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Yeast Arf3p modulates plasma membrane PtdIns(4,5)P2 levels to facilitate endocytosis

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Abstract

Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) is a key regulator of endocytosis. PtdIns(4,5)P2 generation at the plasma membrane in yeast is mediated by the kinase Mss4p, but the mechanism underlying the temporal and spatial activation of Mss4p to increase formation of PtdIns(4,5)P2 at appropriate sites is not known.

Here we show that Arf3p, the yeast homologue of mammalian Arf6, is necessary for wild-type levels of PtdIns(4,5)P2 at the plasma membrane. Arf3p localises to dynamic spots at the membrane and the behaviour of these is consistent with it functioning in concert with endocytic machinery. Localisation of Arf3p is disrupted by deletion of genes encoding an ArfGAP homology protein Gts1p, and a GEF, Yel1p. Significantly, deletion of *arf3* causes a reduction in PtdIns(4,5)P2 at the plasma membrane, while increased levels of active Arf3p, caused by deletion of the GAP Gts1, increases PtdIns(4,5)P2 levels. Furthermore, elevated Arf3p correlates with an increase in the number of endocytic sites. Our data provides evidence for a mechanism in yeast to positively regulate plasma membrane production of PtdIns(4,5)P2 levels and that these changes impact on endocytosis.

Keywords

Saccharomyces cerevisiae; actin; Arf3; Arf6; ArfGAP; synaptojanin

Introduction

The ADP-ribosylation factor (Arf) proteins of mammalian cells have been extensively studied and have been shown to play a number of roles in membrane trafficking pathways (reviewed in (1). As with other members of the Ras-related GTP binding protein family, the Arf proteins cycle between their active (GTP-bound) and inactive (GDP-bound) forms. Hydrolysis of GTP to GDP is mediated by GTPase activating proteins (GAPS), and the exchange of GDP for GTP is performed by guanine nucleotide exchange factors (GEFs). There are six mammalian Arfs, though it is Arf6 that is postulated to play the primary role in endocytosis (1). Evidence to date indicates that active Arf6 plays an important role in modulation of phospholipid metabolism and in particular, activation of phosphatidylinositol kinases, which leads to increased production of plasma membrane phosphatidylinositol(4,5)bisphosphate (PtdIns4,5P2) (2, 3). It is well established in mammalian cells and in yeast that PtdIns(4,5)P2 plays a key role in endocytosis (4-7) and a number of proteins involved in endocytosis contain domains (e.g ENTH) which are able to interact with the lipid head group of PtdIns(4,5)P2. In yeast, the sole phosphatidylinositol 4-

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phosphate 5-kinase Mss4p is essential to generate PtdIns(4,5)P2 (8). Interestingly, genetic interactions have been observed between an Mss4 temperature sensitive allele and a number of genes encoding actin regulatory proteins (*cap1*, *cap2*, *myo5*) as well as with deletions in genes for several membrane trafficking proteins (9). These interactions highlight the importance of PtdIns(4,5)P2 for actin filament assembly and endocytosis.

The role of the yeast homologue of Arf6, called Arf3p, has been less extensively analysed. While it is found at the plasma membrane, earlier reports suggested that Arf3p was not involved in endocytosis (10). This conclusion was based largely on the fact that deletion of *arf3* was not accompanied by an apparent reduction in uptake of the fluid phase marker Lucifer yellow. However, it was noted that overexpression of the active mutant form of Arf3 (Arf3Q71L) did cause defects in cortical actin and that the budding pattern of these expressing mutants was also defective (10). Since cortical actin patches are now known to be sites of endocytosis, mutations that affect these structures seem likely to also affect endocytosis. However, the recognised level of redundancy among endocytic proteins could easily explain why deletion of a single gene might not affect the endosomal uptake process. In addition, the Lucifer yellow fluid phase assay cannot detect subtle changes in the endocytic process. Support for an endocytic role for Arf3p was shown in our lab when we identified an interaction of Arf3p with a protein Lsb5p which itself has a role in yeast endocytosis (11, 12). Lsb5p shows homology with the Gga family of proteins having an N-terminal VHS domain and a central GAT domain. VHS domains are protein-protein interaction domains at the N-terminus of many membrane-trafficking molecules, while GAT domains bind to Arf and also to ubiquitin. Unlike the Gga proteins that function in membrane trafficking events through the Golgi and endosomes, the C-terminal half of Lsb5p does not contain a detectable clathrin binding motif nor a C-terminal gamma adaptin domain. Instead at its extreme C-terminus Lsb5p contains an NPFXD motif that interacts with the endocytic adaptor protein Sla1p (11-13). Lsb5 also interacts with Las17, the major actin-nucleating activity for endocytosis (11, 14). The interaction of Arf3p with proteins having roles in endocytosis gave strong circumstantial evidence for a function for Arf3p within the endocytic process. A recent study (15) has provided further evidence for effects of Arf3 on the actin cytoskeleton though the effect of Arf3 on endocytosis was not reported. Significant effects on key actin regulatory proteins involved in endocytosis were observed. Most notably, that overexpression of Arf3p alleviated cortical patch defects caused by deletion of the yeast WASP and WIP homologues Las17 and Vrp1 respectively. These data strongly indicate that yeast Arf3p may interact with, or regulate proteins important for endocytosis in yeast.

In this study we aimed to take advantage of more sensitive assays for functions in endocytosis using live cell imaging. This approach has demonstrated roles for actin-binding proteins in endocytosis where previous approaches had suggested no apparent role (16, 17). We reasoned that use of this approach, in combination with the generation of various mutant strains, should be able to determine whether such a function of Arf3p exists and also whether there are GAP and GEF proteins that function to modulate it. As well as demonstrating that increased Arf3 levels correlate with an increase in plasma membrane PtdIns(4,5) P2 levels, we show co-localisation of Arf3 with GAP and GEF-homology proteins. The GAP homology protein Gts1 shows clear co-localisation and interactions with a number of components of the endocytic machinery The increased levels of PtdIns(4,5) phosphate appear to enhance the number of endocytic events. Furthermore, loss of Arf3 leads to reduced plasma membrane PtdIns (4,5) P2 levels and an inability of the GEF-homology protein Yel1p to localise to the plasma membrane.

Results

Arf3p localises to foci at the plasma membrane which represent sites of endocytosis

While previous studies have revealed Arf3p localising to the bud and neck region of cells (10, 11), we wanted to address whether the relatively uniform distribution of the protein in these regions was partially due to use of lower resolution microscope systems. We visualised Arf3 protein tagged with GFP at the 3′ end using the DeltaVision deconvolution microscope system. Functionality of the tagged protein was verified as described in materials and methods. Using this approach we could see that in addition to Arf3p throughout the membrane of the bud and neck region, we could detect very dynamic foci of brighter fluorescence. These patches formed and disappeared within the plane of the membrane (Figure 1A, supplementary Movie1). We postulated that these foci could represent sites of endocytosis and so, using dual colour imaging, we determined whether any of these spots also co-localised with known endocytic proteins. We generated yeast strains carrying Arf3-GFP and co-expressing either Sac6p-RFP, clathrin (Chc1) – RFP, Las17p-mRFP, or Sla1p-mRFP. As shown in figure 1B, many, but not all of the Arf3 spots co-localise with known endocytic proteins. In particular, we see a high level of co-localisation with the adaptor protein Sla1p indicating that this might represent the stage of the process during which Arf3p plays its role. We do not however, observe Arf3 moving inwards with the vesicle, consistent with the behaviour of Arf6 in mammalian cells. Co-localisation with Sac6p is relatively low, suggesting that this might be the stage at which Arf3p is leaving the site of endocytosis. In addition, Sac6p appears to be on the cytosolic side of Arf3p further demonstrating that Arf3p does not leave the plane of the membrane and enter into formed vesicles. Thus, Arf3 does localise at appropriate sites to be involved in endocytosis. It has also been shown that addition of latrunculin-A (LAT-A) to cells results in a disassembled actin cytoskeleton and an abrogation of endocytosis (18). Disruption of Arf3p behaviour by LAT-A addition would further support an endocytic role for Arf3p. LAT-A was added to Arf3-GFP expressing cells for 5-30 minutes. This treatment was seen to cause a shift in Arf3p localisation over the time course, from the bud cortex to the neck and membrane of the mother cell, and for about 25% of cells, localisation was observed in the proximity of the vacuole membrane (Figure 1C). However, the Arf3GFP spots remain dynamic in the presence of the drug. Deletion of *SLA1* encoding an endocytic adaptor protein also causes a shift in Arf3p localisation away from the bud, to give an even mother-bud distribution (71% of cells show even mother-bud Arf3 distribution) (Figure 1C). These results suggest that Arf3p requires endocytosis to maintain its polarised localisation. The behaviour of Arf3 patches indicates that it may not form part of the main endocytic complex that invaginates with the forming vesicle.

