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Vrp1p–Las17p interaction is critical for actin patch polarization but is not essential for growth or fluid phase endocytosis in *S. cerevisiae*

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ABSTRACT

Vrp1p (yeast WIP) forms a protein complex with Las17p (yeast WASP), however the physiological significance of the interaction has not been fully characterized. Vrp1p residues, $_{788}$MPKPR$_{792}$ are essential for Vrp1p–Las17p interaction. While C-Vrp1p$_{364–817}$ complements all the defects of the *vrp1Δ* strain, C-Vrp1p$^{5A}_{364–817}$ ($_{788}$AAAAA$_{792}$) does not complement any of the defects, due to its inability to localize to cortical patches. Targeting C-Vrp1p$^{5A}_{364–817}$ to membranes using CAAX motif (C-Vrp1p$^{5A}_{364–817}$-CAAX) rescued the growth and endocytosis defect but not the actin patch polarization defect of *vrp1Δ*. Vrp1p can localize to cortical patches, either by binding to Las17p through LBD (Las17 Binding Domain, Vrp1p$_{760–817}$) or independent of Las17p through residues in N-Vrp1p$_{1–364}$. Unlike Vrp1p, Vrp1p^{5A} localizes poorly to cortical patches and complements all the defects of *vrp1Δ* strain except actin patch polarization at elevated temperature. N-Vrp1p$_{1–364}$ complements all the defects of *vrp1Δ* strain except the actin patch polarization defect while N-Vrp1p$_{1–364}$–LBD fusion protein complements all the defects. Thus our results show that while both Vrp1p and Las17p are essential for many cellular processes, the two proteins do not necessarily have to bind to each other to carry out these cellular functions. However, Las17p–Vrp1p interaction is essential for actin patch polarization at elevated temperature.

1. Introduction

The actin cytoskeleton in *Saccharomyces cerevisiae* is organized into cortical actin patches and cytoplasmic actin cables. Actin patches are polarized towards sites of surface growth during the cell cycle, i.e. nascent bud sites, the tips of small buds, isotropically in large buds and on either side of the bud neck during cytokinesis. Actin cables are thick filaments that align along the mother–bud axis [1–5]. Actin patches undergo rapid movement at the cortex and this movement correlates with internalization of endocytic cargo consistent with participation of cortical actin patches in the internalization step of endocytosis at the plasma membrane [6–9].

Vrp1p (verprolin/End5p) is the functional homologue of mammalian Wiskott–Aldrich Syndrome Protein (WASP)-Interacting Protein (WIP) and is critical for cortical actin patch distribution and endocytosis in *S. cerevisiae* [10–15]. Vrp1p localizes to cortical patches that partially co-localizes with cortical actin patches [12,13]. Loss of Vrp1p (*vrp1Δ*) leads to a loss of cortical actin patch polarization, defects in both receptor-mediated and fluid-phase endocytosis at all temperatures and in-viability at elevated temperature [10,11,13].

Like Vrp1p, Las17p and type I myosins localize to cortical patches with a polarized distribution and partially co-localize with cortical actin patches [16–18]. Las17p localizes to cortical actin patches independent of Vrp1p but Vrp1p is required for polarization of Las17p patches [19,20]. Similarly, type I myosins localize to cortical patches independent of Vrp1p, however polarization of type I myosin patches is dependent on Vrp1p [16]. Las17p and type I myosins promote assembly of G-actin into short actin filaments through the Arp2/3 complex [19,21–24] and Vrp1p is essential for activation of the Arp2/3 complex by type I myosins in vitro [25].

Vrp1p can be divided into two functional modules, N-Vrp1p$_{1–364}$ and C-Vrp1p$_{364–817}$ [20]. C-Vrp1p$_{364–817}$ localizes efficiently to cortical actin patches and complements all the defects of *vrp1Δ* cells [20,26] while N-Vrp1p$_{1–364}$ localizes poorly to cortical patches and complements only the growth and endocytosis defect of *vrp1Δ* cells [20,27]. N-Vrp1p$_{1–364}$ binds with actin monomers [13,20] while C-Vrp1p$_{364–817}$ binds with Las17p, yeast WASP [12,17,18,21,23,28]. Regions in both N-Vrp1p$_{1–364}$ and C-Vrp1p$_{364–817}$ also bind type I myosins [16,21,22,29].

The mammalian homologues of the yeast proteins, WASP (Las17p) and WIP (Vrp1p) form a stable complex and the WASP-WIP interaction is essential for the function and stability of WASP in both animal cells and in *S. cerevisiae* [30–32]. Recently, we identified a mutant of Las17p which does not interact with Vrp1p and this mutant Las17p^{TL} (L80T and H94L) was able to complement all the defects of *las17Δ* strain except actin patch polarization at 37 °C, even when the mutant protein was expressed from a 2 μ plasmid to compensate for the protein instability at 37 °C [33]. It is possible that the mutation (L80T and H94L) may have abolished interaction(s) with other uncharacterized proteins. Human

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WIP interacts with WASP through three distinct motifs [34] and it is not clear whether Vrp1p forms similar interaction with Las17p. Thus the identification of residues in Vrp1p which are critical for Vrp1p–Las17p interaction will lead to a better understanding of the role of Vrp1p–Las17p interaction in cellular functions.

In order to further characterize the role of Las17p–Vrp1p interaction in cellular function, we sought and identified the residues ($_{788}$MPKPR$_{792}$) in Vrp1p which are essential for Vrp1p–Las17p interaction. The full length protein with the mutation Vrp1p^{5A} ($_{788}$AAAAA$_{792}$) restored all the defects of *vrp1Δ* cells except actin patch polarization at 37 °C while C-Vrp1p$^{5A}_{364–817}$ was not able to restore any of the defects of *vrp1Δ* cells. C-Vrp1p$^{5A}_{364–817}$ targeted to membrane using the CAAX motif restored growth at 37 °C and endocytosis at all temperatures tested but not actin patch polarization. Thus efficient localization of Vrp1p to cortical patches mediated by interaction with Las17p is essential for cortical actin patch polarization at 37 °C.

2. Materials and methods

2.1. Yeast strains and techniques

The *S. cerevisiae* strains used in this study are: AMY88 (*MAT***a** *lys2 his4 leu2 ura3 vrp1Δ::KanMx bar1* [20] and PJ69-4A (*MAT***a** *his3 leu2 ura3 trp1 gal4Δ gal80Δ met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3*) [35], IDY166 (*MAT***a** *lys2 his4 leu2 ura3 1as17Δ::URA3*) [12]. YPUAD is 1% yeast extract, 2% peptone, 2% glucose supplemented with 40 μg/ml adenine and 20 μg/ml uracil. SD minimal medium is described in [36]. Plasmid DNA was introduced into yeast cells using a modification of the lithium acetate protocol [11]. Yeast two-hybrid interactions were tested using PJ69-4A as previously described [35].

2.2. DNA Techniques

Standard DNA techniques were performed as described [37]. DNA sequences encoding fragments of Vrp1p were expressed from a YCplac111-based low-copy-number plasmid [38] under the control of the *VRP1* promoter (nucleotides −240 to the *VRP1* ATG start codon). Sequences encoding various fragments of Vrp1p were amplified by PCR and inserted into this plasmid. For GFP-tagged Vrp1p fragments, a sequence encoding GFP was fused in-frame downstream of the *VRP1* coding sequence. The sequences encoding the Ras1p CAAX box were fused downstream of the *VRP1* coding sequence [39]. The sequences of all constructs were confirmed by automated DNA sequencing. Further details of the constructions are available upon request.

