



Class III PI 3-kinase is the main source of PtdIns3P substrate and membrane recruitment signal for PIKfyve constitutive function in podocyte endomembrane homeostasis

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ABSTRACT

The evolutionarily conserved PIKfyve, which synthesizes PtdIns5P from PtdIns, and PtdIns(3,5)P₂ from PtdIns3P, requires PtdIns3P as both an enzyme substrate and a membrane recruitment signal. Whereas the PtdIns3P source is undetermined, class III PI3K (Vps34), the only evolutionarily conserved of the eight mammalian PI3Ks, is presumed as a main candidate. A hallmark of PIKfyve deficiency is formation of multiple translucent cytoplasmic vacuoles seen by light microscopy in cells cultured in complete media. Such an aberrant phenotype is often observed in cells from conditional Vps34 knockout (KO) mice. To clarify the mechanism of Vps34 KO-triggered vacuolation and the PtdIns3P source for PIKfyve functionality, here we have characterized a podocyte cell type derived from Vps34^{fl/fl} mice, which, upon Cre-mediated gene KO, robustly formed cytoplasmic vacuoles resembling those in PIKfyveKO MEFs. Vps34^{wt}, expressed in Vps34KO podocytes restored the normal morphology, but only if the endogenous PIKfyve activity was intact. Conversely, expressed PIKfyve^{wt} rescued completely the vacuolation only in PIKfyveKO MEFs but not in Vps34KO podocytes. Analyses of phosphoinositide profiles by HPLC and localization patterns by a PtdIns3P biosensor revealed that Vps34 is the main supplier of localized PtdIns3P not only for PIKfyve activity but also for membrane recruitment. Concordantly, Vps34KO podocytes had severely reduced steady-state levels of both PtdIns(3,5)P₂ and PtdIns5P, along with PtdIns3P. We further revealed a plausible physiologically-relevant Vps34-independent PtdIns3P supply for PIKfyve, operating through activated class I PI3Ks. Our data provide the first evidence that the vacuolation phenotype in Vps34KO podocytes is due to PIKfyve dysfunction and that Vps34 is a main PtdIns3P source for constitutive PIKfyve functionality.

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1. Introduction

PIKfyve is an evolutionarily conserved kinase that is a product of a single gene in metazoans. Mammalian PIKfyve, synthesizing phosphatidylinositol (PtdIns)¹ 5P from PtdIns, and PtdIns(3,5)P₂ from

PtdIns3P, represents a major node in PI signaling and membrane trafficking downstream of PtdIns3P [1]. PIKfyve controls both constitutive and regulated endocytic trafficking as evidenced by the presence of its lipid products in quiescent cells and their elevation upon activation of endosome dynamics by cues such as insulin, EGF or viral infection [2–8]. A hallmark of PIKfyve cellular inactivation, achieved by means of PIKfyve dominant-negative kinase-deficient mutants [9,10], pharmacological inhibition [5,11,12], siRNA-mediated silencing [13] or Cre-mediated gene disruption [14,15], is progressive dilation of endosomal membranes, culminating in the appearance of multiple translucent cytoplasmic vacuoles readily seen by light microscopy in dividing cells grown in complete media. Because the vacuoles are selectively rescued by cytoplasmic microinjections of PtdIns(3,5)P₂ but not PtdIns5P [10], the aberrant phenotype has been widely used as a sensitive intracellular indicator for selective PIKfyve-dependent PtdIns(3,5)P₂ deficiency [reviewed in [1,2]].

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¹ Abbreviations: PtdIns, phosphatidylinositol; PI, phosphoinositide; PIKfyve, phosphoinositide kinase for position five containing a fyve domain; PAS, PIKfyve-ArPIKfyve-Sac3; Ad-Cre, adenovirus with Cre-recombinase; MEFs, mouse embryonic fibroblasts; MOI, multiplicity of infection; eGFP, enhanced green fluorescence protein; GroPIns, glycerophosphorylinositol; wt, wild-type; KO, knockout; GDI1, GDP dissociation inhibitor 1; and RIPA, radioimmune precipitation assay buffer.

These and other morphological and biochemical data have suggested a rate-limiting function of the PIKfyve-catalyzed PtdIns3P to PtdIns(3,5)P₂ conversion in the course of early endosome maturation, controlling cargo exit from early endosomes and progression of endocytic trafficking [1,2].

The PtdIns3P-producing enzyme(s) supplying the PtdIns3P substrate for the PIKfyve lipid kinase activity is (are) not yet delineated. Likewise, whereas it is well established that PtdIns3P is a major targeting signal for PIKfyve recruitment to Rab5^{GTP}-organized endosome platforms [16,17], whether it is generated by the same enzyme supplying the substrate awaits clarification. Based on the fact that, like PIKfyve, human Vps34, a class III phosphatidylinositol 3-kinase (gene symbol PIK3C3) is evolutionarily conserved, with the orthologous Vps34 being the only PtdIns3P-synthesizing enzyme in *Saccharomyces cerevisiae* [18], its role as a main PtdIns3P source for both PIKfyve activity and localization has been presumed but never studied. Indeed, the vesicular localization pattern of PIKfyve is rendered diffuse in several cell types treated with low concentrations of wortmannin, a powerful catalytic inhibitor of Vps34 [13,16,17]. However, in metazoans, PtdIns3P is also produced by Vps34-independent mechanisms operating through class I and class II PI3Ks. For example, PtdIns(3,4,5)P₃, made at the internal leaflet of the plasma membrane by activated wortmannin-sensitive class I PI3Ks [19,20], might serve as a source of endosomal PtdIns3P subsequent to two consecutive dephosphorylation reactions by the PI 5- and 4-phosphatases [21,22]. Likewise, class II PI3Ks produce PtdIns3P directly from PtdIns and, indirectly, by 3-phosphorylation of PtdIns4P followed by 4-dephosphorylation of PtdIns(3,4)P₂ [23–25], with class II PI3Kβ also being wortmannin-sensitive [26].

Strikingly, Vps34 perturbation by siRNA silencing or delivery of inhibitory antibodies causes cytoplasmic vacuolation resembling that seen upon PIKfyve dysfunction [27–29]. Similarly, primary cells derived from recently developed mouse models with conditional *Vps34* gene disruption also demonstrate the aberrant phenotype of translucent cytoplasmic vacuoles, which often occurs in the absence of autophagic abnormalities [30–32]. Hence, an outstanding question awaiting clarification is whether the aberrant endomembrane phenotype in cells with ablated Vps34-catalyzed PtdIns3P pool is due to arrested downstream signaling by PIKfyve.

Here we used podocytes with *Vps34* Cre recombinase-mediated gene disruption in combination with a rescue approach to unravel the functional relationship between PIKfyve and Vps34 lipid kinases and their role in endomembrane homeostasis. MEFs with *Pikfyve* Cre recombinase-mediated gene disruption were used as reference. We identified that PtdIns3P generated by Vps34 serves as both a major precursor for PtdIns(3,5)P₂ synthesis and a membrane localization target of PIKfyve in resting cells. We clarified that disrupted endomembrane homeostasis in *Vps34* KO podocytes is due to an ablated Vps34-controlled PtdIns3P pool and consequent abrogation of PIKfyve-catalyzed PtdIns(3,5)P₂ and PtdIns5P production. We further revealed that activated class I PI3Ks could contribute to some extent to the regulation of endomembrane homeostasis.