Gts1p, a putative ArfGAP for Arf3p interacts with known endocytic proteins

As Arf3p partically co-localises with sites of endocytosis, we reasoned that there may be regulatory GAPs and GEFs for the protein, also associated with the plasma membrane. We identified Gts1p through a database search for proteins that show partial co-localisation with actin in the GFP localisation database (19). A recent publication also reports Gts1 in cortical patches as well as in the nucleus - though in this study Gts1 was expressed from a plasmid, and not from its own promoter (20). Gts1p, has also over the years, been ascribed a number of functions from nutritional sensing oscillator for ultradian rhythm to a transcriptional activator (21, 22).

As well as containing an ArfGAP homology domain at its N-terminus, Gts1p also contains a UBA (ubiquitin associated) domain, and a glutamine-rich region potentially able to form a coiled coil at its C-terminus (figure 2A). A region just upstream of this has been shown to be important for homodimerisation (22). Within the UBA region is a sequence that has

homology to the central region of human ArfGAP1 protein (Figure 2A, supplementary figure1) which folds into a motif which acts as ArfGAP1 Lipid Packing Sensor (ALPS). The ALPS motif forms an amphipathic helix on highly curved membranes and is necessary for coupling ArfGAP1 activity with the membrane curvature (23).

To investigate interactions between Gts1p and the endocytic machinery we initially used a two-hybrid approach using a Gts1 bait and a number of prey plasmids in which different endocytic proteins were fused to Gal4 activation domain. Interactions were detected with several well characterised endocytic proteins including the endocytic adaptor Sla1p; clathrin light chain Clc1p; a synaptojanin interacting protein Bsp1p; the essential protein Pan1p; the actin associated protein Ysc84; Yap1802 (member of the YAP180 family of endocytic proteins); and a region of the yeast homologue of the Huntingtin interacting protein-1R (HIP1R), Sla2p. (Table 1). Interestingly, a particular region of Sla2p lying between residues 206 and 310 was found to be particularly important for the interaction. This region contains part of the ANTH domain of Sla2p. We attempted to purify Gts1p to confirm these interactions but the protein was very insoluble and prone to precipitation making direct binding studies difficult to interpret. Other genetic and live cell imaging approaches were therefore used to further our studies on the endocytic role of Gts1p. Cells expressing Gts1-GFP were observed and localisation of the protein was examined. As shown in figure 2B and supplemental movie 1, Gts1GFP localises in dynamic punctate structures at the plasma membrane and has an average lifetime in these patches of 24 seconds. During the initial stages of localisation the protein is relatively non-motile. The end of its lifetime is marked by a short inward movement with an average distance of 0.27μm. (supplementary Movie 1). In this respect, the behaviour of Gts1p is most similar to proteins such as Sla1p though its total lifetime is shorter (16, 24). We also generated strains that co-expressed both Gts1p-GFP and either Sla1-mRFP, Las17-mRFP, Chc1-RFP or Sac6-RFP. As shown in figure 2C there is substantial co-localisation of Sla1p-mRFP and Gts1p-GFP. However, the presence of spots that do not co-localise and the fact that Sla1p lifetime on the membrane is about 34 seconds (about 10 seconds longer that Gts1p) suggest, that these proteins are not recruited to and/or released from the membrane at the same time. Kymographs comparing Gts1 and Sla1 appearance in, and disassembly from spots are shown in figure 2B. We also observed co-localisation of Gts1p with an early actin-filament organiser Las17p and actin bundling protein Sac6p. Again, as in the case of Sla1p, there were spots present that do not co-localise. In the case of clathrin heavy chain (Chc1-RFP), we were able to see some co-localisation with Gts1-GFP after magnification (x3) of the region of plasma membrane, which reduced noise from clathrin that localises to the Golgi membranes. These co-localisation data demonstrate that Gts1 is clearly in the forming endocytic patches and within these localises most closely with Sla1p.

We also determined the effect of LAT-A, and deletion of *SLA1 and RVS167* genes, on the appearance and dynamics of Gts1p at the cortex. Time-lapse movies revealed that Gts1p-GFP localises to the cell cortex in an actin-independent manner and does not require the presence of Sla1p or Rvs167p to achieve this (data not shown). However, deletion of amphiphysin Rvs167p, or the adaptor protein Sla1p and inhibition of actin polymerisation all increase lifetime of Gts1 at the membrane. *Rvs167* deletion increases lifetime of Gts1p on the membrane from 24 sec to 39 sec. In the case of LAT-A treatment (5 - 30 min) and *sla1* deletion the changes in Gts1p lifetime are more dramatic, with an average time of 79 sec and 117 sec respectively (in both cases we observed some spots with lifetime longer than 3 min). Using this approach we also analysed the effect of *arf3* deletion on Gts1GFP lifetime. Significantly, we observed a reduction in lifetime at the plasma membrane by over 25% (23.9±0.36 seconds to 17.7±0.4 seconds). Overall, this data strongly supports a role for Gts1 in endocytosis as the behaviour of Gts1 in the patches reflects that of other well characterised proteins.

To address the importance of domains within Gts1 we generated 2 mutants - I224S which disrupt the ALPS motif (Supp fig 1) and C50S,C53S which disrupts function of the GAP domain. Gts1 I224S-GFP localises in cortical spots just as the wild-type protein. However, its lifetime at the membrane is reduced to 17.1 sec (±0.45). Thus, the ALPS motif appears to play a role in stabilising Gts1 at the plasma membrane The Gts1C50S,C53S-GFP protein was also still able to localise to punctate spots at the plasma membrane (data not shown) and its lifetime within these spots was slightly reduced to 20.81 ±0.36 seconds (figure 2D) demonstrating that the GAP domain does not need to be active for Gts1p to localise.

Genetic interactions with *gts1* were also investigated with crosses being made to mutants harbouring deletions in several genes for proteins involved in actin organisation and endocytosis including Δ*abp1,* Δ*lsb5,* Δ*clc1,* Δ*sla1* and Δ*apl3* (encoding α-adaptin, large subunit of the clathrin associated protein complex). Only in the case of Δ*gts1*Δ*sla1* was a marked genetic effect observed. As single mutations Δ*gts1* was able to grow at 37°C and Δ*sla1* at 34°C. The double mutant Δ*gts1*Δ*sla1* displayed slow growth at 30°C and no growth at 37°C, both on YPD and synthetic medium. Notably, this strain was not able to grow on YPD medium at 34 °C, although we still observed slower growth on synthetic medium (data not shown). Single deletions did not show any differences that were dependent on the growth medium.

Yel1p contains Sec7 and PH domains and localises to the plasma membrane in an Arf3 dependent manner

In yeast there are 5 proteins that contain the Sec7 homology domain that is characteristic of GEF proteins. Function is ascribed to 4 of these: Sec7p, Gea1p and Gea2p are GEFs functioning in ER-Golgi and intra-Golgi membrane trafficking (25, 26). Syt1 is an ArfGEF homology protein that was identified as a suppressor of Ypt31/32 mutations, which themselves function within the Golgi apparatus (27). The remaining protein, which like Syt1p contains both a Sec7 and a PH domain is encoded by the gene *YBL060w* (Figure 3A). From the GFP localisation database, this protein showed weak localisation to the bud neck. As we observed this to be a site of significant levels of Arf3p, we determined that this might be a GEF that could be in the appropriate place to activate the Arf3 protein. As Ybl060w-p shows significant homology to other GEFs, and because of the effects described below we and others have suggested the protein be named Yel1p, for yeast EFA-like protein 1. This name also reflects the observation that Yel1 has regions of homology with the EFA-6 protein (Figure 3A). Most recently Yel1p has been shown to be a bona fide and specific GEF for Arf3p (28).