2.3. Protein extracts and immunoblotting

Yeast cells growing in exponential phase were harvested and the cell pellet (7 OD_{600} units) was resuspended in 240 μl lysis solution

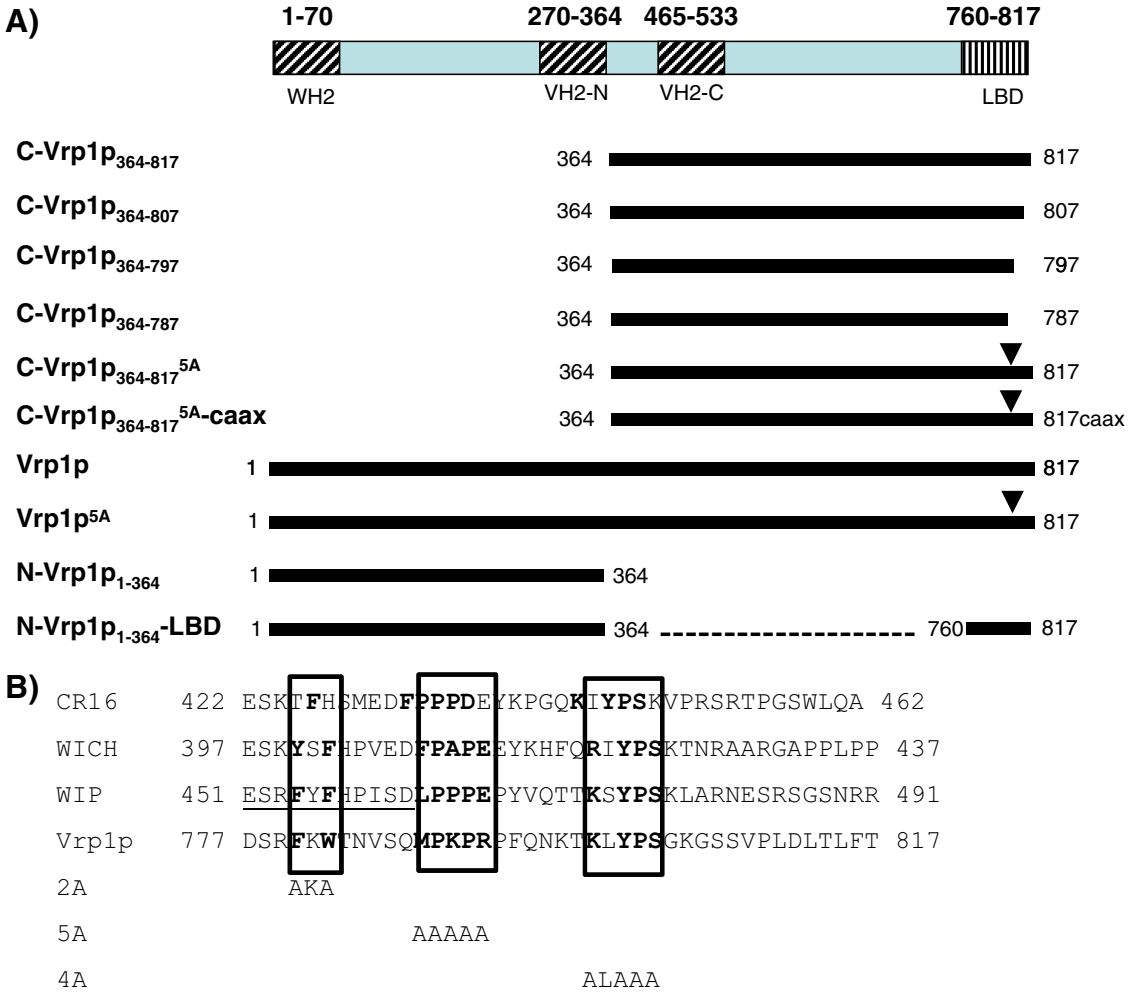


Fig. 1. Vrp1p domain structure and the constructs used. (A) Schematics of Vrp1p and its deletion constructs. Vrp1p contains an N-terminal WH2 domain (1–70) and two additional actin binding domains, VH2-N (270–364) and VH2-C (465–533). Truncated proteins used for functional studies are shown as black bars. (B) Alignment of the tail regions of the three mammalian verprolins with Vrp1p. The three site direct mutants used in this study are 2A (F780A, W782A), 4A (K799A, Y801A, P802A, S803A) and 5A ($_{788}$MPKPR$_{792}$ $_{788}$AAAAA$_{792}$).

