Modulation of phosphatidylinositol 4-phosphate levels by CaBP7 controls cytokinesis in mammalian cells

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ABSTRACT  Calcium and phosphoinositide signaling regulate cell division in model systems, but their significance in mammalian cells is unclear. Calcium-binding protein-7 (CaBP7) is a phosphatidylinositol 4-kinase-IIβ (PI4KIIβ) inhibitor required during cytokinesis in mammalian cells, hinting at a link between these pathways. Here we characterize a novel association of CaBP7 with lysosomes that cluster at the intercellular bridge during cytokinesis in HeLa cells. We show that CaBP7 regulates lysosome clustering and that PI4KIIβ is essential for normal cytokinesis. CaBP7 depletion induces lysosome mislocalization, extension of intercellular bridge lifetime, and cytokinesis failure. These data connect phosphoinositide and calcium pathways to lysosome localization and normal cytokinesis in mammalian cells.

INTRODUCTION
Phosphoinositides (PIs) constitute <1% of cellular lipid in mammalian cells but are important mediators of many signaling pathways (Balca, 2013). Phosphatidylinositol 4-phosphate (PI4P), one of seven possible PIs, can exert biological effects through either induction of local membrane curvature (Furse et al., 2012; important for transport vesicle biogenesis) or recruitment of signaling proteins harboring PI4P-selective binding domains (Lenoir and Overduin, 2013).

Golgi-localized PI4KIIβ is the most extensively studied PI4K isoform and, along with its yeast homologue pik1, functions in secretory cargo trafficking from the trans-Golgi network (TGN; Walch-Solimena and Novick, 1999; Haynes et al., 2005). PI4KIIβ is a soluble enzyme recruited to the TGN by the small GTPase ADP-ribosylation factor 1 (ARF1; Godi et al., 1999). ARF1 activates PI4KIIβ to drive PI4P production and biogenesis of secretory vesicles. PI4KIIβ regulation at the TGN in mammalian cells is further influenced by two Ca²⁺-binding proteins, neuronal calcium sensor-1 (NCS-1; a Ca²⁺-dependent activator) and CaBP7 (a Ca²⁺-independent inhibitor; Zhao et al., 2001; Haynes et al., 2005; Mikhaylova et al., 2009).

CaBP7 is an EF-hand containing, Ca²⁺-binding protein (McCue et al., 2010a,b, 2012). It is involved in the biogenesis of vesicular carriers from the TGN in neuronal cells, consistent with its function as a PI4KIIβ regulator (Mikhaylova et al., 2009). CaBP7 also appears to regulate cytokinesis in mammalian cells (Neumann et al., 2010), although how it achieves this remains to be determined. Cytokinesis is fundamental for normal cellular growth, development, and aging. Defects in this process can ultimately lead to a state of aneuploidy and cellular transformation (Ly et al., 2000; Baker et al., 2005; Pfau and Amon, 2012; Ricke and van Deursen, 2013).

Studies in model organisms have demonstrated PI4KIIβ activity is important during cytokinesis (Polevoy et al., 2009; Brill et al., 2011; Echard, 2012). Evidence that PI4P is required during mitosis in mammalian cells derives from studies examining knockdown of the sole PI4P phosphatase, Sac1. Sac1 depletion in HeLa cells disrupts Golgi morphology and generates mitotic spindle defects (Liu et al., 2008). PI4P is an important factor during mitosis, and evidence from nonmammalian systems indicates that PI4KIIβ may be the enzyme regulating its production.