We visualised Yel1p-GFP using the DeltaVision microscope system and were able to see clear localisation of fluorescence spots mainly to the bud and neck region (figure 3B). Based on time-lapse movies the lifetime was relatively short and we estimated the lifetime of Yel1p-containing spots to be about 9 seconds. (supplementary Movie 1).

The effect of *arf3* deletion on Yel1p was extremely marked. In the absence of *arf3* instead of localising to the mother-bud neck, localisation of Yel1p to the plasma membrane was lost demonstrating that this GEF homology protein requires Arf3 for its localisation (figure 3C). There did appear to be some redistribution of the protein to internal membranes suggesting that Yel1p can localise to other membranes, possibly mitochondria, in the absence of a suitable plasma membrane environment. Conversely, over-expression of active Arf3(Q71L) caused a dramatic increase of Yel1p at the membrane and caused it to become localised in punctate foci (figure 3D).

A recent paper (28) showed that an N-terminal truncation of Yel1 including its Sec7 domain abrogates Yel1p localisation. The importance of the other domains were not reported. As PH

domains are known to interact with specific membranes we firstly cloned the Yel1PH domain and fused it to GFP. This fusion protein localises mostly to the nucleus.

However, in a significant number of cells spots at the plasma membrane were observed, though only 1-4 spots were observed in a single cell. These data indicate that Yel1-PH does not confer normal plasma membrane localisation for the full length protein. However, some interaction with specific proteins or regions of lipids may still occur. We also generated truncated forms of Yel1p lacking the sequence C-terminal region that contains homology to the EFA-6 protein (Yel1 1-560) or lacking both the PH domain and the C-terminal region (Yel1 1-411). In both cases, localisation to the plasma membrane was markedly reduced and exposure times had to be increased to visualise some faint spots close to the plasma membrane (figure 3 E,F). As the localisations were essentially the same this indicates that the C-terminal region is of primary importance in this effect. We conclude that in addition to the Sec7 domain - the EFA-6 homology region at the C-terminus is also important for Yel1 localisation.

Gts1p and Yel1p co-localise with Arf3p and their deletion affects Arf3p localisation

Using GFP and RFP tags we were able to co-express Arf3p and its potential regulatory proteins and determine their spatial and temporal interactions. As shown in figure 4A both the GAP Gts1p and the GEF Yel1p show some localisation that coincide with the Arf3p foci indicating that the proteins are in a position to regulate Arf3p function. Analysis of spots in multiple cells indicated that Arf3p and Yel1p localise within the same plane as each other, while the Gts1 protein mostly localises on the cytosolic side of Arf3p with a region of co-localisation between (arrows). Neither deletion of *rvs167* or *sla1,* nor treatment with Latrunculin-A abolished the observed Arf3p and Gts1p co-localisation (data not shown). Two-hybrid analysis was also carried out and interactions could be detected between Gts1p and Arf3 and also between Yel1p and Arf3p (figure 4B).

By analogy with the GAP and GEF regulators of mammalian Arf6, one would predict that deletion of a GAP protein should increase the level of active Arf at the membrane, and that deletion of a GEF may decrease its level at the plasma membrane. To test whether this analogy holds for Arf3p and the proposed regulators, we generated strains expressing Arf3-GFP but lacking either *gts1* or *yel1*. Multiple images were recorded for each strain and the distribution of Arf3p in the cells was analysed. In each of 10 cells a line was drawn along the mother bud axis also intersecting the neck region (see figure 4C for representative examples). Data for a larger population of cells is shown graphically in figure 4D. The fluorescence intensity in the cells and along this axis was then assessed (lower panels). In wild type cells as described above, the main fluorescence is at the plasma membrane in the neck region and in the bud. This can be seen as two sharp peaks in the intensity profile graph. Thus, if Gts1p is acting as a GAP protein and inactivating Arf3p, its absence would be expected to lead to an increase in Arf3p at the membrane. As shown in figure 4C, the outcome in the *gts1* deletion strain is as hypothesised and there is an elevated level of Arf3p at the membrane. The elevation in Arf3 is not confined to the bud, but increased levels are now seen over the entire mother cell cortex.

Conversely, in the absence of Yel1p which would activate Arf3p, there is a decrease in plasma membrane fluorescence but an increased cytosolic fluorescence that is absent from the vacuole but often appears to be at a detectable level on the vacuolar membrane. The distribution between mother and daughter plasma membrane is even. This indicates that Yel1p is behaving as one would predict for an Arf3 GEF and plays a role in localising active Arf3p to the plasma membrane of the bud. In addition to determining the effects of *yel1* deletion on Arf3p localisation we analysed whether deletion of another Arf GEF *gea2* also affected Arf3p localisation as a genetic interaction between Δ*arf3* and Δ*gea2* has been

reported (15). While the re-localisation of Arf3p observed in the *gts1* and *yel1* mutants shown in figure 4C are found consistently in the population of cells studied (figure 4D) the effect of *gea2* deletion was similar to wild-type cells with the majority of cells (>60%) showing Arf3p in the bud and neck regions (data not shown). No Δ*gea2* cells showed the Δ*yel1* phenotype with a shift of Arf3-GFP away from the plasma membrane.

Having shown that Δ*gts1* caused redistribution of Arf3p we then wanted to address whether the GAP domain of Gts1p is responsible for this activity. We generated a mutant Gts1 C50S,C53S which is mutated in the key cysteines of the GAP domain. Cells lacking *gts1* were transformed with either a wild type copy of *GTS1* or the Cys mutant. While *GTS1* rescued the Arf3GFP localisation defect such that Arf3p was primarily localised to the bud and neck region of cells, the Cys mutant was unable to do this and behaved as a Δ*gts1* strain (figure 4C,D). This demonstrates that the GAP homology domain of Gts1 is critical for its function towards Arf3.

Changes in Arf3 levels correlate with plasma membrane levels of PtdIns(4,5)P2

Studies in mammalian cells have demonstrated the importance of Arf6 in stimulating levels of PtdIns(4,5)P2 via activation of phosphatidylinositol 4-phosphate 5-kinases. While it seems likely that the Mss4 kinase in yeast can produce PtdIns(4,5) P2 in the absence of Arfp3, as the kinase but not Arf3 is essential, we considered that Arf3p might serve a valuable function if it was able to increase levels at specific sites, for example to facilitate higher levels of endocytosis in growing buds.

In order to determine the effect of Arf3p on levels of PtdIns(4,5)P2 cells (wild-type, Δ*arf3*, Δ*yel1*, Δ*gts1*) were transformed with a GFP reporter construct (PH-PLCδ1) carrying a PH domain that specifically interacts with PtdIns(4,5)P2 (29). Each mutant strain was compared directly alongside wild-type cells for comparison. In order to distinguish the wild-type and mutant cells, the mutant cells were also treated with the vacuolar dye FM4-64. As shown in figure 5 decreasing levels of active Arf3 at the plasma membrane (in Δ*arf3* and Δ*yel1*) resulted in reduced levels of the PH domain at the plasma membrane compared to wild-type cells. Conversely, an increase in active Arf3 levels (Δ*gts1*) caused a very significant increase in the level of the GFP- PH reporter that was bound at the plasma membrane. The experiment was repeated with the wild-type strains being stained with the marker dye. Co-labelling in this way did not alter the observed changes (supplemental figure 2).