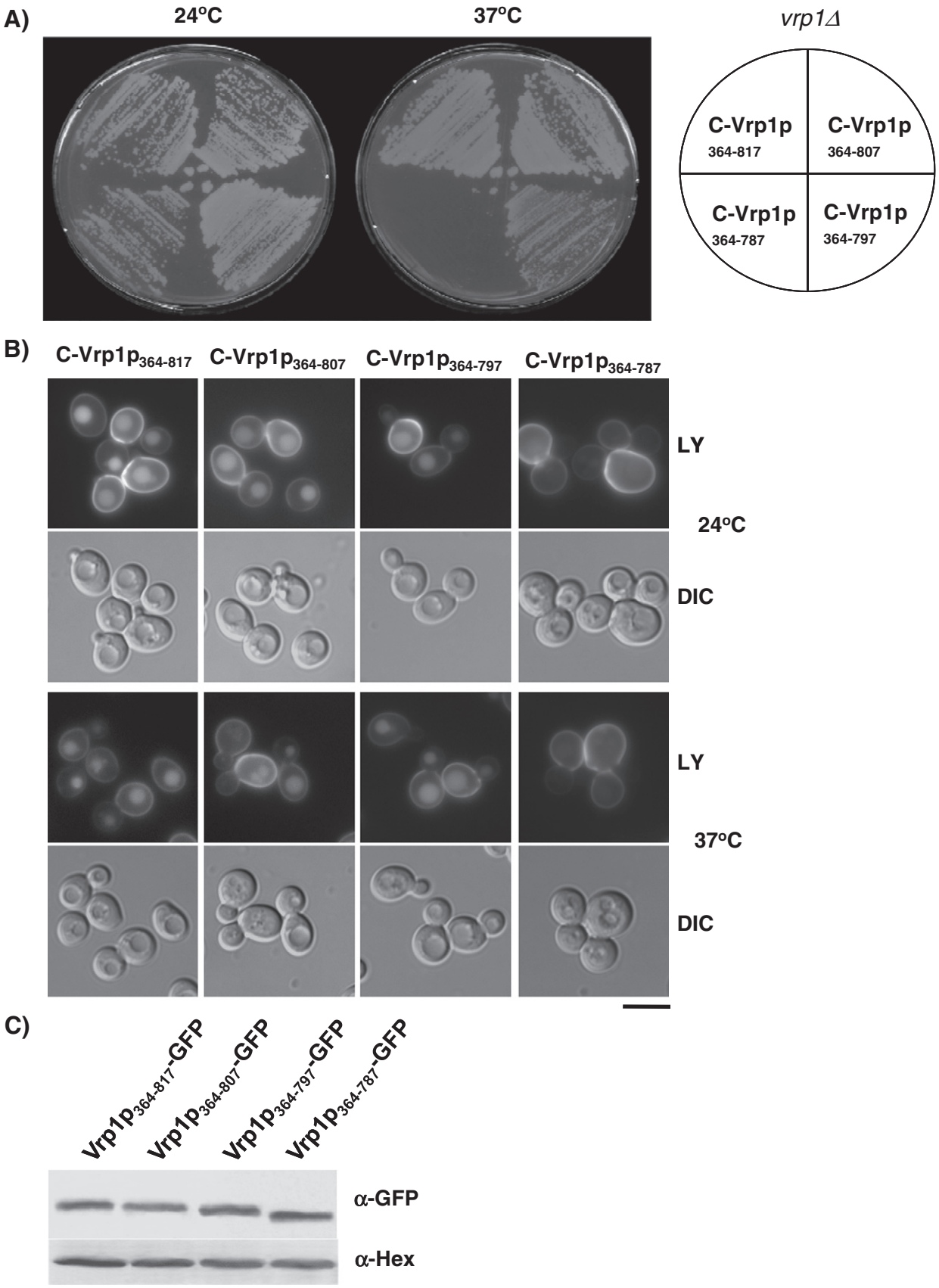
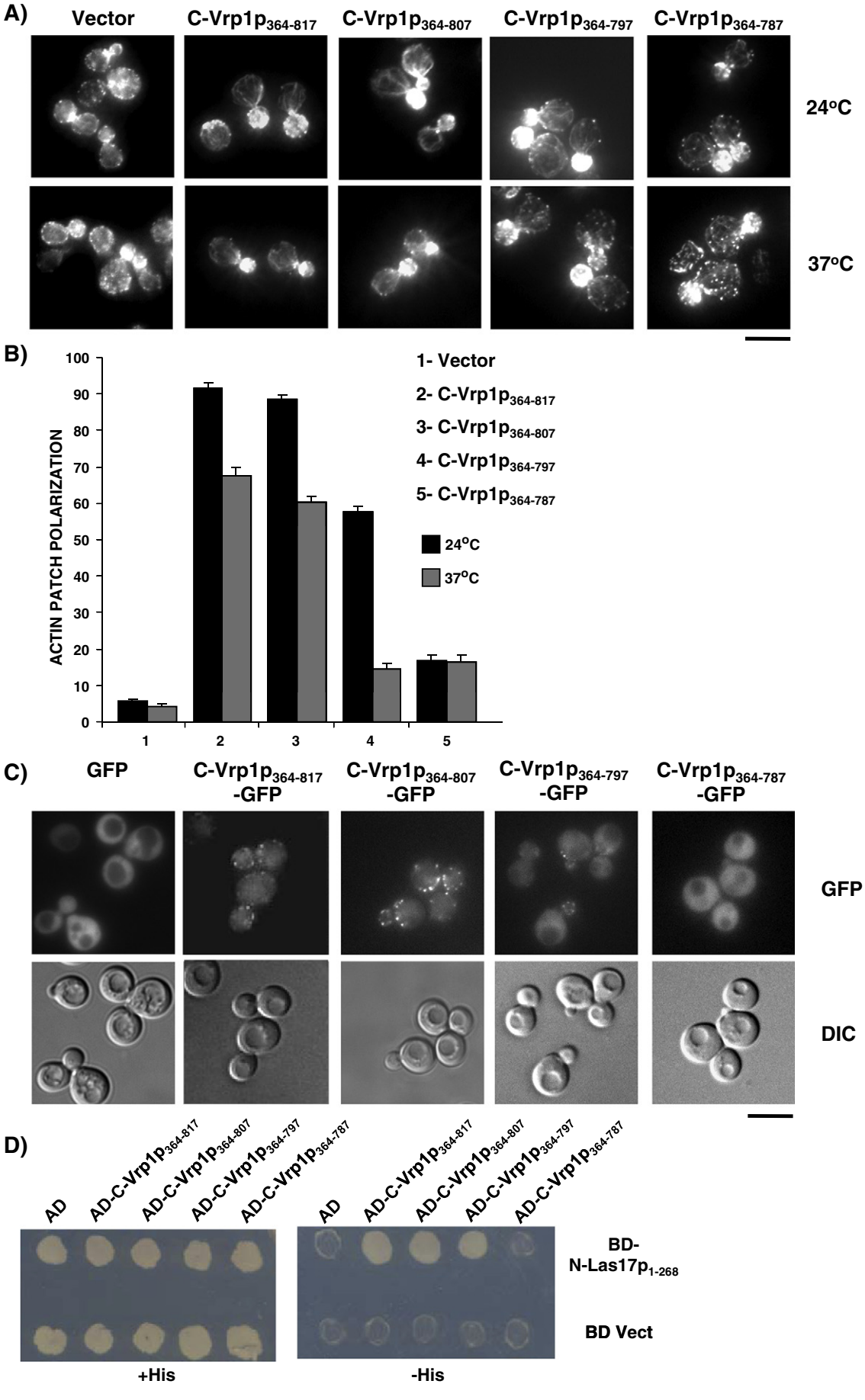


Fig. 2. Residues 788–797 of Vrp1p are essential for the function of C-Vrp1p in growth and endocytosis. (A) Growth of *vrp1Δ* cells harboring centromeric plasmid expressing C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$ or C-Vrp1p$_{364–787}$ at 24 °C and 37 °C. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C and photographed after 3 days. (B) *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$ or C-Vrp1p$_{364–787}$ were grown in YPUAD to exponential phase at 24 °C and LY uptake was carried as described in materials and methods. *Upper panels*: LY. *Lower panels*: DIC. Bar, 5 μm. (C) *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$-GFP, C-Vrp1p$_{364–807}$-GFP, C-Vrp1p$_{364–797}$-GFP or C-Vrp1p$_{364–787}$-GFP were grown to exponential phase at 24 °C in YPUAD. Proteins extracts from the cells were analyzed by immunoblotting using anti-GFP (α-GFP) and anti-hexokinase (α-Hex).


A)
Vector
C-Vrp1p364-817
C-Vrp1p364-807
C-Vrp1p364-797
C-Vrp1p364-787
24°C
37°C
B)
ACTIN PATCH POLARIZATION
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
1- Vector
2- C-Vrp1p364-817
3- C-Vrp1p364-807
4- C-Vrp1p364-797
5- C-Vrp1p364-787
24°C
37°C
C)
GFP
C-Vrp1p364-817 -GFP
C-Vrp1p364-807 -GFP
C-Vrp1p364-797 -GFP
C-Vrp1p364-787 -GFP
GFP
DIC
D)
AD
AD-C-Vrp1p364-817
AD-C-Vrp1p364-807
AD-C-Vrp1p364-797
AD-C-Vrp1p364-787
AD
AD-C-Vrp1p364-817
AD-C-Vrp1p364-807
AD-C-Vrp1p364-797
AD-C-Vrp1p364-787
BD- N-Las17p1-268
BD Vect
+His
-His

(1.85 N NaOH/1.06 M β-mercaptoethanol) and incubated on ice for 10 min. The protein was precipitated with an equal volume of 20% trichloroacetic acid (TCA) on ice for 10 min. The pellet was collected by centrifugation, resuspended in 100 µl of SDS-PAGE loading buffer and heated to 95 °C for 2 min. The proteins were resolved on a 10% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane, probed with appropriate primary antibodies and HRP-conjugated secondary antibodies.

2.4. Lucifer Yellow uptake assay

Fluid phase endocytosis of LY was performed as described [12]. All the transformants were grown to exponential phase in YPUAD and LY uptake was performed at either 24 °C or 37 °C as specified for 1 h. The cells were washed and then visualised using a LY-specific light filter.