Recently it was reported that a pool of PI4KIIβ localizes to lysosomes (Sridhar et al., 2013). Lysosomes were long considered a terminal destination of the secretory pathway where cellular material consigned for recycling was trafficked. Accumulating evidence suggests that these organelles have additional, nondegradative, functions, including acting as Ca²⁺-signaling platforms (Galione et al., 2010; McCue et al., 2013) and as lipid reservoirs during membrane repair events (Holt et al., 2006; Luzio et al., 2007b).
In this article, we show that CaBP7 undergoes a cell cycle–dependent redistribution from the Golgi apparatus at interphase to lysosomes during mitosis and to a mixed Golgi/lysosomal localization at cytokinesis. We report that lysosomes positive for CaBP7 cluster to either side of the intercellular bridge during cytokinesis and that CaBP7 depletion blocks both this clustering and the final abscission step to generate aneuploid cells. More specifically, CaBP7 loss of function appears to extend intercellular bridge lifetime, leading to abscission failure as determined in time-resolved live-cell imaging experiments. Cytokinesis is also inhibited by overexpression of wild-type PI4KIIIβ and by the PI4KIIIβ activators ARF1 and NCS-1 but not by overexpression of wild-type CaBP7 or a catalytically inactive version of PI4KIIIβ. This is the first demonstration that PI4KIIIβ activity regulates cytokinesis in mammalian cells and points to the intriguing possibility that lysosome activity in close proximity to the intercellular bridge plays an essential role in this process.

**RESULTS**

CaBP7 localization in HeLa cells

In a previous study characterizing CaBP7 membrane targeting (McCue et al., 2011), we consistently observed a subpopulation of HeLa cells in which CaBP7 localized to vesicular structures in addition to the TGN. To identify these vesicles, we examined the localization of CaBP7 with defined subcellular markers (Figure 1). Initially, monomeric cherry fluorescent protein (mCh)–tagged CaBP7 was coexpressed with yellow fluorescent protein (YFP)–tagged markers. No colocalization was observed between mCh-CaBP7 and the early endosome marker Rab5a–enhanced YFP (EYFP; Figure 1Ai) or the autophagosome marker LC3β-EYFP (Figure 1Aii). Degrees of colocalization were observed between mCh-CaBP7 and the late endosome/lysosome markers LAMP1-EYFP and green fluorescent protein (GFP)–VAMP7 (Figure 1A, iii and iv). CaBP7 localization in this analysis was variable and ranged from entirely vesicular in some cells to a mixed Golgi/vesicular distribution in others (Figure 1, Ai,

![FIGURE 1: CaBP7 localization in HeLa cells.](image)
CaBP7 exhibits cell cycle–dependent subcellular localization

To understand CaBP7's heterogeneous pattern of localization (see prior discussion), we examined its subcellular distribution with Golgi or lysosome markers during cell division. Because CaBP7 functions during cytokinesis (Neumann et al., 2010), we reasoned that it might traffic in a cell cycle–dependent manner. Initially, the distribution of endogenous CaBP7 with the TGN marker p230 was assessed (Figure 2A). At interphase, there was colocalization of CaBP7 with p230 in a perinuclear region consistent with the TGN. At metaphase, p230 immunoreactivity was lost due to Golgi fragmentation; however, CaBP7 remained associated with vesicles. By early telophase, the Golgi apparatus had begun to reform, and CaBP7 partially colocalized with p230. At late telophase, the Golgi had fully reformed, and CaBP7/p230 partially colocalized to a level comparable to that observed during interphase (Figure 2A and Supplemental Table S1). Cell cycle–dependent localization of CaBP7 with the lysosomal marker LAMP1 was also tested (Figure 2B). At interphase, there was partial colocalization of these proteins (Supplemental Table S1). Colocalization increased at metaphase (explaining CaBP7 localization to vesicles in the absence of the Golgi). Colocalization increased further during early telophase and began to decline at late telophase (Supplemental Table S1). These observations were mirrored in live-cell imaging experiments monitoring mitotic progression in HeLa cells transfected with CaBP7 and LAMP1 (Figure 3 and Supplemental Movie S1). Consistent with these data, PI4KIIIβ exhibited a similar localization profile during mitosis and cytokinesis (Figure 4). PI4KIIIβ concentrated in a perinuclear patch at interphase (the TGN). There was partial colocalization with LAMP1 in interphase cells, and during metaphase and telophase both proteins remained partially colocalized, consistent with a pool of PI4KIIIβ residing on lysosomes during mitosis. The derived Pearson's coefficients further highlight that of all markers assessed, CaBP7 colocalized to the greatest extent with the lysosomal marker LAMP1.