2. Materials and methods

2.1. Cell lines

Pikfyve and *Vps34* (*fl/fl* or *wt/wt*) MEFs were isolated from E12.5 embryos derived from the transgenic mouse models [14,33] following previously described procedures [14]. Glomerular cells were isolated from kidneys of *Vps34*^{fl/fl} mice [33]. Briefly, removed kidneys were chopped on ice into fine pieces and then passed through sieves in a laminar flow hood. Small pieces were first passed through a 70 μm sieve (BD Falcon) and collected in a 50 ml tube. A sterile syringe plunger was used to push the small pieces through the sieve. Following thorough rinses with sterile PBS, the material was collected and then passed through a second 50 μm sieve, followed by rinses with PBS. The material on top of the

50 μm sieve was transferred to a 50 ml tube and then spun down to form a pellet. Cells, resuspended in RPMI supplemented with insulin, transferrin and selenium (each at 1%), and containing 50 U/ml penicillin/50 μg/ml streptomycin, were seeded on 10 cm tissue-culture plates. Plates were incubated at 37 °C for 10–15 days with media change every 3–4 days. Cellular outgrowths were visible at days 6–8 and predominantly consisted of podocytes, with only a minor presence of mesangial and endothelial cells [34,35]. Hence, the glomerular cells are hereafter referred to as podocytes. Cells were then trypsinized and seeded on fresh culture plates for 24–48 h. Cells were then immortalized using SV40 T antigen, packaged in a Retro Viral vector (MMLV-SV40-VSVG), and serum-free DMEM/RPMI media containing polybrene (8 μg/ml) as previously described [34]. Primary cells or transiently transfected cells stopped dividing after a few rounds of sub-culturing and therefore further selection was not required. Podocytes used in this study were derived from two *Vps34*^{fl/fl} mice (#3176 and #3061) and showed similar results. Control podocytes were from the *Vps34*^{wt/wt} mouse (#3175). All experiments were performed with cells between 3 and 5 passages. Unless indicated, all cell types were maintained in complete DMEM, containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin sulfate.

2.2. Adenoviral transduction and transient transfection

Cells were treated with recombinant adenovirus expressing Cre recombinase (Ad-Cre) or empty Ad (titer 1.3×10^{11} ; Vector Core, University of Michigan, Ann Arbor), dissolved in complete media at MOI = 200 and a minimal volume covering the cells. 4 h later, complete media was added to a volume optimal for the dish size. Media was replaced 14 h later with fresh complete media. *Pikfyve* KO MEFs (100 mm confluent dishes) were transfected on day 4 post-Ad-Cre treatment by electroporation using 50 μg of pEGFP-2xYFVE^{Pikfyve} [14] or pTag-YFP-Vps34^{wt} cDNAs under a single pulse of 975 μF and 0.16 kV (Biorad-Gene pulser II) as described previously [36]. *Vps34* KO podocytes were transfected with the above cDNAs by Lipofectamine 3000 transfection kit (Invitrogen) following manufacturer protocols. Where indicated, 24 h posttransfection, *Vps34* KO podocytes were treated (60 min; 37 °C) with 1 μM of the YM201636 inhibitor (Symansis NZ) dissolved in DMSO or only with vehicle.

Recombinant adenoviruses expressing HA-PIKfyve^{wt} and GFP from separate CMV promoters (Ad_{GFP}-PIKfyve^{wt}) or only GFP (Ad_{GFP}) were used at MOI = 10, with MOI = 1, being the lowest dilution reaching 100% infection efficiency in HEK293 cells for 14 h [9]. The chosen MOI delivered infection in ~20–25% of the total *Pikfyve* KO or *Vps34* KO cells, the non-infected cells on the same dish thereby serving as controls.

2.3. Light, fluorescence and electron microscopy analyses

Cells, live or fixed in 4% formaldehyde for 30 min, were viewed at 200× or 400× magnification with a Nikon Eclipse TE200 inverted fluorescence microscope as specified in the figure legends. Images were captured with a SPOT RT Slider charge-coupled device camera (Diagnostic Instruments). Cells expressing eGFP-2xYFVE^{Pikfyve} or YFP-Vps34^{wt} were visualized by phase-contrast and fluorescence microscopy using a standard green channel [14,17]. Cells exhibiting at least 5 perinuclear translucent vacuoles were considered as vacuolated. Under each experimental design, 20–100 cells were counted in 3–4 separate experiments. For electron microscopy analyses, Ad or Ad-Cre-treated *Vps34*^{fl/fl} podocytes grown in complete media (10 cm dish) were trypsinized and pelleted at 4000 g for 5 min. Cell pellets were washed with PBS and fixed in a Sorenson's buffer (0.1 M), containing 2% paraformaldehyde and 2.5% glutaraldehyde, again washed with PBS and fixed using 1% osmium tetroxide. Cell pellets were dehydrated in different strengths of ethanol followed by propylene oxide. After embedding in a resin,

70–100 nm thick sections were collected over copper grids and stained with 2% aqueous uranyl acetate and lead citrate solutions. For imaging, a Philips CM-100 transmission electron microscope equipped with compustage and high resolution (2 K × 2 K) digital camera was used at the University of Michigan core imaging facility.

2.4. Myo-[2-³H] inositol cell labeling, lipid extraction and HPLC analyses

Vps34^{fl/fl} podocytes (35 mm dishes) were treated with Ad-Cre or empty Ad viruses as specified above. On day 4, cells were washed two times with inositol/glucose-free DMEM, containing 5% dialyzed FBS, 0.5% BSA, 5 µg/ml insulin and 5 µg/ml transferrin, 2 mM pyruvate, 25 mM HEPES, pH 7.4, and 100 units/ml penicillin and 100 µg/ml streptomycin for 24 h. Cells were then labeled with 25 µCi/ml myo-[2-³H]inositol (Perkin Elmer) in a fresh media, as detailed above, for 26 h. Cellular lipids were extracted, deacylated, and analyzed by HPLC (Waters 5215) on a 5 µm Partisphere SAX column (Whatman) as we described previously [5,7,14,37]. [³²P]GroPIns5P, [³²P]GroPIns3P, [³²P]GroPIns(4,5)P₂ and [³²P]GroPIns(3,5)P₂ prepared by enzymatic synthesis with [γ-³²P]-ATP were used as HPLC standards as described elsewhere [5,7,14,37]. Fractions were collected every 0.25 min and analyzed for [³H] and [³²P] radioactivity after addition of scintillation mixture. Data evaluation and documentation was performed by Microsoft EXCEL software. Individual peak radioactivity was quantified by area integration and presented as a percentage of the summed radioactivity from the [³H]-GroPIns3P, -4P, -5P, -(3,5)P₂ and -(4,5)P₂ peaks (“total radioactivity”).

2.5. Western blot analysis

Cells were scraped in RIPA + buffer [(composed of 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 and 0.5% Na deoxycholate), supplemented with 1 × protease inhibitors [14]]. Proteins (60–150 µg/lane) were separated by PAGE, transferred onto nitrocellulose membrane and probed with previously characterized polyclonal antibodies against Vps34 [a gift from Jon Backer [38]], Sac3 [37], PIKfyve [R7069 [39]] and anti-GDI1 [40].

2.6. Immunoprecipitation and in vitro PI3K activity assay

Fresh lysates from *Vps34* KO podocytes, treated with or without Ad-GFP for 16 h, were collected in RIPA⁺⁺ buffer containing 1 × protease and 1 × phosphatase inhibitor cocktails as described [41]. Precleared lysates (20,000 ×g, 15 min, 4 °C) were subjected to immunoprecipitation with rabbit polyclonal anti-p85 or mouse monoclonal anti-phosphotyrosine antibodies (4G10), both from EMD Millipore. The in vitro PI3K activity assay was conducted essentially as we described previously [42] and detailed in Supplementary Experimental Procedures.

2.7. Other assays and statistics

Protein concentration was determined by bicinchoninic acid protein assay. Protein and lipid levels on the blots and TLC plates, respectively, were quantified by laser scanning densitometry (Epson V700) and UN-SCAN-IT software (Silk Scientific). Several films of different exposure times were analyzed to assure that the signals were within the linear range of detection sensitivity. Data are presented as mean ± SE. Statistical analysis of variance was performed by t test for independent samples and a one-tail t test for paired samples, with *p* < 0.05 considered statistically significant.