2.5. Localization of Vrp1p

To visualise the sub-cellular distribution of GFP tagged proteins, cells carrying Vrp1p-GFP expressing constructs growing exponentially in SD selective medium at 24 °C were applied to a microscope slide and the GFP signal in living cells was visualised by fluorescence microscopy using a fluorescein isothiocyanate (FITC)-specific light filter.

2.6. F-actin staining

Yeast cells were grown in YPUAD to exponential phase at 24 °C and then fixed by direct addition of 3.7% formaldehyde to the culture and incubated for 30 min at the temperature of growth as described [40]. For visualizing the actin cytoskeleton at 37 °C, the cells growing at 24 °C were shifted to 37 °C for 2 h before fixing. Fixed cells were permeablised using 1% Triton-X-100 in PBS, stained with Alexa-488-conjugated phalloidin and F-actin visualised using fluorescence microscopy, using a Leica RA fluorescence microscope (Leica, Singapore). Images were captured using a CoolSnap^{HQ} cooled charge-coupled device camera.

3. Results

3.1. C-Vrp1p$_{364–817}$ residues 788–797 are essential for cortical actin patch polarisation, endocytosis and growth at elevated temperature

Vrp1p is an actin cytoskeletal protein and deletion of *VRP1* leads a strain with defects in growth at high temperature, defects in endocytosis and actin patch polarization [10,11,13]. We have previously shown that C-Vrp1p$_{364–817}$ can functionally replace the full-length Vrp1p in complementing all the growth defects of *vrp1Δ* strain [20]. The ability of C-Vrp1p$_{364–817}$ to correct the actin patch polarization defect depends on the presence of Las17p Binding Domain (Vrp1p 760–817, LBD) as deletion of LBD results in a peptide, C-Vrp1p$_{364–760}$ which is unable to complement both the growth defect and actin patch polarization defect [26]. However, it is possible that deletion of 57 amino acids may have abolished interaction with other uncharacterized proteins as well as caused changes in the conformation of the protein.

In order to characterize the role of Vrp1p–Las17p interaction in actin patch polarization, we sought to identify the amino acid residues in Vrp1p which are essential for the interaction between Vrp1p and Las17p. To narrow down the amino acid residues in the tail domain of Vrp1p which are important for interaction with Las17p, we constructed deletion mutants initiating at the C-terminus of C-Vrp1p$_{364–817}$ (Fig. 1A). The three deletion constructs were expressed from a centromeric plasmid under the transcriptional regulation of *VRP1* promoter in *vrp1Δ* (AMY88) cells to assess the function of these mutants (Fig. 1A). The two deletion mutants C-Vrp1p$_{364–807}$ and C-Vrp1p$_{364–797}$ were able to rescue the growth and endocytosis defects of *vrp1Δ* strain just like C-Vrp1p$_{364–817}$ (Fig. 2A, B) however C-Vrp1p$_{364–787}$ was not able to rescue any of the defects (growth, endocytosis and actin patch polarization) of *vrp1Δ* strain (Figs. 2A, B, and 3A, B). Deletion of 10 residues had no effect on cortical actin patch polarisation (Fig. 3A, B) thus demonstrating that this region encompassing residues 808–817 are not essential for the function of C-Vrp1p$_{364–817}$. Additional deletion of 10 residues (798–807) from the C-terminus resulted in a protein (C-Vrp1p$_{364–797}$) which was able to restore all the functions just like the C-Vrp1p$_{364–817}$ except in rescuing the actin patch polarization defect of *vrp1Δ* strain at 37 °C (Fig. 3A, B).

In order to analyze the expression and localization of the deletion mutants, we tagged each of the mutants with GFP at the C-terminus. The GFP-tagged proteins (C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$) were functional in restoring growth at elevated temperature when introduced into *vrp1Δ* cells, indicating that addition of the GFP did not affect the function of the GFP fusion proteins (data not shown). The *vrp1Δ* strain expressing the deletion mutants were analysed using live cell imaging. Both C-Vrp1p$_{364–817}$-GFP and C-Vrp1p$_{364–807}$-GFP localized efficiently to cortical patches (Fig. 3C). While C-Vrp1p$_{364–797}$-GFP localized poorly to cortical patches, C-Vrp1$_{364–787}$-GFP showed only diffused cytoplasmic staining without any localization to cortical patches (Fig. 3C). In order to determine whether C-Vrp1p deletion mutants are localised to cortical actin patches, we expressed Arc40-RFP (Red Fluorescent Protein) in *vrp1Δ* cells also expressing GFP-tagged proteins (C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$). Consistent with our previous finding all the C-Vrp1p fragments which localized to cortical patches partially co-localized with actin patches (Fig. S1A). The majority of co-localization with actin were found in the mother cells, this could be due to the assembly of actin patches at sites of polarized growth (bud) and subsequent disassembly associated with movement away from those sites in the mother [41]. We analyzed the interaction of C-Vrp1p$_{364–817}$ and its deletion mutants (C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$, C-Vrp1p$_{364–787}$) with N-Las17p$_{1–268}$ using the yeast two-hybrid assay. While C-Vrp1p$_{364–817}$ and the two functional deletion mutants (C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$) were found to interact with N-Las17p$_{1–268}$, the non-functional deletion mutant C-Vrp1p$_{364–787}$ did not (Fig. 3D). Immunoblot analysis of total cell extracts prepared from *vrp1Δ* (AMY88) cells expressing the corresponding GFP-tagged versions of each protein (Fig. 2C) showed that all the deletion mutants are expressed at comparable levels. Thus the lack of complementation of growth defects of *vrp1Δ* strain by C-Vrp1p$_{364–787}$ is not due to poor expression but rather due to lack of localization of this peptide to cortical patches probably due to loss of interaction with Las17p.

3.2. C-Vrp1p$_{364–817}$–Las17p interaction is essential for actin patch polarization

Based on the deletion analysis, amino acids 788–797 of Vrp1p are essential for the activity of C-Vrp1p$_{364–817}$. The amino acids 408–412

Fig. 3. Residues 788–797 of Vrp1p are essential for cortical patch localization of C-Vrp1p$_{364–817}$ and actin patch polarization. (A) *vrp1Δ* cells harboring empty centromeric plasmid or centromeric plasmids expressing C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$ or C-Vrp1p$_{364–787}$ were grown to exponential phase at 24 °C in YPUAD and either left at 24 °C or shifted to 37 °C for 2 h. Cells were then stained with Alexa-488-conjugated phalloidin and visualized by fluorescence microscopy. Bar, 5 µm. (B) The actin patch polarization of *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$, C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$ or C-Vrp1p$_{364–787}$ was quantified. Actin patches in 100 small budded cells were analyzed and scored as polarized if the mother has less than 5 patches and scored as depolarized otherwise. The experiment was repeated three times with similar results. (C) *vrp1Δ* cells expressing GFP, C-Vrp1p$_{364–817}$-GFP, C-Vrp1p$_{364–807}$-GFP, C-Vrp1p$_{364–797}$-GFP or C-Vrp1p$_{364–787}$-GFP grown in YPUAD to exponential phase at 24 °C. GFP fluorescence was visualized in living cells by fluorescence microscopy. Bar, 5 µm. (D) Yeast two hybrid interactions between Gal4-BD-N-Las17p$_{1–268}$ (Gal4-Binding Domain) with Gal4-AD-C-Vrp1p$_{364–817}$ (Gal4-Activation Domain) or its mutants (C-Vrp1p$_{364–807}$, C-Vrp1p$_{364–797}$, C-Vrp1p$_{364–787}$) was assessed by growth on medium lacking histidine.