CaBP7 reduces PI4P levels and clusters lysosomes

Knowing that CaBP7 and PI4KIIIβ target to lysosomes, we examined whether CaBP7 could modulate PI4P levels in cells. Cells were transfected with mCh-CaBP7 and PI4P levels assessed with a PI4P-specific antibody (Figure 5A). On average, there was 58% reduction in PI4P-positive vesicle number in the presence of mCh-CaBP7 compared with controls (average 20.9 ± 1.6 PI4P particles/cell for mCh-CaBP7–transfected cells vs. average 49.9 ± 3.4 PI4P particles/cell for untransfected cells; Figure 5C). Because PI4P is a major precursor of phosphatidylinositol 4,5-bisphosphate (PIP₂) production, a prominent inositol lipid–signaling species with described functions during cytokinesis (Brill et al., 2011; Echard, 2012), we next addressed whether CaBP7 might indirectly modulate levels of this lipid as a consequence of reducing PI4P production. Cells were transfected with mCh-CaBP7, and PIP₂ levels were assessed with a PIP₂-specific antibody (Figure 5B). Although we observed a small reduction in cellular PIP₂, this was not statistically significant compared with PIP₂ levels observed in untransfected control cells from the same experiment (Figure 5C). We also attempted to confirm the influence of CaBP7 overexpression on cellular PI4P levels by dot blot analysis using a PIP-Strip–based method (Supplemental Figure S1). No discernible difference in total cellular PI4P between control and CaBP7–transfected cells was observable using this approach. This is likely
CaBP7, Ca\textsuperscript{2+}-deficient EF-hand mutants of CaBP7 and a kinase-dead PI4KIII\(\beta\) mutant can induce lysosome clustering, suggesting an effect on lysosomal PI4P (Supplemental Figure S2 and Supplemental Table S2). This was specific to lysosomes, since organelles earlier in the endolysosomal pathway were unaffected (Supplemental Figure S3).

CaBP7 and PI4P function during mitotic cell division (Liu et al., 2008; Neumann et al., 2010; Brill et al., 2011; Echard, 2012). Because CaBP7 lowered PI4P levels and induced lysosome clustering, we examined potential connections between CaBP7, lysosomes, and mitosis. We first examined the distribution of CaBP7 during mitosis (Figure 6A). At interphase, CaBP7, as expected, localized to vesicles and a perinuclear region, consistent with the TGN. From metaphase through to completion of anaphase, CaBP7-positive structures aggregated and moved toward the cell periphery. At telophase, these vesicles redistributed from the periphery to flank the intercellular bridge. These data are consistent with a previous study in which lysosomal clustering during cytokinesis was observed (Matteoni and Kreis, 1987). Colocalization of CD63-positive endolysosomal compartments with CaBP7-positive vesicles was observed during all stages of mitosis (Figure 6B), suggesting that the majority of CaBP7 becomes lysosome associated during this process.

PI4KIII\(\beta\) activity influences normal completion of cytokinesis

Knowing that CaBP7-positive lysosomes exhibit an organized redistribution during cytokinesis and that CaBP7 controls PI4P levels in cells prompted us to examine the requirement for PI4KIII\(\beta\) activity during mitosis in HeLa cells (Supplemental Table S3 and Figure 7A). A previous study uncovered a role for CaBP7 during cytokinesis (Neumann et al., 2010), and we used the same analysis to examine the role of PI4KIII\(\beta\) and its effectors during HeLa cell cytokinesis. First, we confirmed that CaBP7 depletion causes cytokinesis failure. CaBP7 knockdown and control transfected cells were imaged, and those harboring more than one nucleus were scored as abnormal (Figure 7A). Expression of scrambled short hairpin RNA interference (shRNAi) generated an 8.4% abnormal nuclei frequency (ANF), which increased to 22.9% on CaBP7 knockdown (Figure 7B). This 2.7-fold difference in ANF is consistent with the previous study (Neumann et al., 2010). Of importance, the cytokinesis defect generated by CaBP7 protein depletion could be rescued by coexpression of a shRNAi-resistant version of the protein (Supplemental Table S3 and Figure 7B).