3. Results

3.1. Identification of *Vps34* KO cells robustly forming cytoplasmic vacuoles in complete culture media

Along with PIKfyve protein loss, the excision of the floxed alleles in MEFs isolated from *Pikfyve^{fl/fl}* mice triggered progressively exacerbating cytoplasmic vacuolation 3–5 days posttransfection with GFP-Cre recombinase, readily visible by light microscopy of cells cultured in complete (serum-containing) media, as we have previously reported [14]. This result was extended herein for cells transduced with adenovirus expressing Cre recombinase, whereby only *Pikfyve^{fl/fl}* but not the *Pikfyve^{wt/wt}* MEFs showed a profound vacuolation phenotype, reaching ~90% on the 5th day posttransduction (Fig. 1A and B). PIKfyve protein levels were undetectable under these conditions (Fig. 1D). By contrast, control *Pikfyve^{fl/fl}* MEFs infected with empty adenovirus displayed only a very low % of vacuolating cells (<2%, Fig. 1A, panel b) until the end of the 7-day observation.

To begin elucidating the functional relationship between the enzymatic products of Vps34 and PIKfyve as well as their role in endomembrane homeostasis, we aimed at identifying a *Vps34^{fl/fl}* cell type that, upon Ad-Cre treatment would exhibit a robust time-course of cell vacuolation as seen in *Pikfyve^{fl/fl}* – Ad-Cre-treated MEFs. Surprisingly, examination of primary *Vps34^{fl/fl}* MEFs showed only 10 and 25% vacuolating cells on days 5 and 7 posttransduction, respectively (Fig. 1B). Consistently, an independent study [43] in MEFs, derived from the same *Vps34^{fl/fl}* mouse model used herein [33], also revealed aberrant vacuolation only upon prolonged treatment with Cre recombinase. Conversely, Ad-Cre transduction of immortalized *Vps34^{fl/fl}* podocytes resulted in formation of translucent cytoplasmic vacuoles at a pace similar to that observed in Ad-Cre-treated *Pikfyve^{fl/fl}* MEFs (Fig. 1A and B). Indeed, as high as 90% of the *Vps34^{fl/fl}* podocytes cultured in complete media exhibited cytoplasmic vacuoles at day 5 post-Ad-Cre treatment, which were readily seen by light microscopy (Fig. 1A, panel c and B). Our observation for robust cytoplasmic vacuolation in podocytes with *Vps34* ablation confirms and extends previous findings in primary podocytes isolated from podocyte-specific KO mice [31], generated from the same conditional *Vps34^{fl/fl}* mouse model used herein [33]. Of note, control *Vps34^{fl/fl}* or *Vps34^{wt/wt}* podocytes transduced with empty Ad or Ad-Cre, respectively, demonstrated a negligible number of vacuolating cells (<2%; Fig. 1A, panel d, and B).

In addition to a similar pace of vacuole appearance in the two cell types, electron microscopy revealed that, similarly to HEK293 cells with impaired PIKfyve activity [44], the cytoplasmic vacuoles formed in *Vps34^{fl/fl}* podocytes at 5–6 days post-Cre-infection were surrounded by a single-membrane and were largely empty inside (Fig. 1C). This indicates that the swollen vacuoles in podocytes with *Vps34* ablation, as in the case with PIKfyve [44], are distinct from autophagosome structures induced by serum starvation. Even under conditions of 24 h serum starvation, the morphology in *Vps34^{fl/fl}* podocytes remained normal by light microscopy (Fig. S1). Notably, the exclusion of the Oil red O lipid marker from vacuoles in both *Pikfyve^{fl/fl}* MEFs and *Vps34^{fl/fl}* podocytes at 5–6 days post-Ad-Cre treatment indicated that the translucent vacuoles were not lipid droplets (Fig. S2).

Ad-Cre transduction led to drastic and selective decreases of Vps34 protein levels in *Vps34^{fl/fl}* podocytes 5–6 days post-treatment, thus confirming the expected complete gene disruption (Fig. 1D). Importantly, after day 7 of Ad-Cre treatment, vacuolated *Vps34^{fl/fl}* podocytes showed progressively reduced viability, with most of them demising by day 10 likely through apoptosis, as determined in cultured primary podocytes isolated from the podocyte-specific *Vps34* KO mouse model [31].

Considering these similarities and limitations, detailed above, we focused our study on *Vps34^{fl/fl}* podocytes and *Pikfyve^{fl/fl}* MEFs at days 5–6 after Ad-Cre treatment (referred to further as *Vps34*

KO and *Pikfyve* KO). Cross examination of the protein levels under these conditions revealed that PIKfyve was not significantly affected in *Vps34* KO podocytes, nor was Sac3 (Fig. 1D), the 5-

phosphatase that associates with PIKfyve and is potentially able to modulate the PtdIns3P pool [1]. Similarly, Vps34 protein levels were unaltered in *Pikfyve* KO MEFs (Fig. 1D). Likewise, in *Pikfyve*