(FPAPE) of WIRE (WIP-RElated) are essential for interaction with mammalian WASP [42]. The region of Vrp1p which matches with these five amino acids falls within residues 788–797 of Vrp1p and is represented by MPKPR in Vrp1p. In order to analyze the function of this motif we mutated all the five amino acids to alanine and expressed the mutant in *vrp1Δ* cells. It has been proposed that three epitopes in WIP mediate interaction with WASP, epitope 1 comprises of Phe_{454} and Phe_{456}, epitope 2 comprises of residues 462–466 and epitope 3 comprises of Lys_{473}, Tyr_{475}, Pro_{476} and Ser_{477} [34,43,44]. Mutating the two Phenylalanine (F454 and F456) (Fig. 1B) residues to Alanine in WIP abolished WASP-WIP interaction [43] and similarly mutation of the corresponding residues in WIRE (Y400A and F402A) (Fig. 1B) abolished WASP-WIRE interaction [42]. Thus we mutated the corresponding Vrp1p residues (epitope 1: F_{780} and W_{782}) to alanine, C-Vrp1p^{2A} (FKW to AKA), and we also mutated Vrp1p residues (epitope 3: K_{799} and $Y_{801}P_{802}S_{803}$), to alanine, C-Vrp1p^{4A} (KLYPS to ALAAA). Expression of C-Vrp1p$^{2A}_{364–817}$ or C-Vrp1p$^{4A}_{364–817}$ from a centromeric plasmid rescued the growth, endocytic and actin patch polarization defects of *vrp1Δ* (Fig. S2). Mutating epitope 1 or epitope 3 residues did not affect the binding of C-Vrp1p to N-Las17p as determined by yeast two-hybrid assay as well as GST pull down assay (Fig. S2). Thus these two epitopes are not critical for Vrp1p-Las17p interaction. Expression of C-Vrp1p$^{5A}_{364–817}$ mutant (epitope 2) from a centromeric plasmid did not complement the growth defect (Fig. 4A), the endocytic defect or the actin patch polarization defect of *vrp1Δ* cells (Figs. 4B and 5A and B) at both 24 °C and 37 °C. Thus we analyzed the importance of Vrp1p–Las17p interaction using the C-Vrp1p$^{5A}_{364–817}$.

In order to analyze the localization and expression of C-Vrp1p$^{5A}_{364–817}$, we expressed the mutant with the GFP tag at the C-terminus. Live cell imaging showed that, while C-Vrp1p$_{364–817}$-GFP displayed cortical patch localization, C-Vrp1p$^{5A}_{364–817}$-GFP showed diffused cytoplasmic localization (Fig. 5C). The lack of cortical patch localization is not due to poor expression as C-Vrp1p$^{5A}_{364–817}$-GFP is expressed at comparable levels to C-Vrp1p$_{364–817}$–GFP (Fig. 4C).

The effect of the 5A mutation on the interaction between C-Vrp1p$_{364–817}$ and N-Las17p$_{1–268}$ was examined using yeast two hybrid assay and pull-down assay. While C-Vrp1p$_{364–817}$ interacted with N-Las17p$_{1–268}$ in a yeast two hybrid assay, C-Vrp1p$^{5A}_{364–817}$ did not (Fig. 5D). A GST pull down assay using *S. cerevisiae* cells expressing C-Vrp1p$^{5A}_{364–817}$–GFP and N-Las17p$_{1–206}$-GST gave similar results (Fig. S2F). In order to determine whether the LBD of Vrp1p is sufficient to mediate direct interaction with Las17p, we carried out pull down assay using proteins expressed from *Escherichia coli*. Las17p$_{1–206}$-His was able to pull down GST–Vrp1p$_{716–817}$ but not GST–Vrp1p$^{5A}_{716–817}$ (Fig. 5E). This shows that these 5 amino acids are critical for C–Vrp1p$_{364–817}$ interaction with Las17p and this interaction is essential for localization of C–Vrp1p$_{364–817}$ to cortical patches and also for growth at elevated temperatures and actin patch polarization.

We tested the effect of C–Vrp1p$^{5A}_{364–817}$ on the endogenous Las17p–Vrp1p complex by over expressing the mutant in Wild type *S. cerevisiae* cells. Over expression of C–Vrp1p$_{364–817}$ or C–Vrp1p$^{5A}_{364–817}$ did not affect the growth at 37 °C or endocytosis of the wild type *S. cerevisiae* (data not shown). However wild type cells over expressing of C–Vrp1$^{5A}_{364–817}$ were found to have a partial actin patch polarization defect at 37 °C compared to wild type cells over expressing C–Vrp1p$_{364–817}$ (Fig. S3). This suggests that the mutant C–Vrp1p$^{5A}_{364–817}$ might be titrating out some molecules (e.g. type I myosin) critical for actin patch polarization.

3.3. Addition of a CAAX box to C–Vrp1p$^{5A}_{364–817}$ bypasses the requirement for the Las17p mediated localization for endocytosis and growth at 37 °C

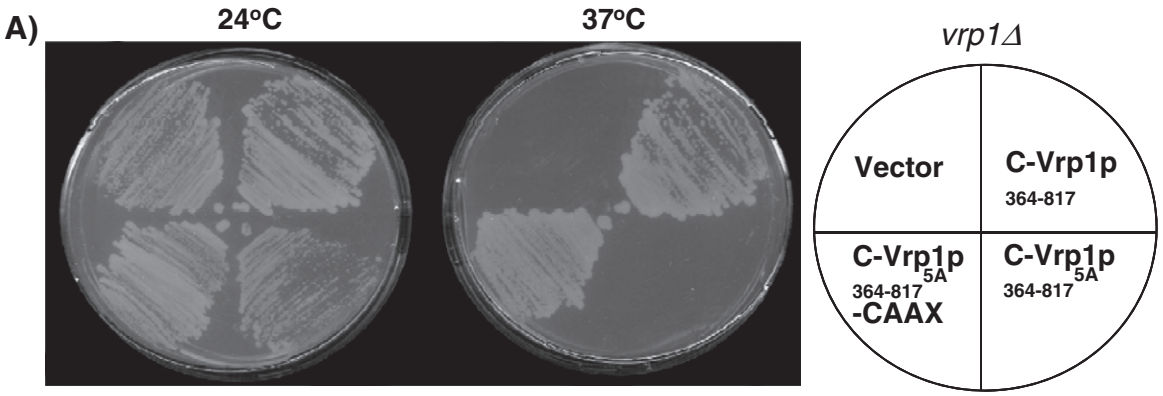
Addition of a CAAX box to the C-terminus of proteins confers covalent lipid attachment and efficient membrane anchoring to proteins [45]. Thus we fused the CAAX motif of Ras1p to C–Vrp1p$^{5A}_{364–817}$ (C–Vrp1p$^{5A}_{364–817}$–CAAX). This protein C–Vrp1p$^{5A}_{364–817}$–CAAX was expressed in *vrp1Δ* (AMY88) cells and its ability to restore various Vrp1p-dependent functions was compared to that of C-Vrp1p$_{364–817}$ (Figs. 4A–B, and 5A). The presence of the CAAX box allowed the mutant C–Vrp1p$^{5A}_{364–817}$–CAAX to restore growth at elevated temperature to *vrp1Δ* cells (Fig. 4A) and endocytosis (Fig. 4B). We tested the possibility that addition of the CAAX motif to the mutant C–Vrp1p$^{5A}_{364–817}$ may confer Las17p binding activity to the mutant by carrying out a pull down assay but did not detect any interaction between N-Las17p$_{1–206}$ and C–Vrp1p$^{5A}_{364–817}$-CAAX (Fig. S4). This suggests that one of the functions of the LBD is to target Vrp1p to cortical patches and that another membrane-targeting sequence can bypass the requirement for the LBD at least in growth at elevated temperature and endocytosis.