If CaBP7 depletion affected cytokinesis through loss of PI4KIII\(\beta\) inhibition, then overexpression of wild-type PI4KIII\(\beta\) or its activators (NCS-1 and ARF1) should elicit the same phenotype. To test this hypothesis, we examined how overexpression of wild-type PI4KIII\(\beta\) and its effectors influenced cytokinesis (Figure 7C). EYFP control protein elicited an 8.2% ANF, similar to that observed with control shRNAi expression (Supplemental Table S3 and Figure 7C). Overexpression of wild-type PI4KIII\(\beta\) and its activators NCS-1 and ARF1 (all predicted to increase PI4P production by PI4KIII\(\beta\)) generated ANFs of 17.9, 19.6, and 13.5%, respectively (Figure 7C and Supplemental Table S2). Overexpression of PI4KIII\(\beta\)D656A or CaBP7, both of which should antagonize endogenous PI4KIII\(\beta\), generated ANFs similar to those observed with control EYFP expression (7.8 and 6.8% ANF; respectively; Supplemental Table S3 and Figure 7C). These data are consistent with the hypothesis that excessive activation of PI4KIII\(\beta\) impairs cytokinesis in mammalian cells.

Depletion of CaBP7 induces loss of lysosomal clustering at cytokinesis

To understand how CaBP7 loss of function elicits cytokinesis failure, we examined lysosome distribution during mitosis in
CaBP7-knockdown cells versus controls (Figure 8, A–C). Lysosomes cluster near the intercellular bridge at cytokinesis (Figures 2B and 6B; Matteoni and Kreis, 1987). In shRNAi control cells, clustering was observed (Figure 8A). CaBP7 shRNAi–expressing cells exhibited a marked loss of clustering at the intercellular bridge during cytokinesis (Figure 8A). This was quantified by calculating LAMP1 fluorescence intensity in the intercellular bridge region (Figure 8C). Consistent data were acquired from live-cell experiments in which LysoTracker Red was monitored during mitosis and cytokinesis in cells depleted of CaBP7 and compared with untransfected cells on the same dish (Figure 9 and Supplemental Movies S2 and S3). Loss of LAMP1 fluorescence at the intercellular bridge was not due to CaBP7 shRNAi expression causing a reduction in lysosome numbers, as total cellular LAMP1 fluorescence was similar in both CaBP7 shRNAi and scrambled control cells (Supplemental Figure S4). Finally, we investigated whether loss of lysosomal clustering on CaBP7 depletion was specific for these organelles by examining the distribution of the TGN at cytokinesis (Figure 8, B and C). No difference in p230 distribution in cells at cytokinesis was observed between scrambled and CaBP7 knockdown conditions (Figure 8, B and C). Because we previously demonstrated that CaBP7 overexpression was able to deplete cellular PI4P levels in interphase cells (Figure 5A), we tested whether this was also observable in mitotic cells (Figure 8D). Indeed, overexpression of CaBP7 qualitatively reduced PI4P staining compared with that observed in untransfected control cells. These data suggest that CaBP7 can modulate PI4P levels during cytokinesis in HeLa cells.

Closer analysis of live-cell imaging data showed that in control, untransfected cells at cytokinesis, the intercellular bridge had an

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**FIGURE 4:** Distribution of PI4KIIIβ with LAMP1 during mitosis. Cells were stained with anti-PI4KIIIβ antibody (red), anti-LAMP1 (green), and DAPI (blue). Inset regions in telophase overlay images are enlargements of the ROI highlighted (white dotted square). Cells are shown at stages of the cell cycle. Colocalization between LAMP1 and PI4KIIIβ appears yellow in overlay images. Scale bars, 10 μm.
DISCUSSION