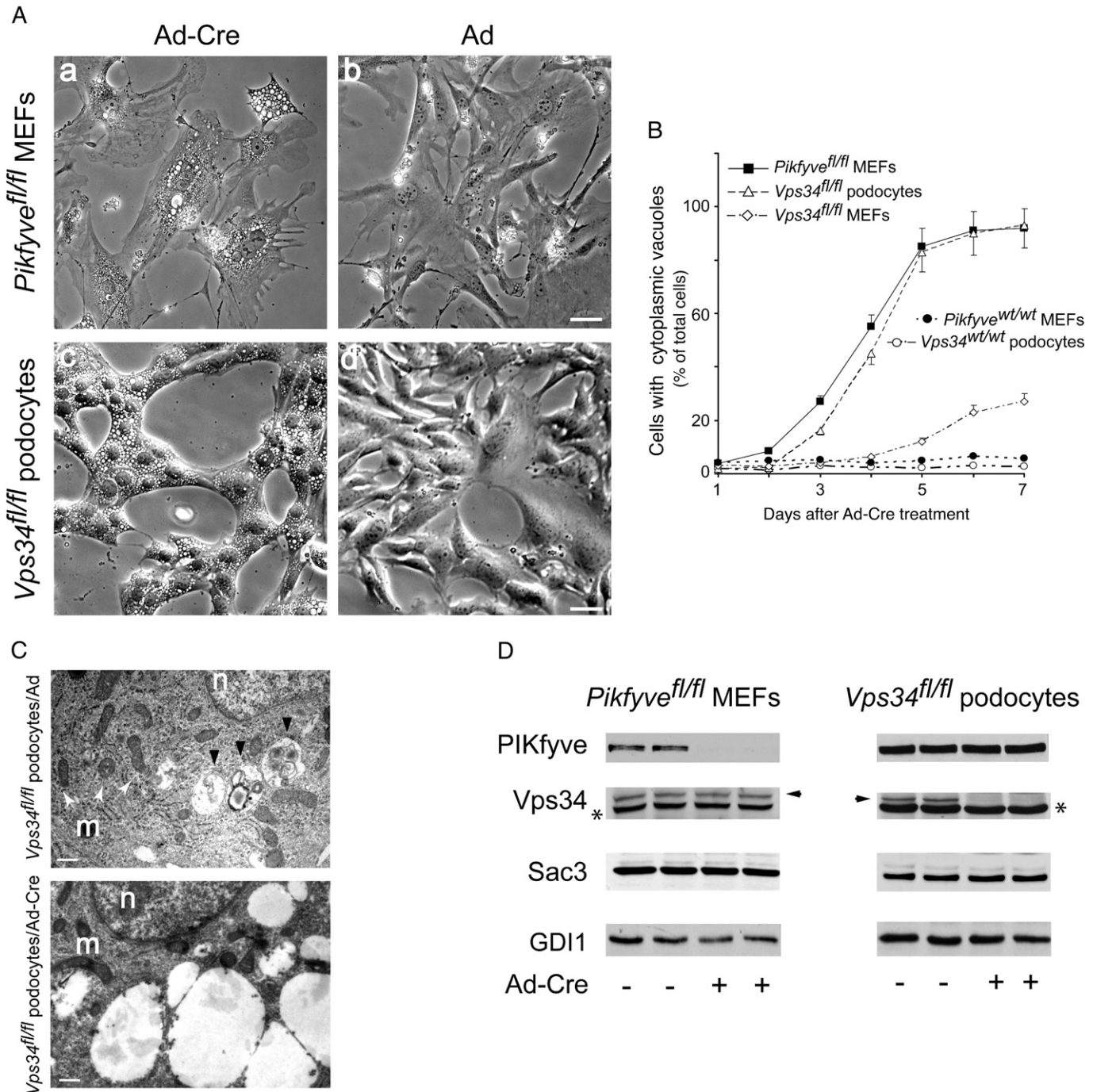


Fig. 1. Gene disruption of *Vps34* in podocytes and of *Pikfyve* in MEFs triggers cytoplasmic vacuolation at a similar pace. *Pikfyve*^{fl/fl} or *Pikfyve*^{wt/wt} mouse embryonic fibroblasts (MEFs), *Vps34*^{fl/fl} or *Vps34*^{wt/wt} podocytes and *Vps34*^{fl/fl} MEFs cultured in complete media were treated with adenovirus, either empty (Ad) or expressing full-length Cre recombinase (Ad-Cre). A, representative phase-contrast images, taken 5 days post-treatment depicting similar multiple translucent cytoplasmic vacuoles in nearly all Ad-Cre-treated *Pikfyve*^{fl/fl} and *Vps34*^{fl/fl} cells (panels a and c). Bar, 10 μ m. B, time-course quantification of cytoplasmic vacuolation subsequent to Ad-Cre treatment showing similar precipitous vacuolation progression in *Pikfyve*^{fl/fl} MEFs or *Vps34*^{fl/fl} podocytes, reaching ~90% of total cells at days 5–6, weak cytoplasmic vacuolation (~25% of total cells at day 7 post-Ad-Cre, but increasing thereafter) in Ad-Cre-treated *Vps34*^{fl/fl} MEFs and no vacuoles in Ad-Cre-treated *Pikfyve*^{wt/wt} or *Vps34*^{wt/wt} cells. Quantification of the vacuolating cells was obtained by counting > 100 cells per condition per cell type in 3 separate experiments (mean $\pm$ SE). C, *Vps34*^{fl/fl} podocytes maintained in complete media and treated with Ad or Ad-Cre (6 days) were observed by electron microscopy. Large-sized dilated empty vacuoles surrounded by a single membrane, containing only few intraluminal vesicles, are observed only in *Vps34* KO podocytes. In contrast, in control *Vps34*^{fl/fl} podocytes, single-membraned MVBs are visible (black arrowheads) containing multilamellar membrane whorls and intraluminal vesicles. Micrographs are representative of 30–60 images out of 2 independent experiments with similar results. m, mitochondria (white arrows), n, nucleus. Bar, 0.5 μ m. D, Western blot analysis of lysates derived from indicated cells on day 5 postinfection using antibodies against the indicated proteins confirms complete and selective ablation of only the targeted gene products by Ad-Cre. Sac3 phosphatase levels are unaltered by *Pikfyve* KO or *Vps34* KO. Shown are representative blots out of three to six independent experiments with similar results. GDI1, loading control; *, unspecific bands recognized by the anti-Vps34 antibodies, also indicating equal loading; Arrowhead, the Vps34 protein band.

KO MEFs, the Sac3 phosphatase was at the control protein levels (Fig. 1D), in agreement with our previous studies for unaltered Sac3 protein levels under PIKfyve perturbations [14,37].

3.2. The cytoplasmic vacuolation in *Vps34* KO podocytes is due to PIKfyve dysfunction

Notably, the morphological defect caused by *Vps34* KO in podocytes was phenocopied by inhibition of the PIKfyve activity in this cell type, as observed upon treatment with the cell permeable PIKfyve inhibitor YM201636 [5,12] (Fig. S3). This raised the question as to whether PtdIns3P ablation in the *Vps34* KO podocytes disrupted endomembrane homeostasis indirectly, i.e., by depleting the substrate for PIKfyve activity and, hence, PtdIns(3,5)P₂ synthesis. We reasoned that if this were the case, then heterologous expression of *Vps34* should prevent the endomembrane defect in *Vps34* KO podocytes, but only if the latter harbor an intact PIKfyve activity. Likewise, increased PIKfyve protein should restore the aberrant phenotype back to normal only in *Pikfyve* KO MEFs but not in *Vps34* KO podocytes. As illustrated in Fig. 2A, panels a and b, morphological inspection of *Vps34* KO podocytes positive for YFP-*Vps34*^{wt} fluorescence signals from three independent experiments revealed the absence of vacuoles in practically all transfected cells (Fig. 2B), consistent with the restored PtdIns3P pool and the downstream PIKfyve function. However, if cells were acutely treated with YM201636, expressed YFP-*Vps34*^{wt} failed to rescue the vacuolation (Fig. 2A, panels c and d), which was observed in ~98% of transfected cells (Fig. 2B). Likewise, similar expression of YFP-*Vps34*^{wt} in *Pikfyve* KO MEFs was ineffective as evidenced by sustained aberrant vacuolation in all transfected cells 24–48 h post-transfection (Fig. 2A, panels e and f, and Fig. 2B). We next increased levels of PIKfyve in *Vps34* KO and *Pikfyve* KO cells by adenovirus-mediated gene delivery of PIKfyve^{wt}. This approach was fully effective only in *Pikfyve* KO cells (see below). These data, together with other published observations (see discussion), suggest that under basal conditions, the cytoplasmic vacuolation in the *Vps34* KO podocytes is due to PIKfyve-dependent loss of PtdIns(3,5)P₂ synthesis and that the *Vps34*-catalyzed PtdIns3P pool is the main source for the PIKfyve function in the maintenance of endomembrane homeostasis in podocytes.

3.3. *Vps34*-produced PtdIns3P is a major membrane recruitment signal for PIKfyve

In addition to serving as an enzyme substrate, endosomal PtdIns3P represents a major determinant for PIKfyve membrane localization that is mediated through the PI3P-binding FYVE domain in PIKfyve [16]. Membrane recruitment is critical for PIKfyve functionality as evidenced by the inability of the kinase-deficient dominant-negative point mutant PIKfyve^{K1831} harboring a disrupted FYVE domain to trigger cytoplasmic vacuolation [9]. To determine whether *Vps34* catalytic activity is also supplying the PIKfyve localization signal, we used the 2xFYVE^{PIKfyve} biosensor that avidly localized to PtdIns3P/EEA1-enriched vesicular structures in *Pikfyve*^{wt/wt} MEFs [14]. In agreement with our previous data, transient transfection of eGFP-2xFYVE^{PIKfyve} in *Pikfyve* KO MEFs or *Vps34*^{fl/fl} podocytes showed a similar vesicular localization (Fig. 3, panels c and d, and not shown). By contrast, the eGFP-2xFYVE^{PIKfyve} fluorescent signals in *Vps34* KO podocytes were mostly diffuse (Fig. 3, panels a and b), although vesicle association could still be observed in some vacuolated cells (~25% of the cells). These data corroborate the notion that PtdIns3P produced by *Vps34* is also a major membrane recruitment signal for PIKfyve. The sparse eGFP-2xFYVE^{PIKfyve}-positive vesicles seen in some cells support the idea for additional sources of localized PtdIns3P production, as also has been recently concluded in *Vps34* KO MEFs by usage of an enhanced FYVE domain probe [43].