As this approach has not been reported widely in yeast before we sought to validate it as a useful method to allow PtdIns(4,5) levels to be assessed. First, we generated the synaptojanin mutant Δ*inp51*Δ*inp52* (Δ*sjl1*Δ*sjl2*). Deletion of synaptojanins has in previous studies been shown to cause elevated PtdIns(4,5)P2 (30). We transformed this strain with the PH reporter. As shown in figure 5A these cells, like the Δ*gts1* strain, have clearly elevated levels of plasma membrane GFP localisation indicating raised PtdIns(4,5)P2. Flow cytometry was also used to measure PH-GFP fluorescence in the cells (figure 5B and supplementary figure 3). Reflecting the visual assay, deletion of Gts1, or of Δ*inp51*Δ*inp52* (Δ*sjl1*Δ*sjl2*) caused an increase in PtdIns(4,5)P2 while there was a slight reduction in the measurement of fluorescence in the absence of *arf3* and *yel1*. This decrease appears smaller than in the visualised cells because it takes into account total cell fluorescence and in these mutants there is a higher cytoplasmic fluorescence which would be included in the flow cytometry measurements.

Overall, our data demonstrate a role for Arf3p, Gts1p and Yel1p in modulating plasma membrane PtdIns(4,5)P2 levels at the plasma membrane and the comparison with Δ*inp51*Δ*inp52* (Δ*sjl1*Δ*sjl2*) cells indicates that this is a valid and useful approach for

assessing changes in PtdIns(4,5)P2. A representative flow cytometry data set is shown in supplementary figure 3.

This assay then allowed us to test whether the marked increase in the Δ*gts1* mutant is mediated through Arf3p. This would provide strong evidence that the role of Gts1 is to activate Arf3 and to regulate its function. To address this we generated a mutant Δ*gts1*Δ*arf3*. As shown in figure 5A and B deletion of *arf3* abolishes the increase in PH-GFP binding observed in the single Δ*gts1* mutant. Therefore, the effect of Gts1 on PtdIns(4,5)P2 is mediated through its activity toward Arf3p.

Increased levels of Arf3 correlate with an increase in endocytic events

While none of the mutants analysed (Δ*arf3*, Δ*gts1*, Δ*yel1*) show defects in uptake of the fluid phase marker lucfier yellow, or of the lipophylic dye, FM4-64, the data presented strongly indicates that these proteins function during the endocytic process. Indeed, several other proteins that are acknowledged to play important roles in actin organisation during endocytosis (Cap1/2 and Abp1) show no defects in these assays (Kaksonen et a, 2005) and it appears that uptake is observed even if the process functions inefficiently.

Importantly, a role for PtdIns(4,5)P2 at specific stages of endocytosis has recently been reported (31). Having demonstrated that levels of Arf3p are able to modulate PtdIns(4,5)P2 levels at the plasma membrane we then aimed to consider the consequences of this to the endocytic process. Of particular interest to us is the observation that the kinase Mss4 is localised all over the plasma membrane while Arf3p is largely polarised to the bud and neck regions (figure 6A). Observations by many labs over the last 20 years has also revealed that the density of cortical patches, which we now believe represent endocytic events, is greater in the bud than in the mother cell. We postulated that Arf3p may act to modulate PtdIns(4,5)P2 levels to facilitate endocytosis in the bud and neck regions of cells while levels of PtdIns(4,5)P2 within the mother cell generated by Mss4p alone may be sufficient for the lower levels of endocytosis that occur here. We therefore aimed to determine the effect of increased Arf3 levels (caused by overexpression of active Arf3 or by deletion of *gts1*) on the number of endocytic events in mother cells. Due to the high density of patches in buds it is difficult to resolve these events sufficiently accurately within the buds themselves. However, as shown in figure 6A,B using Sla1GFP as a marker of endocytic events, increased levels of Arf3 at the plasma membrane have a clear impact on the number of events that can be recorded per minute within mother cells with the number of events increasing from 9 per minute in wild type cells to 16 per minute in cells in the Δ*gts1* cells and 14 per minute in cells overexpressing the constitutively active Arf3Q71L.

While accurate counting of individual endocytic events is difficult in buds of wild-type cells, certain mutants have been characterised by a number of labs to contain fewer, cortical patches making it possible to detect all events within the bud relatively clearly. One of these mutants is Δ*sla1* (32, 33). We postulated that if we could distinguish individual cortical patches in this mutant, then we could determine whether the additional deletion of *arf3* had any further consequences. As shown in figure 6C, in the absence of *sla1* there are clearly definable patches in the bud containing Abp1-GFP. The deletion of *arf3* in this mutant to generate the Δ*sla1*Δ*arf3* double strain has markedly fewer patches indicating that Arf3p has a role in facilitating the generation of endocytic sites. Counting of cortical patches from cells ($n>25$) revealed average patch number in Δ*sla1* cells 13.68±0.7 and in Δ*sla1*Δ*arf3* cells 6.93±0.7. t tests indicate P value of $P<0.0001$. Furthermore, the patches appear morphologically aberrant often being elongated or tubular suggesting that the perturbation in lipids has effects on the actual ability of cells to form the appropriate vesicle structure.

Discussion

Budding yeast has been used extensively to further our understanding of a number of stages in membrane trafficking pathways both in endocytosis and exocytosis. Key to the endocytic process is the generation of an appropriate lipid environment necessary to allow the curvature and invagination process to occur. PtdIns(4,5)P2 has been proposed to play a central role in facilitating binding of proteins, which in turn promote endocytosis. The kinase responsible for PtdIns(4,5)P2 production at the plasma membrane has been proposed to be Mss4p, while a number of phosphatases, the synaptojanins (Inp51/sjl1,Inp52/sjl2, Inp53/sjl3) are required for turnover of PtdIns(4,5)P2, a key event later in endocytosis. In this study we have demonstrated that Arf3p does indeed function in the endocytic process and that altered levels of Arf3 affect plasma membrane PtdIns(4,5)P2 levels and, correlate with changes in numbers of endocytic events. We have also identified an ArfGAP homology protein Gts1p and a GEF, Yel1p, which appear to affect Arf3p localisation in a manner consistent with their predicted functions.

Based on our data we propose that Arf3p functions to regulate levels of PtdIns(4,5)P2 production within the inner leaflet of the plasma membrane. Arf3p on the plasma membrane is activated by its GEF Yel1p. When it is active, Arf3p is able to bind to certain components of the endocytic coat complex such as the Gga related protein Lsb5p (11). At these sites Arf3p most likely acts to increase Mss4p kinase activity to direct PtdIns(4,5)P2 production. Phospholipid asymmetries, caused by local changes in PtdIns(4,5)P2 composition, facilitate endocytosis by interacting with, and activating a number of endocytic and actin-regulatory proteins. The interactions of these proteins with the phospholipid bilayer and between each other cause physical deformation of the membrane, and may initiate invagination (34). Cargo binding may then facilitate changes in conformation of proteins within the endocytic coat that allows Gts1p to trigger Arf3 inactivation. In this regard, cargo is known to bind to the adaptor protein Sla1p which in turn interacts with both Gts1 and with actin polymerisation machinery (table 1; (32)). Gts1p remains with the coat during invagination, most likely until release of the coat immediately following vesicle scission. This model explains our data revealing that Arf3 levels modulate PtdIns(4,5)P2 levels at endocytic sites on the plasma membrane and why co-localisation is not required between the kinase Mss4p and endocytic machinery. It also fits with our observation that there is some co-localisation between Arf3 and both Gts1p and the endocytic component Sla1p, but that localisation with the actin-polymerisation machinery is very limited.

Another significant result from our data is that the activity of Arf3p affects localisation of its proposed GEF protein Yel1p. In the absence of *arf3* we observe no plasma membrane localisation of Yel1p indicating that Arf3 provides a feedback mechanism to control localisation of its own GEF and thereby its own activation. Furthermore, overexpression of active Arf3Q71L leads to an increase in Yel1p levels at the membrane substantiating the link between Arf3 and Yel1p localisations.