This study was initiated by the novel observation that in addition to association with the TGN, CaBP7 was present on various uniformly distributed cytoplasmic vesicles in some but not all cells (McCue et al., 2009). On closer inspection, we were able to identify the CaBP7-positive vesicles not as secretory cargo carriers but, somewhat unexpectedly, as late endosomes/lysosomes. CaBP7 has been implicated as a factor required for normal completion of cytokinesis in mammalian cells (Neumann et al., 2010). This led us to examine whether CaBP7 might exhibit cell cycle–dependent localization, which might also explain the heterogeneity in CaBP7 distribution between cells within a population. Our data indicate that during interphase, CaBP7 is Golgi and lysosome resident, but that as cells enter mitosis, the protein relocates to become more lysosomal. This shift in CaBP7 distribution begins to reverse during cytokinesis, consistent with a proportion of CaBP7 returning to the TGN at reestablishment of interphase. A recent study in mammalian cells reported that a biochemically distinct pool of PI4KIIIβ is lysosomally associated and its activity there is essential for lysosomal content sorting (Sridhar et al., 2013). Based on our cell cycle–dependent localization data, a candidate PI4KIIIβ interaction partner localized to lysosomes was CaBP7. PI4KIIIβ is the only documented target for CaBP7, and we therefore reasoned that lysosomal CaBP7 might regulate the PI4KIIIβ pool resident at these organelles. Consistent with this hypothesis, we demonstrated that overexpression of CaBP7 significantly reduced cellular PI4P levels in both interphase and mitotic cells. These observations were corroborated and linked specifically to lysosomal PI4P by using a simple lysosomal clustering assay developed by Sridhar et al. (2013). We were able to show that CaBP7 overexpression elicited a similar lysosomal clustering phenotype observed when endogenous PI4KIIIβ activity is reduced through RNAi-mediated protein depletion (Sridhar et al., 2013) or overexpression of the dominant-negative, kinase-dead version of the enzyme (Sridhar et al., 2013; Supplemental Figure S1). Collectively these data are consistent with a model in which PI4KIIIβ activity, regulated at least in part by CaBP7, is able to control lysosomal positioning, a property that is misregulated through inhibition of the enzyme and depletion of a lysosomal pool of PI4P.

PI4KIIIβ has also been implicated as an important factor regulating mitosis and cytokinesis in a number of model organisms; however, the importance of PI4KIIIβ activity during the process of cell division in mammalian cell systems has yet to be assessed. We sought to determine whether CaBP7 influenced cytokinesis via PI4KIIIβ, in light

average lifetime of 25.2 ± 2.4 min (Supplemental Movie S4 and Supplemental Figure S5). In contrast, cells depleted of CaBP7 exhibited a 3.4-fold increase in the lifetime of the intercellular bridge (86.7 ± 9.0 min; Supplemental Movie S5 and Supplemental Figure S5). This could indicate that loss of CaBP7 induces a problem with bridge stability/assembly or abscission (Barr and Gruneberg, 2007; Mierzwa and Gerlich, 2014).
of experimental evidence that disruption of PI4P levels is detrimental to this process in model organisms. We addressed potential functional consequences of PI4KIIIβ activity and alterations in the pool of PI4P controlled by this kinase during HeLa cell mitosis and provided new evidence suggesting that it is an important factor in mammalian cell division. We were able to demonstrate that factors elevating PI4P production by PI4KIIIβ, including shRNAi-mediated depletion of endogenous CaBP7 protein or overexpression of PI4KIIIβ or its activators, all disrupt cytokinesis and generate binucleate or multinucleate cells likely through stabilization of the intercellular bridge, incomplete bridge assembly, or defective abscission. The precise defect requires further examination of bridge-specific structures and whether their appearance is disrupted by loss of CaBP7 activity. Conversely, factors inhibiting PI4P production, including overexpression of exogenous CaBP7, have no effect on normal progression through mitosis and successful cytokinesis. Our findings are in contrast to those from Drosophila, in which PI4KIIIβ Fwd has been reported to be required for cytokinesis (Polevoy et al., 2009). Note, however, that this applied only to spermatocytes and not somatic cells and that part of the requirement for Fwd was in a nonenzymatic capacity.