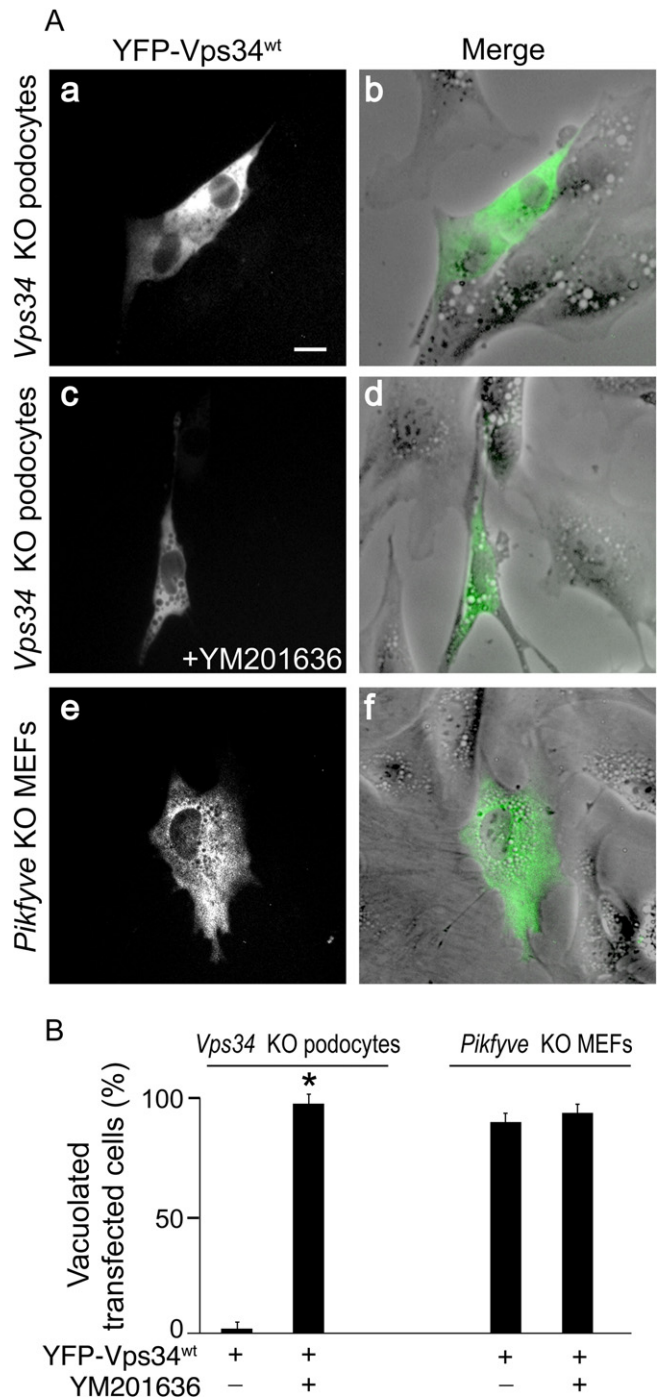


Fig. 2. PIKfyve dysfunction in *Vps34* KO podocytes underlies the aberrant vacuolation. On day 4 post-Ad-Cre-treatment, *Vps34* KO podocytes (panels a–d) and *Pikfyve* KO MEFs (panels e and f) were transfected with pTag-YFP-*Vps34*^{wt} cDNA. 24 h post-transfection cells were treated with vehicle (panels a, b, e and f) or 1 μ M YM201636 for 1 h (panels c, d and not shown). A, live cells were observed by fluorescence microscopy and phase-contrast. Shown are representative images of transfected cells (panels a, c and e) and the overlay with the phase-contrast images (panels b, d and f). Rescue of the vacuolation phenotype occurs in *Vps34* KO podocytes upon YFP-*Vps34*^{wt} expression (panels a and b) in practically all transfected cells (see B). If endogenous PIKfyve activity is inhibited by YM201636 such rescue is not observed (panels c and d). As expected, YFP-*Vps34*^{wt} expression in *Pikfyve* KO MEFs did not prevent the cytoplasmic vacuolation (panels e and f). Bar, 10 μ m. B, Quantification of vacuolated *Vps34*^{wt}-expressing cells in the presence or absence of the inhibitor was obtained by inspecting >20 transfected cells per condition per experiment in three separate experiments and presented as percentage of the total number of transfected cells. *, different vs. (–) inhibitor, $P < 0.001$.

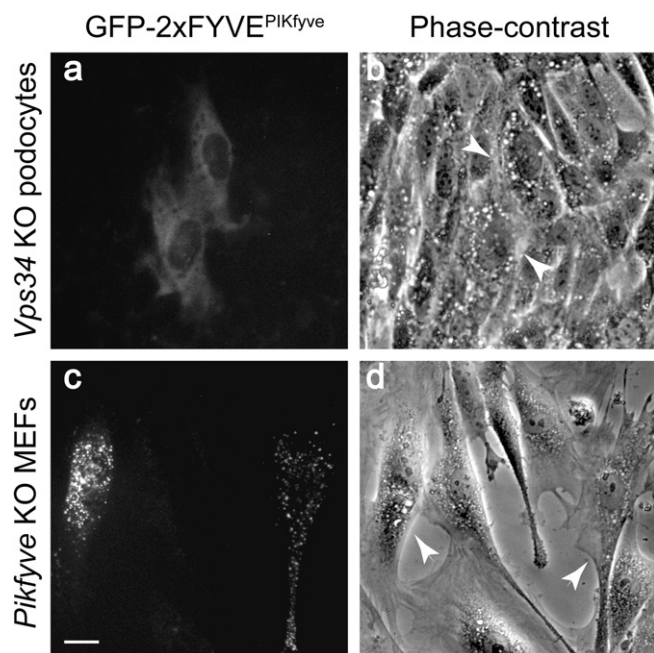


Fig. 3. 2xFYVE vesicular pattern is rendered diffuse in *Vps34* KO podocytes. On day 4 post-Ad-Cre-treatment, *Vps34* KO podocytes and *Pikfyve* KO MEFs were transfected with pEGFP-2xFYVE^{PIKfyve} cDNA for visualizing PtdIns3P-enriched endosomal structures. 24 h post-transfection cells were fixed for fluorescence microscopy (panels a and c) and phase-contrast inspection (panels b and d). White arrows denote transfected cells. The 2xFYVE^{PIKfyve} fluorescence signals were diffuse in the transfected *Vps34* KO podocytes (panel a), which was observed in $77 \pm 4\%$ of transfected *Vps34* KO podocytes. The fluorescence appearance of 2xFYVE^{PIKfyve} in the transfected *Pikfyve* KO MEFs was vesicular (panel c), seen in $93 \pm 3\%$ of transfected cells. Quantitation was obtained by counting >20 transfected cells per condition per experiment in 3 separate experiments. Bar, 10 μ m.

3.4. Commensurate decreases in steady-state levels of PtdIns3P, PtdIns5P and PtdIns(3,5)P₂ in *Vps34* KO podocytes

The data presented above indicate that PIKfyve is a major downstream effector of *Vps34*-catalyzed localized PtdIns3P production in the maintenance of endomembrane homeostasis. However, a *Vps34*-independent PtdIns3P pool, generated directly or indirectly by class I and class II PI3Ks could be quite substantial (up to 65% of total PtdIns3P by the combined two activities) [6,22,23,43,45]. To reveal the extent of *Vps34* contribution to the PtdIns3P pool in *Vps34* podocytes and the proportion of *Vps34*-catalyzed PtdIns3P used by the PIKfyve catalytic activity, we measured steady-state levels of PIs by HPLC inositol head-group analysis subsequent to cell labeling with myo-[2-³H]inositol. As illustrated in Fig. 4, selective and significant changes were observed only in steady-state levels of PtdIns3P, PtdIns5P and PtdIns(3,5)P₂, consistent with the key role of *Vps34* in PIKfyve functionality. Steady-state levels of PtdIns(4,5)P₂ and PtdIns4P remained unaltered (Fig. 4). In agreement with the HPLC profiles of PIs in other quiescent cells [5,14], PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ were not detected. Data from three separate labeling experiments in *Vps34* KO vs. *Vps34*^{fl/fl} control podocytes revealed that steady-state levels of PtdIns3P were reduced to ~45% of the control levels, with comparable diminution in PtdIns(3,5)P₂ (~40%) and PtdIns5P (~41%) (Fig. 4). Of note, during the labeling procedure, a relative increase (up to 20–25%) of non-vacuolated cells (presumably, expressing some *Vps34*) in parallel with preferential death of vacuolated *Vps34* KO podocytes has occurred. Therefore, if we normalize for the total number of vacuolating cells seen at the end of cell labeling, the values of the residual amounts of these lipids are overestimated by ~20–25%.