The Sec7 domain of Yel1 has been shown to be important for localisation (27). Here we show that deletion of the C-terminal EFA6 homology region also reduces localisation to the plasma membrane. Rather we see labelling of internal bright spots that appear similar to Golgi or endosomes. At these sites Yel1 may be interacting with other Arfs and suggests that this C-terminal region may be important for the specificity of binding to Arf3, and that Sec7 domain confers more general Arf binding. Localisation of the PH domain of Yel1 was mostly to the nucleus (figure 4E).

As mentioned in the introduction, overexpression of Arf3p can rescue patch defects caused by deletion of the yeast WASP and WIP homologues Las17p and Vrp1 respectively (15).

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We believe that these results may partly be explained by our data demonstrating the effects of Arf3 on PtdIns(4,5)P2 levels. A regulatory interaction between profilin and PtdIns(4,5)P2 is well documented (35, 36), and profilin has also been suggested to bind to Vrp1 which in turn regulates Las17p (37). Other actin-binding proteins in yeast involved in endocytosis such as capping protein and twinfilin also appear to be regulated by PtdIns(4,5)P2 in the membrane (38-40). Thus, a large increase in PtdIns(4,5)P2 in cells overexpressing Arf3 may be expected to have substantial effects on factors regulating and organising actin which could result in the effects reported by Lambert and colleagues (15).

Another finding from our studies is a role in endocytosis for the ArfGAP homology protein Gts1p. We demonstrate the importance of the Gts1 GAP homology domain for its effects on Arf3p localisation (fig4). We also show that the effects of *gts1* deletion on PtdIns(4,5)P2 levels are mediated through arf3. Mutation of the I224 residue in the putative ALPS motif reduces the lifetime of Gts1 at the plasma membrane indicating that this residue may form part of an amphipathic helix with a role in stabilising Gts1 localisation at the membrane. Gts1 has previously been studied as a regulator of yeast ultradian rhythm (22). This is the oscillatory mechanism that couples fermentation with redox state in addition to the transcriptome and cell-division-cycle progression. Ultradian rhythm has been postulated to be a basic universal necessity for maintaining intracellular coherence (41). Our research suggests the possibility that Gts1p may serve to link the process of endocytosis to the metabolic oscillator and growth machinery. Future studies will aim to investigate this possible link further.

Finally, our data suggest that this Arf module comprising Arf3p, Gts1p and Yel1p is of particular importance on the plasma membrane of the bud and neck region rather than in the mother. This could be because the kinase Mss4p is able to generate sufficient PtdIns(4,5) P2 itself in the mother to support the level of endocytosis that is required there. However, to facilitate the higher level of endocytosis that normally occurs within the bud, additional PtdIns(4,5)P2 is required. Arf3p may function to activate Mss4p further to produce an increased level of PtdIns(4,5)P2 in this region. Our data in which we determine the number of endocytic events per minute in cells expressing wild-type and elevated levels of active Arf3 indicate that increased Arf3p levels correlate with increase numbers of endocytic events (figure 6). This also suggests that Arf3p functions as part of a mechanism to generate the polarity in endocytosis observed in mother and bud regions of yeast cells.

Materials and Methods

Yeast strains and cell growth

Yeast strains carrying complete gene deletions, and expressing GFP- or mRFP- tagged proteins, with the exception of KAY752 were constructed by PCR-based integrative transformations as described by Longtine *et al.* (42). KAY752 was derived by transformation of KAY725 (Gts1GFP) with a linearised pKA57 to delete *sla1*. Functionality for tagged proteins was verified. For Gts1GFP we demonstrated that when combined with the *sla1* deletion the strain grew similarly to the *sla1* delete alone and better than in the combined Δ*gts1*Δ*sla1* mutant. Since Yel1GFP is able to localise in the presence of absence of tagged Arf3, but not in the absence of *arf3*, we reasoned that tagged Arf3 was also functional. Sla1GFP was functional by the same criteria as described for Gts1GFP above. Strains used in this study are listed in Supplementary Table I. Plasmids and oligonucleotides used to generate PCR products for direct deletions and epitope tagging are given in Supplementary Table II and Table III respectively. All integrants were verified by colony-PCR amplification to confirm the replacement occurred at the expected locus. Plasmids were also generated to express Gts1 mutations. Gts1 and its promoter region was cloned into a

YCplac vector and expressed either alone or fused to GFP. Mutations I224S and C50S,C53S were introduced using oligos listed and using Quick Change mutagenesis (Stratagene).

Cells were grown with rotary shaking at 30°C in liquid YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose supplemented with 40 μg/ml adenine) or in synthetic medium (0.67% yeast nitrogen base, 2% glucose) with appropriate supplements. Zygote isolation and tetrad dissection was performed using a Singer Instruments MSM Manual micromanipulator. Transformations were performed using lithium acetate as previously described (43).

Yeast Two-Hybrid assays

The yeast two-hybrid screen used bait and activation plasmids, and a yeast strain pJ69-4A designed and constructed by P. James (44). Matα strains carrying the binding domain constructs and MATa strains carrying the activation domain constructs were crossed and diploids were selected on selective synthetic media (lacking either uracil and leucine or lacking tryptophan and leucine depending on plasmids used). Diploid strains were grown overnight in selective liquid culture and plated as serial (10x) dilutions onto plates lacking leucine, tryptophan, (or uracil), adenine and histidine to allow combinations that trigger activation of reporter genes to be detected. Further stringency was made by adding 4 mM 3-aminotriazole to the plates. Growth was scored after plating as spots on plates lacking leucine, tryptophan, histidine and adenine; ± 3-amino triazole. In all cases negative controls with empty binding domain plasmids were carried out to check for self-activation.

Strains carrying the Gal4 activation domain fusion plasmids (Yel1AD, Arf3AD, Sla1AD, Ysc84 AD, Lsb3AD, Bsp1AD, Pan1AD, Yap1801AD, Yap1802AD, Ark1AD, and Clc1AD) were a gift from P. Piper. (University of Sheffield) The Sla2 AD plasmids were a gift from David Drubin (University of Berkeley). The Arf3BD plasmid was a kind gift from Sean Munro (LMB Cambridge). Gts1 binding domain plasmid was made using the pBDC plasmid (a gift from P. Piper, Sheffield) and oligonucleotides oKA390 and oKA391 and the method as described (45).

Flow Cytometry

Cell expressing PH(PLCδ1)GFP were grown overnight and then re-inoculated into fresh medium for 3-4 hours to an OD600 of about 0.5. Fluorescence was analysed using a Dako Cyan flow cytometer (Dako, Carpinteria, CA). Flow cytometry parameters were set at excitation and emission settings of 304 and 551 nm (filter FL-1), respectively. Each strain gave a single peak and the median of the range was plotted. 10,000 cells were analysed in each case and each strain was analysed through at least 5 independent experiments.

Microscopy

Fluorescence microscopy was performed with a DeltaVision RT Restoration Microscopy System running SoftWoRx™ image analysis and model-building application (Applied Precision Instruments, Seattle). For live-cell imaging, cells expressing tagged proteins were visualized after growing to early log phase in synthetic medium with appropriate supplements. When the effect of actin depolarization was examined, cells were treated for 5-30 min with 200μM latrunculin-A (LAT-A) by the addition of a 50 mM stock in DMSO to the medium as described before (18). FM4-64 (3-triethylammoniumpropyl-4-p-diethylaminophenylhexatrienyl pyridinium dibromide) labelling of cells was carried out in co-staining experiments with cells carrying the GFP-PH reporter construct in synthetic media lacking uracil. Cells were grown to log phase, 1 ml of cells was harvested and cells were incubated with 16 nM FM4-64 for 15 minutes, followed by a chase without the dye for 1 hour. Cells were then viewed. *Z*-stack images were collected with step sizes of 0.2 μm.