We showed that alterations in PI4KIIIβ activity can affect lysosomal dynamics and that loss of CaBP7 function prevents organized lysosome clustering at the intercellular bridge during cytokinesis. It has been known for >25 yr that lysosomes cluster in a cell cycle–specific manner (Matteoni and Kreis, 1987), but why has remained a mystery. Lysosomes could conceivably mediate a degradative role to remove cytoskeletal components before membrane fission can proceed or provide a membrane reservoir required for completion of abscission, as shown for endosomes (Boucrot and Kirchhausen, 2007). Lysosomes do exhibit membrane donor behavior during plasma membrane repair (McNeil and Kirchhausen, 2005; Luzio et al., 2007a). Lysosomes have also been recognized to mediate another key cellular role by acting as focal Ca²⁺-signaling platforms (Galione and Chuang, 2012; McCue et al., 2013). The role of Ca²⁺ during mitosis has been established in model cell systems (Miller et al., 1993; Whitaker, 2006); however, the significance of intracellular Ca²⁺-signals during mammalian cell mitosis remains unclear. This report characterizes the novel finding that a specific Ca²⁺-binding protein directly affects normal cytokinesis through regulation of phosphoinositide signaling. Our observations raise a number of interesting possibilities, and further work will be required to ascertain whether one or a combination of lysosomal activities (Ca²⁺ handling, recycling of cellular debris, membrane donation) contribute to cytokinesis in mammalian cells.

FIGURE 6: Distribution of CaBP7-positive vesicles during HeLa cell mitosis. (A) Cells were stained with DAPI (blue), anti-CaBP7 (red), and anti-α-tubulin (green). CaBP7 distribution was monitored at interphase and at defined points during mitotic cell division (metaphase through to late telophase—cytokinesis, arrowhead in anti-tubulin image highlights the intercellular bridge). CaBP7 and tubulin colocalization is yellow in overlay images. (B) Cells stained with DAPI (blue) were costained with anti-CaBP7 (red) and anti-CD63 (green) antibodies. CaBP7/CD63 distribution was characterized at interphase and in cells at metaphase, early telophase, and late telophase. CaBP7 and CD63 colocalization is yellow in overlay images. Scale bars, 10 μm.
made by QuikChange site-directed mutagenesis according to the manufacturer’s protocol (Agilent, Santa Clara, CA). For mCh-CaBP7 EF1 and mCh-CaBP7 EF2 mutations, mCh-CaBP7 was used as template with sense primer 5’-TTTATCCAAGCA-GCAGCTGGGCACAGCCATG-3’ and antisense primer 5’-CATG-GCTGTGCCCAGCTGCTGCTTGGAGATGAA-3’ and sense primer

MATERIALS AND METHODS

Plasmid constructs

mCherry-CaBP7, CaBP7-mCherry (mCh-CaBP7/CaBP7-mCh), and EYFP-CaBP7 were generated as previously described (McCue et al., 2009). mCh-CaBP7 EFx2 (E56Q, E93Q) double mutation and EF 1 (E56Q) and EF2 (E93Q) single mutations were all

FIGURE 7: PI4KIIIβ activity influences cytokinesis. (A) Cells transfected with constructs able to modulate PI4KIIIβ activity were stained with DAPI (red). Samples were scored for the number of multinucleate cells and cells at cytokinesis connected by an intercellular bridge. Cells were transfected with CaBP7 or scrambled control shRNAi plasmids. Silencing plasmids expressed GFP as a transfection marker (green). Overexpression of PI4KIIIβ, NCS-1, ARF1, PI4KIIIβD656A, and CaBP7 was also tested in this assay with EYFP as a control (all overexpressed constructs are green in overlays). Multinucleate cells are highlighted with an asterisk and cells undergoing cytokinesis with an arrowhead. Scale bars, 10 μm. (B) Quantification of shRNAi data from A. All data are plotted as mean ± SEM. Students unpaired t test analysis was performed for silencing data sets where CaBP7 knockdown and rescue were compared with scrambled control (p < 0.0001 for both conditions). (C) Quantification of overexpression conditions from A. Students unpaired t test analysis comparing each data set to the EYFP control condition generated p < 0.0001 in all instances, with the exception of ARF1, for which p = 0.0127. Statistical data are summarized in Supplemental Table S3.
GTCAAGTGGACTT-3′. The plasmids encode a 29-mer shRNAi under control of the human U6 promoter and a separate GFP reporter under the control of the CMV promoter for verification of transfection. shRNAi-resistant mCh-CaBP7 was generated by introduction of three silent point mutations using site-directed mutagenesis with sense primer 5′-GACATAGAGAACATATATGACAGAGGAG-3′ and antisense primer 5′-CTCCTCCTCCTTCATGTCCTCTATGTC-3′. Point mutations are highlighted in boldface. All new plasmid constructs were verified by automated sequencing (Sequencing Service, University of Dundee, Dundee, United Kingdom).