Consistent with the failure of PIKfyve membrane recruitment under localized depletion of PtdIns3P, a step critical for formation of productive complex of PIKfyve with its protein partners and,

hence, activation of PIKfyve lipid kinase [1,46], we observed similar reduction in both PIKfyve lipid products PtdIns5P and PtdIns(3,5)P₂ (Fig. 4). Taken together, the data for residual PtdIns3P levels even after normalization and roughly commensurate residual levels of PtdIns(3,5)P₂ and PtdIns5P by *Vps34* KO suggest that: 1) in podocytes, the *Vps34*-independent pathway(s) could synthesize a small proportion of basal PtdIns3P and, 2) this alternatively synthesized PtdIns3P pool could, at least in part, serve as a catalytic precursor and localization signal for PIKfyve.

3.5. Sustained activation of class I PI3Ks delays the vacuolation phenotype in *Vps34* KO podocytes

Our findings that the vacuolation phenotype in *Vps34* KO podocytes is mainly due to PIKfyve dysfunction provided a paradigm to address the question as to whether PIKfyve could utilize PtdIns3P produced by class I PI3Ks, found previously to indirectly feed into the endosomal PtdIns3P pool [47]. We took advantage of the fact that activation of class I PI3Ks is a common cellular survival response to diverse viral infections [48], with the non-replicating E1/E3-deleted adenoviral vector type 5 causing sustained activation, beginning 10 min subsequent to virus binding to its receptors and persisting for over 24 h [49–52]. We reasoned that if class I PI3Ks were to provide PtdIns3P for PIKfyve function, then adenovirus infection of *Vps34* KO should mitigate the susceptibility of cytoplasmic vacuole formation. To this end, we used E1/E3-deleted adenoviral vector type 5 harboring GFP (Ad_{GFP}) to facilitate detection of infected cells by fluorescent microscopy (Fig. 5). Remarkably, Ad_{GFP} infection of *Vps34* KO cells on day 4 post-Ad-Cre treatment, a time point when the vacuolated cells were ~50% (see Fig. 1), led to a statistically significant time delay of vacuole formation at the 16 h, and less so, at the 36 h post-infection vs. non-infected neighboring *Vps34* KO cells on the same dish (Fig. 5A and C). Intriguingly, if wortmannin was present during the 16 h Ad_{GFP} infection, such a delay was not apparent (Fig. S4). Direct measurement of class I PI3K activity in *Vps34* KO podocytes subsequent to the 16 h Ad_{GFP} infection revealed on average 15 and 50% elevation in PtdIns3P product in p85 and phospho-tyrosine immunoprecipitates, respectively, in comparison to non-treated *Vps34* KO podocytes (Fig. S5), consistent with activated class I PI3K underlying the vacuolation delay.

Contrary to *Vps34* KO cells, similar Ad_{GFP} infection of *Pikfyve* KO cells failed to delay the vacuolation phenotype (Fig. 5A and C), consistent with lack of PtdIns(3,5)P₂ production in PIKfyve's absence. Conversely, introduction of Ad_{GFP} that simultaneously expresses PIKfyve^{wt} from an independent promoter (Ad_{GFP}-PIKfyve^{wt}) on day 4 of the KO resulted in nearly complete and sustained rescue of the vacuolation phenotype in *Pikfyve* KO MEFs (Fig. 5B and D), in agreement with studies utilizing other approaches for PIKfyve perturbation/vacuolation rescue [9,11]. However, Ad_{GFP}-PIKfyve^{wt} infection of *Vps34* KO podocytes produced only a transient delay of cytoplasmic vacuolation, quantitatively similar to that observed by the Ad_{GFP} infection (Fig. 5C and D), further reinforcing the conclusion from Fig. 2 that PtdIns3P substrate deprivation and reduced PtdIns(3,5)P₂ synthesis by PIKfyve are causing the endomembrane defect. Combined data support the notion that under induced conditions, class I PI3Ks could deliver to some extent the PtdIns3P precursor for PIKfyve function.

4. Discussion

The observation that perturbations of the PIKfyve kinase trigger aberrant cell morphology in the form of translucent cytoplasmic vacuoles, readily reversed by exogenous PtdIns(3,5)P₂ delivery, together with unavailability of sufficiently specific probes for PtdIns(3,5)P₂ cellular detection, has promoted the cytoplasmic vacuolation phenotype as a sensitive functional measure of localized PtdIns(3,5)P₂ reduction [1, 2]. Intriguingly, similar translucent vacuoles accompanied by

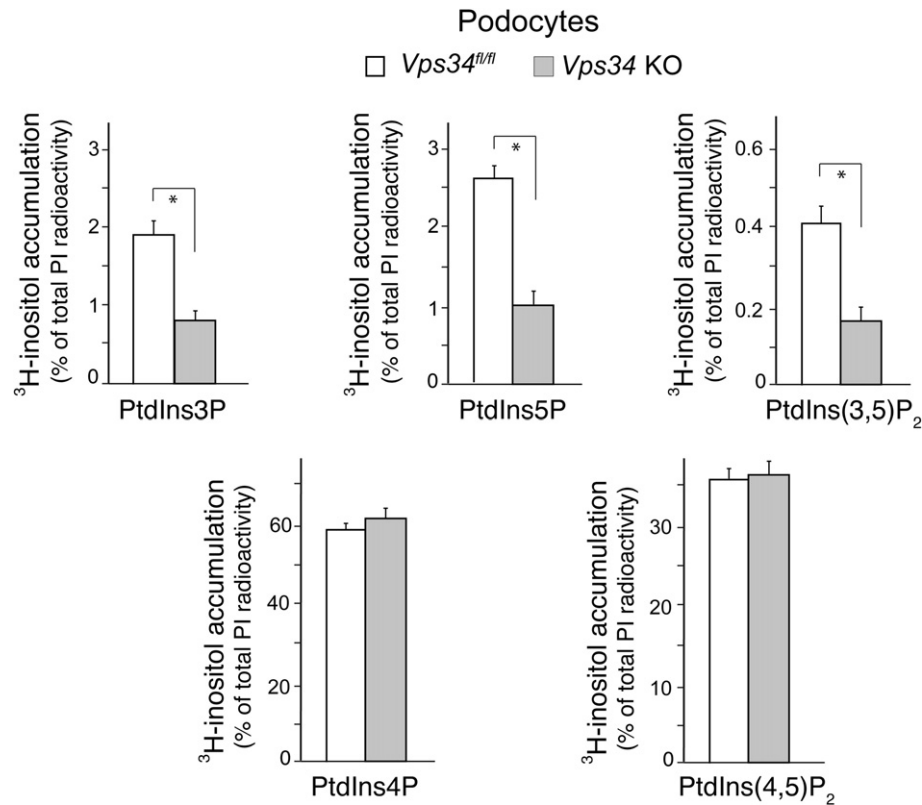


Fig. 4. Reduced PIKfyve lipid products in *Vps34* KO podocytes. *Vps34^{fl/fl}* podocytes, seeded on 35 mm dishes, were treated with Ad-Cre or Ad in complete culture media, as described under “Materials and Methods”. On day 4 post-treatment media was replaced with inositol/glucose-free media for 24 h, followed by myo-[2-³H]inositol labeling for 26 h. Lipids were extracted, deacylated and fractionated by HPLC. Shown is quantitation from three independent experiments, calculated as a percentage of the total PI radioactivity and presented as mean $\pm$ SE. The total PI radioactivity was obtained by summing the counts within the elution times corresponding to the indicated [³H]GroPIs peaks. *, PtdIns(3,5)P₂, PtdIns5P and PtdIns3P in *Vps34* KO vs. those in *Vps34^{fl/fl}* podocytes, $P < 0.05$.

