAG–conjugated agarose (Sigma-Aldrich), and rinses were performed using alk-16 IP buffer (150 mM NaCl, 50 mM triethanolamine, and 1% Triton X-100, pH 7.4, 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-coupling click chemistry. Where indicated, an aliquot of this solution was incubated with 2.5% neutral hydroxyamine (NH2OH). The samples were then boiled and analyzed by Western blotting, detecting with an infrared imager (Odyssey; LI-COR Biosciences).

Generation of PI4KIIIβ conditional KO mice
The PI4KA conditional targeting strategy flanked exons 48–52 with loxP sites and inserted a neomycin resistance gene flanked by flippase 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 recipient female CD1 mice. Male offspring were subsequently crossed to a enhanced flippase recombinase-expressing deleter strain (B6.Cg-Tg(ACTFLPe)9205Dym/J; The Jackson Laboratory) to remove the neomycin resistance gene. Obtained conditional mutant mice were then crossed with either a β-actin-Cre mouse (FVB/N-Tg(Actb-cre)2Mtr/J, The Jackson Laboratory) to delete the PI4KA gene ubiquitously or a strain expressing an Esr1-Cre fusion protein (B6.129-Gt(Rosa)26Sortm1(cre/ERT2)J, The Jackson Laboratory) to generate mutants in which PI4KA gene ablation could be induced pharmacologically by the addition of 4-OHT. Animal care and use was performed in accordance with our institutional guidelines. Genotyping of the mutant mice was performed by PCR using the following primers: wild-type allele, 5′-GTCGCCAGACTGACTTTGGCATGTATG-3′ and 5′-GGATGCTGTGCAGCACCAC-3′; KO allele, 5′-GGATGCTGTGCAGCACCAC-3′; KO allele, 5′-CCGCCAGACTGACTTTGGCATGTATG-3′, and 5′-GGATGCTGTGCAGCACCAC-3′; KO allele, 5′-GGATGCTGTGCAGCACCAC-3′; KO allele, 5′-GGATGCTGTGCAGCACCAC-3′; KO allele, 5′-GACCAGGAGGTAGACTCTGAGTAGTCAAG-3′ and 5′-GACCAGGAGGTAGACTCTGAGTAGTCAAG-3′.

Generation of PI4KIIIβ conditional KO MEFs
Primary MEFs were generated from embryonic day 15 to postnatal day 1 from the appropriate strains. Some populations of MEFs were immortalized using the standard NIH 3T3 protocol (Ferguson et al., 2009). To generate cells lacking PI4KIIIβ, recombination was induced by treatment of cells with 0.3–1.5 µM 4-OHT for 24–36 h followed by replacement of the media and subsequent growth for a total period of 7–10 d. Cells were grown in DMEM containing glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. In this study, three different sets of control and KO cells were used interchangeably, as indicated in Table S2, and no differences were observed between sets of control/KO pairs in all assays in which multiple sets were tested.

Quantification of phosphoinositide levels
For the quantification of PtdIns4P and PtdIns(4,5)P₂ shown in Fig. 5, control and PI4KIIIβ KO MEFs were metabolically labeled with [3H]myo-inositol for days 7–10 after treatment with 4-OHT (in the case of PI4KIIIβ KO MEFs). Cells were lysed in 4.5% perchloric acid, and the pellet was rinsed three times with 0.1 M EDTA. The pellet was deacylated with a mixture of methylene/water/n-butanol/methanol (36:8:9:47) for 1 h at 50°C and dried in a SpeedVac (SVC 100; Savant), and the residue was extracted using 500 µl of a mixture of n-butanol/petroleum ether/ethyl formate (20:40:1) and 500 µl of water. The aqueous phase was resolved using anion-exchange HPLC with an ammonium phosphate gradient (LC-20AT ultrafast liquid chromatography [Shimadzu] equipped with a Partisil 5 SAX 4.6 x 125-mm column [Whatman] and identified using a radiometric detector (β-RAM 4B, LabLogic). The identity of each peak was determined by comparison with known compounds, and the values reported in Fig. 5 are shown as the amount of the desired lipid as a percentage of total detectable inositol phospholipids (PtdIns, PtdIns4P, and PtdIns(4,5)P₂). For the time course of phosphoinositide measurements shown in Fig. S4, total anionic lipids were isolated by 1:1 methanol/aqueous 1 M HCl and 2 mM AlCl₃, followed by addition of chloroform, extraction of the organic layer with 1:1 methanol/2 mM oxalic acid, and evaporation of the solvent. Decaylation 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) equipped 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-CAXX, mCherry-CRY2-PH(0.4), and IRFP-PH(0.4) and kept in the dark before the experiment. PtdIns(4,5)P₂ depletion was initiated by illumination with 488-nm light after a baseline image of the mCherry and IRFP channels was acquired. Constant PtdIns(4,5)P₂ depletion was ensured by repeated 200-ms illuminations with 488-nm light at 1-min intervals for 1 h, whereas mCherry and IRFP images were acquired at 15-min intervals to minimize photobleaching.

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(0.4) 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-26,26,26,27,27-d₅) was obtained from C/D/N Isotopes Inc. In brief, total cellular lipids were dissolved in a 1:1 mixture of chloroform/methanol, spiked with 2.5 µg/ml cholesterol-d₅, 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-d₅ (375.4/161.0) were monitored in positive-ion electrospray mode on a QTRAP 3200 with nitrogen gas as the collision gas. Mass spectrometric cholesterol levels were first normalized to the cholesterol-d₅ 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 [3H]-myo-inositol and 1-[14C] cyclohextrin (Sigma). Total internalized cholesterol was calculated as the difference between the amount of extracted cholesterol using an enzyme-coupled assay, normalizing to total protein content determined after cell lysis (Zidovetzki and Levitan, 2007). In brief, control and PI4KIIIβ 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 determined using the enzyme-coupled assay kit (Amplex Red; Invitrogen) according to the manufacturer’s instructions.
with fluorescence detection on a fluorescence microplate reader (infinite 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 [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-PHOsh2 and GFP-PH PLC, Image analysis was performed using Volocity and ImageJ (National Institutes of Health for each sample was normalized to protein concentration (n = 29 cells for control; n = 30 cells for KO for GFP-PH0sh2 and n = 22 cells for control; n = 23 cells for KO for GFP-PH PLC)). For quantification of GFP-M1-Pi4KIIIa 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 EFR3B KO for GFP-PH PLC with fluorescence detection on a fluorescence microplate reader (Infinite M1000).Fig. S2 shows that palmitoylation of EFR3B is required for recruitment of TTC7B and PI4KIII KO for GFP-PH PLC with fluorescence detection on a fluorescence microplate reader (Infinite M1000).

References