Cell culture and plasmid transfections

HeLa cells were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) penicillin/streptomycin, and 1% (vol/vol) nonessential amino acids. All cells were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were

5′-CAAGTGGACTTTGAGCAGTTTGTGACCCTTCTG-3′ and antisense primer 5′-CAGAAGGGTCACAAACTGCTCAAAGTCCACTTGTGC-3′. The plasmids encode a 29-mer shRNAi under control of the human U6 promoter and a separate GFP reporter under the control of the CMV promoter for verification of transfection. shRNAi-resistant mCh-CaBP7 was generated by introduction of three silent point mutations using site-directed mutagenesis with sense primer 5′-GACATAGAGAACATATATGACAGAGGAG-3′ and antisense primer 5′-CTCCTCCTCCTTCATGTCCTCTATGTC-3′. Point mutations are highlighted in boldface. All new plasmid constructs were verified by automated sequencing (Sequencing Service, University of Dundee, Dundee, United Kingdom).

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clonal anti–α-tubulin (1:500; AbCam); rabbit polyclonal anti–mannose-6-phosphate receptor (1:500; a kind gift from Paul Luzio, University of Cambridge, Cambridge, United Kingdom) and rabbit polyclonal anti-HRS (1:1000; a kind gift from Sylvie Urbé, University of Liverpool, Liverpool, United Kingdom). Primary antibodies were applied overnight at 4°C with gentle agitation. Cells were washed three times with 1 ml of PBS and twice with 1 ml of blocking solution and incubated with secondary goat anti-mouse immunoglobulin G (IgG) conjugated to Alexa Fluor 488 or 405 for 1 h at room temperature (with the exception of FITC-conjugated anti-CD63). Coverslips were washed three times with 1 ml of PBS and then air dried and mounted onto microscope slides using Prolong. For anti-PI4P staining, the staining protocol was modified as follows: Cells were fixed with 4% (vol/vol) formaldehyde/Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 20 min at room temperature and washed subsequently three times with TBS. Permeabilization was achieved by incubating the cells with 0.5% (wt/vol) saponin in TBS at room temperature for 15 min. Cells were washed three times with TBS and then air dried and mounted onto microscope slides using Prolong. For anti-PI4P staining, the staining protocol was modified as follows: Cells were fixed with 4% (vol/vol) formaldehyde/Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 20 min at room temperature and washed subsequently three times with TBS. Permeabilization was achieved by incubating the cells with 0.5% (wt/vol) saponin in TBS at room temperature for 15 min. Cells were washed three times with TBS and then blocked with 10% (vol/vol) goat serum in TBS for 30 min at 37°C. Anti-PI4P antibody diluted in TBS was added to cells and incubated for 60 min at 37°C. Antibody was removed and cells washed three times with 1% (vol/vol) goat serum in TBS before secondary antibody incubation (30 min at 37°C). Secondary antibody was also diluted in TBS. Cells were rinsed thoroughly with distilled water, air dried, and mounted onto microscope slides with Prolong. Fixed cells were imaged using a Leica TCS-SP-MP confocal system (Leica Microsystems, Heidelberg, Germany) with pinhole set to 1 Airy unit and a 63x oil immersion objective with a numerical aperture of 1.3. Images were exported as TIFF files and compiled, processed, and analyzed with ImageJ and CorelDraw X6 applications.
Particle counting analysis
A representative untransfected control cell stained with anti-Pi4P antibody was chosen and 25 randomly distributed Pi4P positive particles selected. The diameters of these particles was determined using ImageJ, and the lower and upper limits of the range (8 and 15 pixels, respectively) was subsequently used to set a range (8–15 pixels) of particle diameters that would be detected by automated ImageJ counting. Particles having diameters smaller or larger than the limits of this range were therefore excluded from the analysis. For lysosomal clustering analysis, a minimum particle diameter of 10 pixels was used, with the maximum value set to infinity.