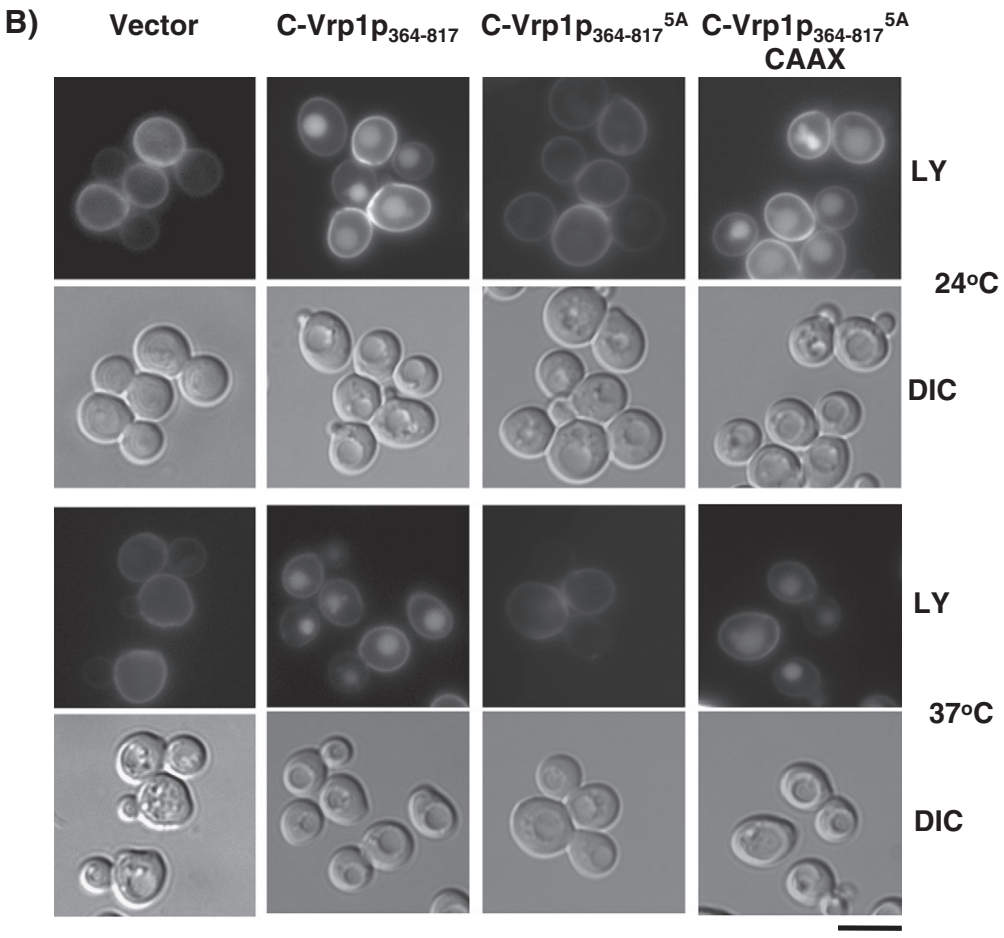
We examined C–Vrp1p$^{5A}_{364–817}$–CAAX ability to restore cortical actin patch polarisation to *vrp1Δ* cells by staining *vrp1Δ* cells expressing C–Vrp1p$^{5A}_{364–817}$–CAAX with Fluorescein -conjugated phalloidin (Fig. 5A and B). C–Vrp1p$^{5A}_{364–817}$–CAAX was not able to restore a polarised cortical actin patch distribution to *vrp1Δ* cells (Fig. 5A, B). In order to visualize the localization of C–Vrp1p$^{5A}_{364–817}$–CAAX, the DNA sequence encoding C–Vrp1p$^{5A}_{364–817}$ was fused in-frame to sequences encoding GFP-CAAX and expressed in *vrp1Δ* cells (Fig. 5C). The CAAX sequence targeted C–Vrp1p$^{5A}_{364–817}$ to membranes including the plasma membrane (Fig. 5C) consistent with our earlier findings with N-Vrp1p$_{1–364}$ [20]. Immunoblot analysis showed that the ability of C–Vrp1p$^{5A}_{364–817}$–CAAX to rescue the growth and endocytic defect of *vrp1Δ* cells is not due to higher expression as C–Vrp1p$^{5A}_{364–817}$–GFP and C-Vrp1p$^{5A}_{364–817}$–GFP–CAAX are expressed at comparable levels (Fig. 4C). Cdc42 localizes to cellular membranes at sites of polarized growth [46] thus we tested the ability of CAAX motif from Cdc42p to restore the function of C–Vrp1p$^{5A}_{364–817}$ mutant by making similar construct C–Vrp1p$^{5A}_{364–817}$–CAAX$_{Cdc42}$. This construct was functional just like the C–Vrp1p$^{5A}_{364–817}$–CAAX (Ras1p), restored growth and endocytosis but not actin patch polarization (data not shown).

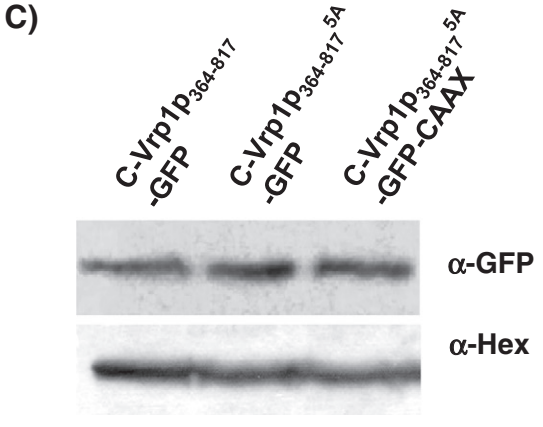
3.4. Las17p binding motif of Vrp1p is essential for actin patch polarization at 37 °C

In order to analyze the role of Las17p binding motif for the function of the full length protein, we mutated the Las17p binding motif in Vrp1p and expressed the mutant, Vrp1p^{5A} in *vrp1Δ* strain to analyze its function. The *vrp1Δ* cells expressing Vrp1p^{5A} were able to grow at 37 °C (Fig. 6A) as well as carry out fluid phase endocytosis of LY at both 24 °C and 37 °C, unlike the *vrp1Δ* cells harbouring empty vector (Fig. 6B). Although Vrp1p^{5A} rescued the actin patch polarization defect of *vrp1Δ* cells at 24 °C, it did not rescue the actin patch polarization defect at 37 °C (Fig. 7A and B). In order to determine the sub-cellular localization of the mutant, we tagged the mutant with GFP and expressed Vrp1p^{5A}–GFP in *vrp1Δ* cells and *las17Δ* cells. Unlike Vrp1p–GFP, the Vrp1p^{5A}–GFP did not localize to cortical patches in *vrp1Δ* cells (Fig. 7C, I)) and the expression of the mutant was not compromised (Fig. 6C). We also checked for Las17p