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Except where noted otherwise, time-lapse live cell imaging of GFP-tagged proteins was performed with 1 sec time-lapse. All image data sets were deconvolved, the distance of moving fluorescence spots were measured, and the arbitrary profile of intensity values, image coordinates and tracking of patch movements were established using the SoftWoRx application. Where enlargement is shown, the image was depixellated using the interpolated zoom facility. Images were exported as TIFF files and image size was adjusted to 300 dpi and assembled using Adobe Photoshop CS2. Movies were assembled using ImageJ software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LAT-A	latrunculin-A
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GGA	Golgi localised, gamma ear homology domain, ADP-ribosylation factor (Arf) binding
GAT	GGA and Tom1
VHS	Vps27, Hrs and STAM
PtdIns(4,5)P2	phosphatidyl inositol 4,5, bisphosphate

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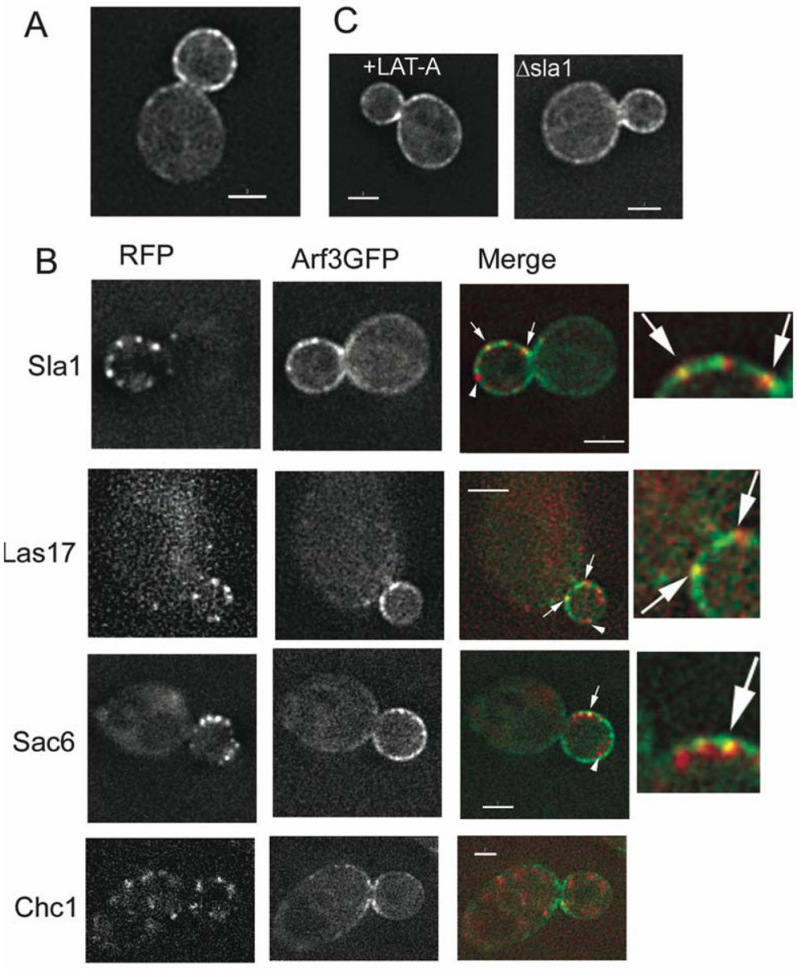

Figure 1.

Localisation and behaviour of Arf3p protein at the plasma membrane. (A) Fluorescence of Arf3p-GFP in a wild-type cell. Arf3p localises to the plasma membrane in patch-like structures and its localisation is polarised mainly to bud and neck of the cell. This image is taken from supp. movie 1 (B) Arf3p co-localisation study - insets shown are taken from the merged images. Arf3p shows partial co-localisation with endocytic proteins Las17p, Sac6p and Sla1p. Co-localisation with clathrin heavy chain was not detected. Arrows mark the spots that co-localise and arrow-heads mark spots that do not co-localise. (C) Arf3p loses its bud polarised localisation in the presence of LAT-A and in cells lacking *sla1* expression. Both wild-type cells expressing Arf3p-GFP treated with the actin-disrupting drug latrunculin-A (200μM for 5-30 min) and Δ*sla1* cells expressing Arf3pGFP were visualised by DeltaVision microscope. Bar, 2 μm.

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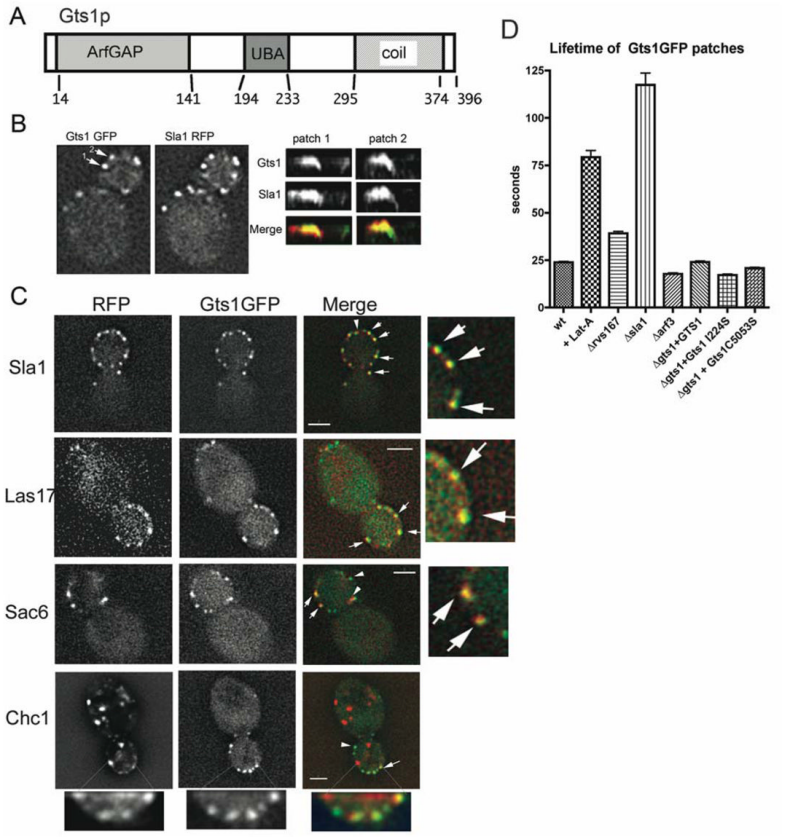

Figure 2.

Structure and localisation of the Gts1 protein. (A) Domain structure of Gts1p. Identifiable domains and their positions are depicted. (B) Gts1p localisation at the membrane. Visualisation of GFP-tagged Gts1 and kymograph show an average 24 second lifetime on the membrane. Co-localisation is shown with Sla1-mRFP. (C) Gts1p co-localisation with endocytic proteins. Gts1-GFP was co-expressed with Las17p-mRFP, Sac6p-RFP, Chc1p-RFPC and Sla1p-mRFP. Co-localisation of Gts1p with all of these proteins was observed, although the highest co-localisation rate is clearly visible for Gts1p and Sla1p proteins. Box is magnified 3 times. Arrows mark the spots that co-localise and arrow-heads mark spots that do not co-localise. Bar, 2μm. (D) Bar graph showing average patch lifetime of Gts1p-GFP in *WT,* Δ*rvs167,* Δ*sla1,* Δ*arf3* and after treatment with 200 μM LAT-A for 30min. Lifetimes of Gts1 mutants Gts1 I224S and Gts1 C50S C53S are also shown. All lifetimes are significantly different from wt Gts1GFP lifetimes ($P<0.0001$) and the mutants Gts1 I224S and Gts1C50S,C53S are significantly different from cells transformed with wild type GTS1 ($P<0.0001$). Error bars show standard errors of the mean (n> 35 patches).