endocytic trafficking defects and severe in vivo phenotypes have been recently reported in cells derived from mouse models with conditional *Vps34* gene disruption [30–32]. Importantly, in podocyte-specific *Vps34* KO mice, the aberrant vacuolation phenotype and disrupted endocytic trafficking precede the early-age severe proteinuria and glomerulosclerosis that lead to premature death [31,53]. As deficient autophagy is reportedly not the primary cause of the severe defects in podocytes or in other cells and tissues with *Vps34*-deficiency [30–32], elucidating the molecular mechanism(s) causing the faulty performance of the endosomal system is of critical importance. The current study was undertaken to determine, first, if the *Vps34* KO-triggered endomembrane defect in podocytes is secondary to PIKfyve dysfunction and, hence, loss of PtdIns(3,5)P₂ production and, second, the degree to which *Vps34* activity is responsible for providing PtdIns3P as a catalytic substrate and membrane localization signal for the constitutive PIKfyve function in this cell type. The key finding we presented here is that in podocytes, *Vps34* is the main supplier of PtdIns3P that serves as both a substrate for PIKfyve lipid kinase activity and an endosome targeting signal for PIKfyve membrane recruitment. We further revealed that the cytoplasmic vacuolation seen upon *Vps34* KO in podocytes results from loss of PIKfyve downstream function due to substrate deprivation and enzyme mislocalization. We also demonstrated that induction of class I PI3Ks in the background of *Vps34* KO in podocytes can transiently restore the PIKfyve function. Our results provide the first experimental evidence that the *Vps34* catalytic activity is the main PtdIns3P supplier for PIKfyve functionality, at least in podocytes, and that the aberrant cytoplasmic vacuolation seen by *Vps34* disruption is secondary to PIKfyve dysfunction.

Several lines of evidence in the current study support the conclusion that dysregulated endomembrane homeostasis in *Vps34*-deficient cells

is due to deficiency of PtdIns(3,5)P₂ synthesis by PIKfyve. Thus, unlike PIKfyve, increased protein levels of *Vps34* were able to restore the normal morphology in *Vps34* KO podocytes, but only if the endogenous PIKfyve activity was intact (Fig. 2). Conversely, increased protein levels of PIKfyve but not of *Vps34* in *Pikfyve* KO MEFs produced a complete and sustained restoration of the normal endomembrane morphology (Fig. 5). Concordantly, PtdIns(3,5)P₂ levels were profoundly reduced in *Vps34* KO podocytes (Fig. 4). Additional support that reduced PtdIns(3,5)P₂, rather than PtdIns3P synthesis, causes the cytoplasmic vacuolation is provided by findings for marked decreases of PtdIns3P under increased PIKfyve activity and elevated PtdIns(3,5)P₂ synthesis, yet vacuole formation is not apparent as seen in HEK293 cells infected with Ad-PIKfyve^{wt} [9]. In the same vein, unlike with PtdIns(3,5)P₂, delivery of exogenous PtdIns3P in COS cells, vacuolated due to ectopically expressed PIKfyve dominant-negative kinase-deficient point mutant K¹⁸³¹, did not rescue the phenotype [10].

Our observations for similar and substantial reduction in steady-state levels of PtdIns3P and PtdIns(3,5)P₂ in *Vps34* KO podocytes (Fig. 4) allow the conclusion that *Vps34* provides a considerable subfraction of PtdIns3P as a substrate for PIKfyve enzymatic activity (~65–70% after normalization for the total *Vps34* KO cell number, see text to Fig. 4). The observed diffuse cytosolic pattern of the PtdIns3P reporter 2xFYVE^{PIKfyve} (Fig. 3) indicates that *Vps34*-generated PtdIns3P also serves as a major targeting signal for PIKfyve membrane recruitment. The ramification of the lost PIKfyve endosome localization is a commensurate reduction of PtdIns5P production (Fig. 4), a finding consistent with reported direct PIKfyve-catalyzed synthesis of PtdIns5P [46,54]. The concomitant PtdIns5P diminution suggests that the cytosolic PIKfyve fails to gain access to the PtdIns substrate on membranes and/or form a productive heterooligomeric complex