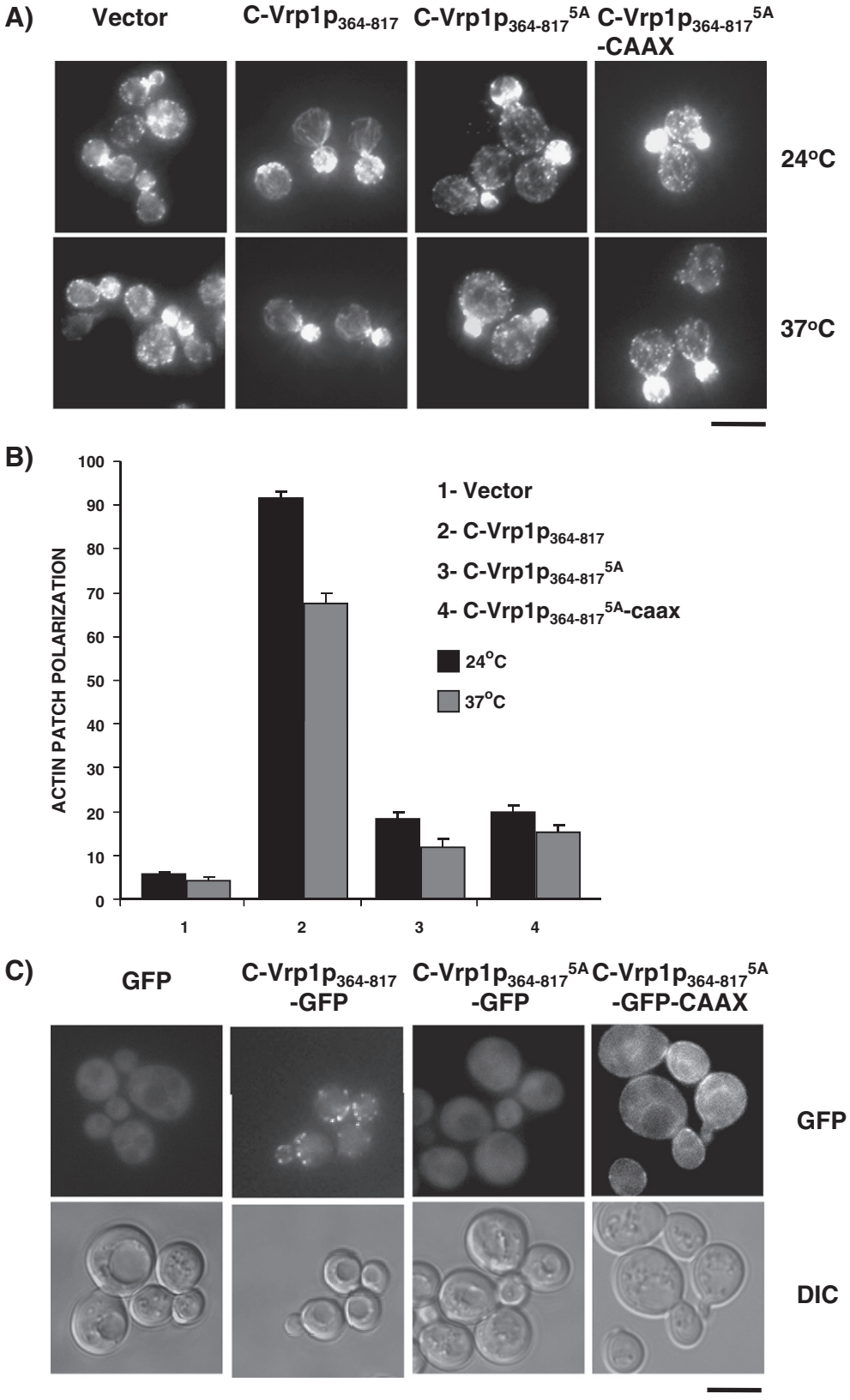
Fig. 4. Residues 788–792 are essential for the function of C-Vrp1p$_{364–817}$ in growth and endocytosis. (A) Growth of *vrp1Δ* cells harboring empty centromeric plasmid or centromeric plasmid expressing C-Vrp1p$_{364–817}$, C-Vrp1p$^{5A}_{364–817}$ or C-Vrp1p$^{5A}_{364–817}$-CAAX at 24 °C and 37 °C. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C and photographed after 3 days. (B) *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$, C-Vrp1p$^{5A}_{364–817}$ or Vrp1p$^{5A}_{364–817}$-CAAX were grown in YPUAD to exponential phase at 24 °C and LY uptake was examined by fluorescence microscopy. *Upper panels*: LY. *Lower panels*: DIC. Bar, 5 μm. (C) *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$-GFP, C-Vrp1p$^{5A}_{364–817}$-GFP or C-Vrp1p$^{5A}_{364–817}$GFP-CAAX were grown in YPUAD to exponential phase at 24 °C. Proteins extracts from the cells were analyzed by immunoblotting with anti-GFP (α-GFP) and anti-hexokinase (α-Hex).


A)
24°C
37°C
vrp1Δ
Vector
C-Vrp1p 364-817
C-Vrp1p 364-817 5A -CAAX
C-Vrp1p 364-817 5A


B)
Vector
C-Vrp1p364-817
C-Vrp1p364-817 5A
C-Vrp1p364-817 5A CAAX
LY
24°C
DIC
LY
37°C
DIC


C)
C-Vrp1p364-817 -GFP
C-Vrp1p364-817 5A -GFP
C-Vrp1p364-817 5A -GFP-CAAX
α-GFP
α-Hex

independent localization of Vrp1p–GFP and Vrp1p^{5A}–GFP in *las17Δ* cells (Fig. 7C, II)) and found that both Vrp1p–GFP and Vrp1p^{5A}–GFP localizes to cortical patches in *las17Δ* cells. We had previously shown that Vrp1p–GFP localizes to cortical patches in *las17Δ* cells [20] and it was also shown that Vrp1p–GFP can localize to cortical patches without interacting with Las17p [19]. However it was reported that Vrp1p–GFP does not localize in *las17Δ* strain [25]. Thus we analysed the localization of Vrp1p and fragments of Vrp1p in *las17Δ* strain by expressing Vrp1p–GFP, N–Vrp1p$_{1-364}$–GFP, C–Vrp1p$_{364-817}$–GFP and C–Vrp1p$^{5A}_{364-817}$–GFP in *las17Δ* strain (Fig. 7D). Both Vrp1p–GFP and N–Vrp1p$_{1-364}$–GFP localized to cortical patches in *las17Δ* strain while C–Vrp1p$_{364-817}$–GFP did not. The localization of Vrp1p–GFP to cortical