Fluorescence colocalization analysis with JACoP
The ImageJ plug-in JACoP (Bolte and Cordelieres, 2006) was used to derive Pearson’s correlation coefficients for pairwise sets of image data. The Pearson’s coefficient under these circumstances measures the strength of relationship between pixels at the same location in two images being compared. Perfect correlation (colocalization) generates a correlation coefficient, R, of 1. Briefly, each image was first subjected to background subtraction before analysis with JA-CoP. Costes’ randomization was simultaneously applied for each pair of images analyzed to ensure the validity of the correlation data. For all image pairs analyzed, the randomized control Pearson’s coefficient was 0 ± 0.003.

Analysis of lysosome/Golgi localization during cytokinesis
GFP-positive cells (a transfection marker present in CaBP7 shRNAi and scrambled shRNAi plasmids) at cytokinesis (determined through α-tubulin intercellular bridge staining) were analyzed with ImageJ software and the plot profile application. A straight line across the intercellular bridge extending to the edge of the nucleus of each daughter cell was drawn on p230/LAMP1-stained sample images and the fluorescence profile along this line analyzed. The area under the curve for these profiles was calculated, which represents the average fluorescence intensity of p230/LAMP1 in the regions of the cytoplasm and intercellular bridge directly beneath the line. Data from scrambled shRNAi samples were normalized to 100% (control) and CaBP7 shRNAi: fluorescence intensity calculated as a percentage of this. Whole-cell fluorescence data were also analyzed from the same cells by tracing a region of interest around the plasma membrane of each cell and quantifying the average p230/LAMP1 fluorescence intensity over this area.

Lipid extraction and dot blot analysis
HeLa cells were plated onto 10-cm dishes and transfected with 5 μg of mCherry-N1 or 5 μg of mCherry-CaBP7. At 24 h posttransfection, cells (~2.2 × 10^6 cells/dish) were trypsinized, collected, and homogenized in 1 ml of chloroform/methanol 2:1 (vol/vol). After dispersion, the mixture was agitated at room temperature) on an orbital shaker (30 rpm) for 15 min. The homogenate was centrifuged at 2400 × g for 3 min to recover the aqueous phase and the organic solvent (SpeedVac SC210A). Lipid pellets were resuspended in 50 μl of chloroform/ methanol 2:1 (vol/vol). From this sample, two further dilutions (twofold and fourfold) were prepared. A 1-μl sample of each was spotted onto a PIP-Strip (Echelon Biosciences) between the lipid standards and allowed to dry completely. Once dried, the filter was incubated with blocking buffer (BB; 3% [wt/vol] BSA in 0.1% [vol/vol] Tween-20/ PBS [PBS-T]) overnight at 4°C. The filter was then incubated with 0.5 μg/ml Pi4P-Grip protein (Echelon Biosciences) in BB for 1 h at room temperature with gentle agitation. After three washes with PBS-T, the filter was incubated with anti–glutathione S-transferase antibody (rabbit polyclonal; Sigma) diluted 1:500 in BB for 1 h at room temperature with gentle agitation. The filter was subsequently washed three times with PBS-T and incubated with horseradish peroxidase–conjugated anti-rabbit IgG (Sigma) diluted 1:400 in BB for 1 h at room temperature with gentle agitation. Bound protein was visualized with enhanced chemiluminescence reagents.

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