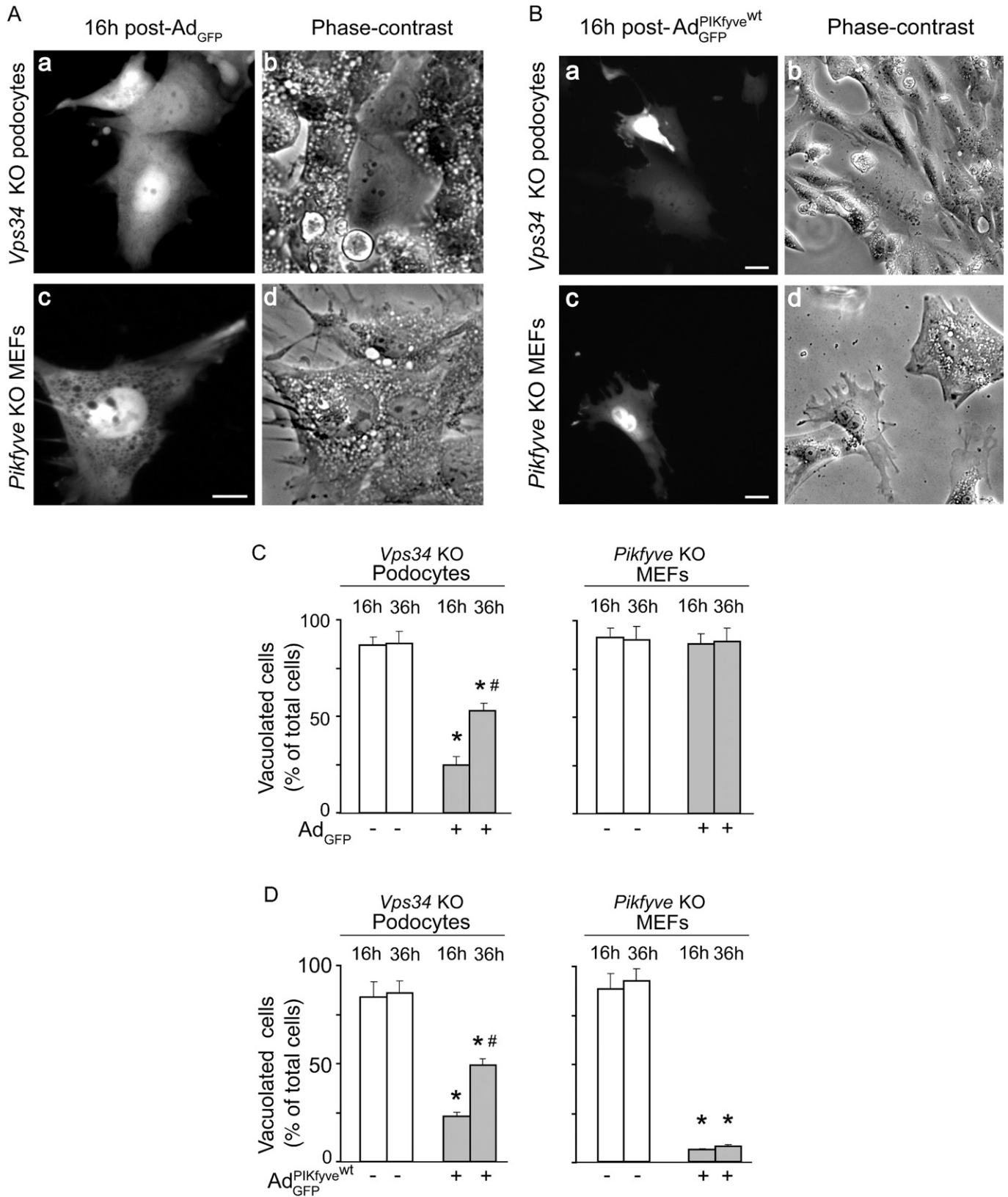


Fig. 5. Transient activation of class I PI3Ks selectively delays the cytoplasmic vacuolation only in *Vps34* KO podocytes. A and B, on day 4 post-Ad-Cre treatment *Vps34* KO podocytes and *Pikfyve* KO MEFs were infected with either Ad_{GFP} (A) or Ad_{GFP}-PIKfyve^{wt} (B) at MOI = 10, as described under “Materials and methods”. 16–36 h post-infection, cells were observed live by fluorescence and phase-contrast microscopy monitoring the GFP signals and the vacuolation phenotype in both infected [GFP(+)] and noninfected [GFP(-)] cells. A, fluorescence and phase-contrast images of representative cells at 16 h post-infection showing Ad_{GFP}-infected *Vps34* KO podocytes (panels a and b) that did not exhibit cytoplasmic vacuoles in contrast to the GFP(-) neighboring cells and Ad_{GFP}-infected *Pikfyve* KO MEFs, in which the vacuolation phenotype was unaltered (panels c and d). See C for quantification. Bar, 10 μ m. B, fluorescence and phase-contrast images of representative cells at 16 h post-infection with Ad_{GFP}-PIKfyve^{wt} showing the absence of vacuolation in Ad_{GFP}-PIKfyve^{wt}-expressing *Vps34* KO podocytes (panels a and b) and *Pikfyve* KO MEFs (panels c and d). In both cell lines the aberrant vacuoles persisted in the GFP(-) neighboring cells. See D for quantification. Bar, 10 μ m. C and D, quantification of vacuolation in the non-infected GFP(-) control cells (white bars) and GFP(+)-infected condition (gray bars) as percentage of total GFP(-) control cells and total GFP(+) cells, respectively by counting > 100 cells per condition in three separate experiments. *, different vs. GFP(-) cells at the same time point; #, different vs. the 16 h time point, $P < 0.001$.

(PAS complex) with the two partner proteins, i.e., the scaffolding regulator ArPIKfyve and the antagonistic PtdIns(3,5)P₂ phosphatase Sac3 [1,37,55]. Both membrane recruitment and the PAS complex formation are crucial steps for PIKfyve activity, as evidenced by the inability of the dominant-negative kinase-deficient point mutant PIKfyve^{K1831} to trigger the vacuolation phenotype if incapacitated for PtdIns3P binding or ArPIKfyve–Sac3 association through truncated mutations in the FYVE domain or ArPIKfyve–Sac3 interaction sites, respectively [9,56]. Concordantly, gene disruption of ArPIKfyve or Sac3 impairs PtdIns5P synthesis, further reinforcing the prerequisite of intact PAS complex in PIKfyve-catalyzed PtdIns5P production [reviewed in [46,54]].

Previous studies in cells of kidney origin have indicated the key role of PIKfyve and Vps34 in the progression of endocytic trafficking, with fluid-phase endocytosis being particularly sensitive to PIKfyve or Vps34 dysfunction [31,44]. In addition to similar endomembrane vacuolation and endocytic trafficking defects upon Vps34 or PIKfyve perturbations, cells with genetic Vps34 KO may exhibit defects in autophagy. Reportedly, the latter could precede or follow the endomembrane dysfunction, with differences likely related to the cell type, the strategy in the Vps34 gene targeting, and/or the mouse genetic background. For example, MEFs isolated from two independent conditional Vps34 KO mouse models [30,33] exhibit different phenotypes. Indeed, whereas MEFs from the Vps34^{fl/fl} model used by Jaber et al. form severe cytoplasmic vacuoles at early stage of excision with Cre recombinase (5–6 days) [30], those derived from the Vps34^{fl/fl} model of Zhou et al. [33] vacuolate only after prolonged ablation of Vps34 [herein, Fig. 1 and [43], 14–15 days]. Intriguingly, in the latter case, the MEFs exhibit predominant autophagic defects [43]. By contrast, Vps34 disruption causes predominantly endocytic dysfunction in sensory neurons, podocytes (Fig. 1) or lens cells [31–33]. Consistent with this, our observations in Vps34 KO podocytes at the ultrastructural level failed to reveal double-membrane autophagic structures (Fig. 1C). Instead, large swollen empty vacuoles were the most pronounced defect in these cells. These data suggest that the autophagic and endocytic phenotypes seen upon Vps34 KO have separate molecular mechanisms and are consistent with the Vps34 endocytic functions being delivered predominantly by PIKfyve. Whereas our data indicate that the latter mechanism operates in podocytes, it is conceivable that it has a more general significance. Future studies should define plausible cell specificity in the requirement of Vps34-dependent PtdIns3P synthesis for PIKfyve function in maintaining endomembrane homeostasis.

Class II PI3Ks that regulate key processes in different cell types, including autophagy, hormonal and immune responses, cilia movements and glomerular filtration, also supply the constitutive PtdIns3P pool [6, 23,43,45,57]. The observations for residual PtdIns3P, as documented in Vps34 KO podocytes (Fig. 4) or MEFs [43], derived from the same Vps34^{fl/fl} mouse model, together with the ubiquitous distribution of class II PI3K–C2α/β, are consistent with the latter feeding endosomal PtdIns3P for PIKfyve function. Although this has not been addressed in our study, evidence for such a pathway has been recently provided by findings for concurrent, though small, decreases in PtdIns3P and PtdIns(3,5)P₂ upon PI3K–C2α knock-down in 3T3L1 adipocytes [6].

Whereas the degree of PtdIns3P contribution by class II PI3Ks is a matter of future investigation, in the framework of this study we have provided evidence for a Vps34-independent pathway, comprised of activated class I PI3Ks, supplying the substrate for PIKfyve in Vps34 KO podocytes. Intriguingly, a Rab5-regulated enzyme cascade, comprised of class I PI3Kβ and two phosphatases (PI 5- and PI 4-phosphatase), has been suggested to generate as much as 30% of the endosomal PtdIns3P pool [22]. Under our experimental paradigm of temporary activation of class I PI3Ks, we observed a transient delay of the endomembrane defects in Vps34 KO podocytes in parallel with elevated class I PI3K activity (Fig. 5 and Fig. S5), corroborating the notion of a class I PI3K-related endosomal PtdIns3P pool being used for PtdIns(3,5)P₂ production. That this pathway could be physiologically

significant is inferred by the comparable phenotypes between mice with muscle-specific KO of *Pikfyve* (MPIfKO) and those with muscle-specific inactivation of *Pik3r1* combined with global KO of *Pik3r2* (*pik3r1 mKO pik3r2^{-/-}*) [58,59] (note that both models are generated by the same muscle–creatine kinase (MCK) promoter-driven Cre recombinase and on C57Bl6 background). The two mouse models share remarkably similar metabolic abnormalities, including systemic glucose intolerance, muscle insulin resistance and increased adiposity. This suggests that the metabolic defects in the *pik3r1 mKO pik3r2^{-/-}* mice might be related, in part, to ablation of a class I PI3K-dependent PtdIns3P pool that serves as a precursor for PIKfyve function. These data become more prominent in light of findings for distinct phenotypes in mice with muscle-specific Vps34 KO [30,60] (also controlled by the same MCK promoter-driven Cre recombinase and on Bl6 background) and MPIfKO [59]. Indeed, MPIfKO mice have normal life span and size of heart or striated muscles [59], whereas muscle-specific Vps34 KO causes premature death at 2–4 months of age, enlarged heart and striated muscular dystrophy [30,60]. The more severe mouse phenotypes upon muscle-specific Vps34 KO suggest that if the Vps34-catalysed PtdIns3P pool is also a main substrate supplier for PIKfyve functionality in muscle cells, as observed herein for podocytes, only a subfraction of this pool undergoes the PAS complex-dependent PtdIns3P–PtdIns(3,5)P₂ cycling.

In conclusion, our study demonstrates that podocytes lacking Vps34 produce an abnormal vacuolation phenotype that is due to inadequate Vps34-dependent PtdIns3P synthesis on endosomes and consequent PtdIns(3,5)P₂/PtdIns5P reduction due to PIKfyve dysfunction. These data are consistent with an important physiological relationship of Vps34 and PIKfyve in podocyte endomembrane homeostasis and maintenance of glomerular filtration.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2015.01.008>.

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