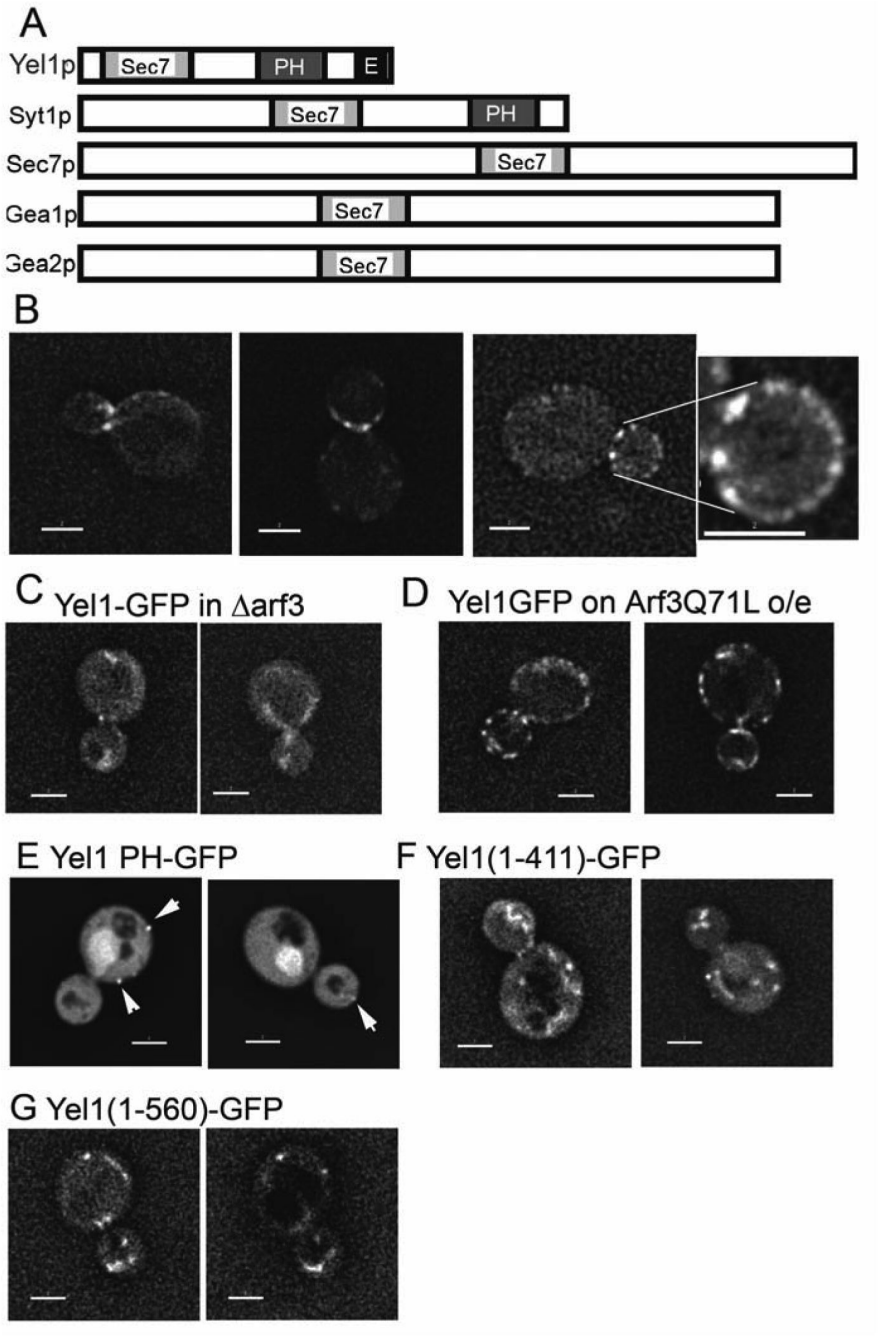

Figure 3.

Domain structure and localisation of Yel1p. (A). Five proteins in *S. cerevisiae* contain the Sec7 domain (Sec7p, Gea1p ,Gea2p, Syt1p and Ybl060w/Yel1p). Of these Syt1p and Yel1p also contain a PH domain. E - denotes a region of homology (607-685) with the EFA-6 protein. (B) Yel1p localises mainly to the neck and bud membrane. Cells expressing GFP-tagged Yel1p were visualised using a DeltaVison microscope as described. (C) Deletion of Δ*arf3* causes delocalisation of the Yel1p GEF. The *ARF3* gene was deleted in cells expressing Yel1-GFP and cells were analysed as described. Yel1p was no longer observed at the plasma membrane, though some localisation on internal membranes is seen. (D) Overexpression of the active Arf3 mutant Arf3Q71L causes an increased level of Yel1GFp

at the plasma membrane and the protein becomes localised in punctate foci. Bar, 2 μm. (E) The PH domain of Yel1 fused to GFP localises mostly to the nucleus, though some cortical spots can be observed - arrows. (F) Yel1(1-411) lacking the PH and EFA-6 homology region fused to GFP (G) Yel1(1-560) lacking the EFA-6 homology region fused to GFP. In all images Bar, 2 μm.

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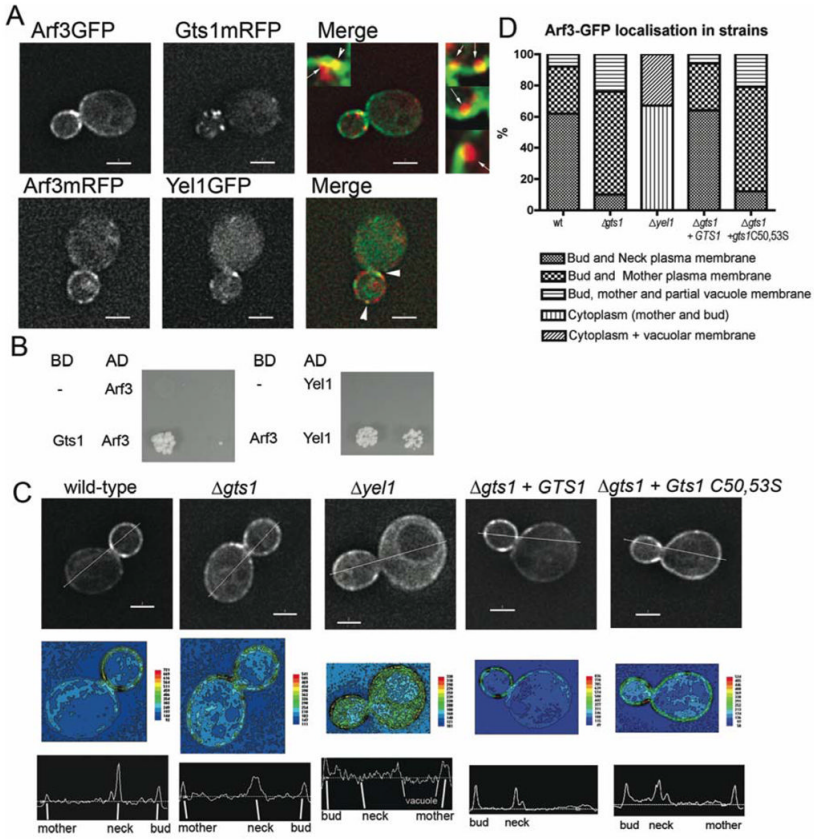

Figure 4.

Gts1p and Yel1p interact and co-localise with Arf3p. (**A**) Upper panels - localisation of Arf3GFP and Gts1-mRFP in wild-type cells. Both proteins localise to the plasma membrane and spots of co-localisation can be seen (arrowhead), though more often the spots appear to partially overlap with Gts1p on the cytosolic side and Arf3p within the plane of the membrane (arrows). Further examples are shown with three merge figures at the right. Lower panels - localisation of Yel1-GFP and Arf3-mRFP and Arf3p in wild-type cells. Spots of co-localisation can be observed (arrowheads). Bar, 2μm. (B) Two hybrid assays were performed as described in materials and methods to investigate possible interactions between Arf3, Gts1 and Yel1. Adjacent spots are 10 fold dilutions of strains (C) Deletion of either *yel1*, *gts1* or expression of the gts1 C50,53S mutant affect Arf3p localisation consistent with their activity. Fluorescence image, graph of Arf3p-GFP intensity and cell profile are shown for *WT, Δyel1, Δgts1* and *gts1C50,53S* strains. Profiles display a plot of intensity values along a line marked on every cell. In wild-type strains Arf3p localisation is polarised mainly to bud and neck of the cell. Deletion of *GTS1* causes increased of fluorescence intensity in the mother cell plasma membrane. Deletion of *YEL1* causes a shift of fluorescence to the mother cell plasma membrane, the cytosol and the vacuolar membrane. Cells expressing Gts1 C50S,C53S have a similar profile to the Δgts1 strain. Bar, 2μm. (D) Quantitation of Arf3GFP localisation in the mutant strains. >100 cells counted for each mutant.

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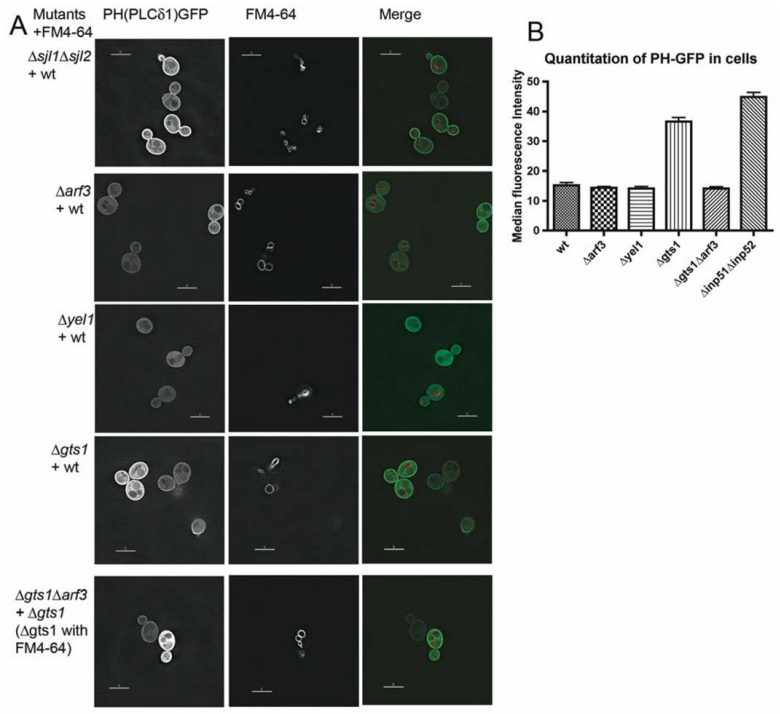

Figure 5.

Changes in levels of Arf3 modulate PtdIns(4,5)P2 levels at the plasma membrane. (A) Cells (wild-type, Δ*arf3*, Δ*yel1*, Δ*gts1, inp51Δinp52, Δgts1Δarf3*) were transformed with a reporter construct expressing a PH domain (PLCδ1) fused to GFP. To allow direct comparison with wild type cells for intensity levels of the PH localisation each mutant was co-incubated with FM4-64 so it could be distinguished (see materials and methods). Loss of *arf3* or *yel1* causes a reduction in PtdIns(4,5)P2 compared to wild-type cells, while Δ*gts1* and Δ*inp51*Δ*inp52* cells have increased PtdIns(4,5)P2. Deletion of Δ*arf3* in Δ*gts1* cells reduces PH-GFP at the membrane indicating the effect of Gts1 is mediated through Arf3. Bar = 2 μm. (B) Cells expressing the PH reporter construct were also analysed by flow cytometry as described in materials and methods. The data depicted represents >5 independently performed experiments for each strain. The graph shows the median fluorescence intensity for each strain. Error bars are standard error.

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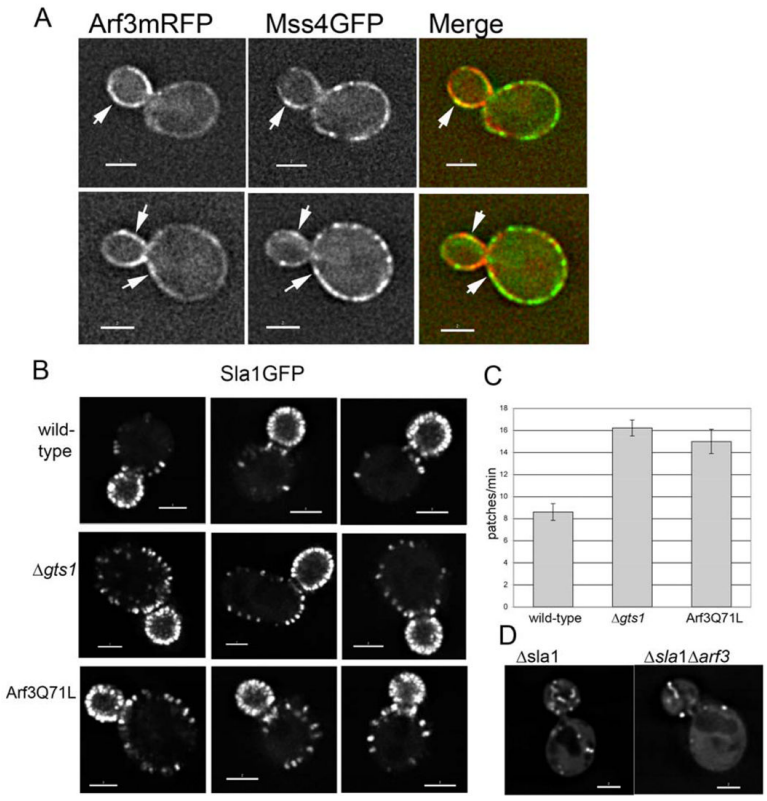

Figure 6.

Arf3 and Mss4 show some co-localisation in the bud and neck region. (A) Arf3mRFP and Mss4GFP were expressed in cells and localised as described. As shown Arf3 localises primarily to the bud and neck region, while Mss4 is on both the mother and bud membrane. Arrowheads indicate spots of co-localisation. Bar, 2 μm.(B) Levels of Arf3p correlate with numbers of endocytic events. Sla1GFP was expressed in wild-type cells or in cells containing elevated active Arf3p (Δ*gts1* and cells transformed with a Arf3Q71L plasmid). Patch movement was tracked during 90 second time-lapse movies. Note the increased number of spots from sites within the mother in cells with elevated Arf3 activity. (C) The number of endocytic patches in 10 mother cells was counted over the 90 second period and expressed graphically as number of patches in mother cells observed per minute for each strain. Bars = 2 μm. Error is standard error of the mean. (D) Deletion of *arf3* can reduce observe patch number in cells. Δ*sla1* cells have fewer patches than wild-type cells and so were used as a starting point to determine whether there was any added effect of *arf3* deletion. As shown deletion of *arf3* in an *sla1* deletion background does causes a reduction in the number of patches observed. Average patch number in Δ*sla1* cells 13.68±0.7 and in Δ*sla1*Δ*arf3* cells 6.93±0.7. t tests indicate P value of $P<0.0001$. Bar, 2 μm.

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Table I

Interactions of Gtslp by Two Hybrid Analysis

Binding Domain	Activation Domain	Strength of Interaction [a]
Gtsl	empty	−
empty	Gts1	−
empty	empty	−
Gtsl	Ysc84	+
Gtsl	Lsb3	+
Gtsl	Sla1	++
Gtsl	Clc1	+
Gtsl	Sla2(aa310-769)	+
Gtsl	Sla2(aa206-769)	+++
Gtsl	Bsp1	+++
Gtsl	Pan1	+++
Gtsl	Yap1801	++
Gtsl	Yap1802	++
Gtsl	Ark1	+

[a]Strength of interaction: − no growth on any interaction plates; + weak growth only on plates lacking leucine, tryptophan, histidine and adenine; ++ moderate/strong growth on plates lacking leucine, tryptophan, histidine and adenine, no or very poor growth on plates with 3-amino triazole; +++ strong growth on plates lacking leucine, tryptophan, histidine and adenine + 3-aminotriazole. In all cases negative controls with empty binding domain plasmids were carried out to check for self-activation.