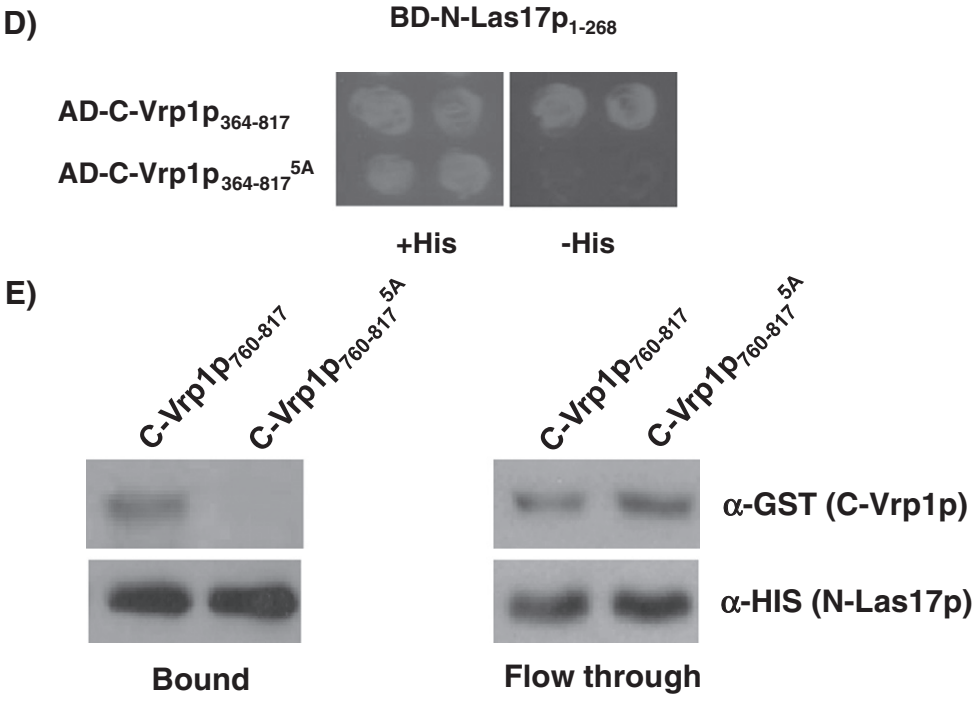


Fig. 5. Residues 788–792 are essential for localization of C-Vrp1p$_{364–817}$ to cortical patches and actin patch polarization. (A) *vrp1Δ* cells harboring centromeric plasmid or plasmids expressing C-Vrp1p$_{364–817}$, C-Vrp1p$^{5A}_{364–817}$ or C-Vrp1p$^{5A}_{364–817}$-CAAX were grown to exponential phase at 24 °C in YPUAD and either left at 24 °C or shifted to 37 °C for 2 h. The actin cytoskeleton was visualized as described in materials and methods. Bar, 5 μm. (B) Quantification of actin patch polarization of *vrp1Δ* cells expressing C-Vrp1p$_{364–817}$, C-Vrp1p$^{5A}_{364–817}$ or C-Vrp1p$^{5A}_{364–817}$-CAAX from a centromeric plasmid. Actin patches were analyzed as described in Fig. 3B. (C) *vrp1Δ* cells expressing GFP, C-Vrp1p$_{364–817}$-GFP, C-Vrp1p$^{5A}_{364–817}$-GFP or C-Vrp1p$^{5A}_{364–817}$-GFP-CAAX were grown in YPUAD to exponential phase at 24 °C. GFP fluorescence was visualized in living cells by fluorescence microscopy. Bar, 5 μm. (D) Yeast two hybrid interactions between Gal4-BD-N-Las17p$_{1–268}$ (Gal4-Binding Domain) with Gal4-AD-C-Vrp1p$_{364–817}$ or Gal4-AD-C-Vrp1p$^{5A}_{364–817}$ (Gal4-Activation Domain) was assessed by growth on medium lacking histidine and containing 2 mM 3-amino 1,2,4-triazole. Plates were photographed after 3 days. (E) *E. coli* lysate from cells expressing N-Las17p$_{1–206}$-His was mixed with either lysate from cells expressing GST-C-Vrp1p$_{716–817}$ or GST-C-Vrp1p$^{5A}_{716–817}$. The mixed lysate was incubated with Ni-NTA beads to pull down the His-tagged proteins and proteins associated with N-Las17p$_{1–206}$. The bound proteins and flow through fractions were analyzed by immunoblotting with anti-GST (α-GST) and anti-His (α-His) antibodies.

patches in *las17Δ* strain was not affected by the presence of N-Las17p$_{1–368}$, however the localization of N–Vrp1p$_{1–364}$–GFP to cortical patches in *las17Δ* strain was reduced by the presence of N–Las17p$_{1–368}$ (Fig. 7D). This is probably due to the ability of Vrp1p but not N–Vrp1p$_{1–364}$ to interact with N–Las17p$_{1–368}$. C–Vrp1p$_{364–817}$–GFP does not localize to cortical patches in *las17Δ* strain but localized to cortical patches in *las17Δ* strain in the presence of N–Las17p$_{1–368}$ (Fig. 7D) and this localization were abolished by the 5A mutation as C–Vrp1p$^{5A}_{364–817}$–GFP showed a diffused cytoplasmic staining even in the presence of N–Las17$_{1–368}$. Thus Vrp1p–GFP can localise to cortical patches either through its own localization motif found in the N–Vrp1p$_{1–364}$ or by binding to Las17p mediated by LBD found in C–Vrp1p$_{364–817}$.

3.5. N–Vrp1p$_{1–364}$ fused with the LBD restored cortical actin patch polarization

We had previously shown that N–Vrp1p$_{1–364}$ can rescue the growth defect of *vrp1Δ* strain while C–Vrp1p$_{364–817}$ can rescue both the growth and actin patch polarization defect of *vrp1Δ* strain [20]. This is probably due to the inefficient localization of N–Vrp1p$_{1–364}$ to cortical patches compared to C–Vrp1p$_{364–817}$ which localized efficiently to cortical patches through interaction with Las17p, mediated through the Las17p binding domain (LBD) located at the C-terminus of Vrp1p [12]. In order to test this hypothesis we fused the LBD (Vrp1p$_{760–817}$) to N–Vrp1p$_{1–364}$. The fusion protein N–Vrp1p$_{1–364}$–LBD rescued the growth defect at 37 °C and endocytosis defect of *vrp1Δ* strain similar to N–Vrp1p$_{1–364}$ (Fig. 8A, B). In addition, N–Vrp1p$_{1–364}$–LBD restored actin patch polarization in *vrp1Δ* strain unlike N–Vrp1p$_{1–364}$ (Fig. 9A and B). This is not due to increased expression of N–Vrp1p$_{1–364}$–LBD compared to N–Vrp1p$_{1–364}$ (Fig. 8C) but is due to efficient localization of N–Vrp1p$_{1–364}$–LBD–GFP to cortical patches compared to N–Vrp1p$_{1–364}$–GFP (Fig. 9C). It is possible that Vrp1p stabilizes Las17p in a similar fashion to WIP stabilizing WASP [31]. Thus we analyzed the expression of Las17p–GFP in the presence and absence of C–Vrp1p$_{364–817}$ expressed from a centromeric plasmid and we did not find any enhanced stability of Las17p-GFP at 37 °C in the presence of C–Vrp1p$_{364–817}$ (Fig. 9D).

4. Discussion

The organization of actin cytoskeleton in *S. cerevisiae* is dependent on many cellular proteins such as Vrp1p (yeast WIP) and Las17p (yeast WASP) [10,17]. Las17p and Vrp1p form a complex similar to the mammalian WASP-WIP complex [12,30,47] and deletion of either *LAS17* or *VRP1* results in *S. cerevisiae* strains which are unable to grow at 37 °C and with defects in endocytosis as well as actin patch polarization defect at both 24 °C and 37 °C [10,11,13,15,17]. Thus it is not clear which of the cellular activity requires the formation of Las17p–Vrp1p complex. We have previously shown that a peptide comprising the N-terminal of Vrp1p, N–Vrp1p$_{1–364}$ which does not interact with Las17p can rescue growth at 37 °C without rescuing the actin patch polarization defect of *vrp1Δ* strain [20] suggesting that Las17p/Vrp1p complex is not essential for growth at 37 °C. This conclusion was further supported by the finding that mammalian verprolin proteins WIP [14], CR16 and WIRE [48] can suppress the growth and endocytic defect of *vrp1Δ* strain without interacting with Las17p. A peptide comprising of the C-terminal of Vrp1p, C–Vrp1p$_{364–817}$ can rescue both the growth defect as well as the endocytosis defect of

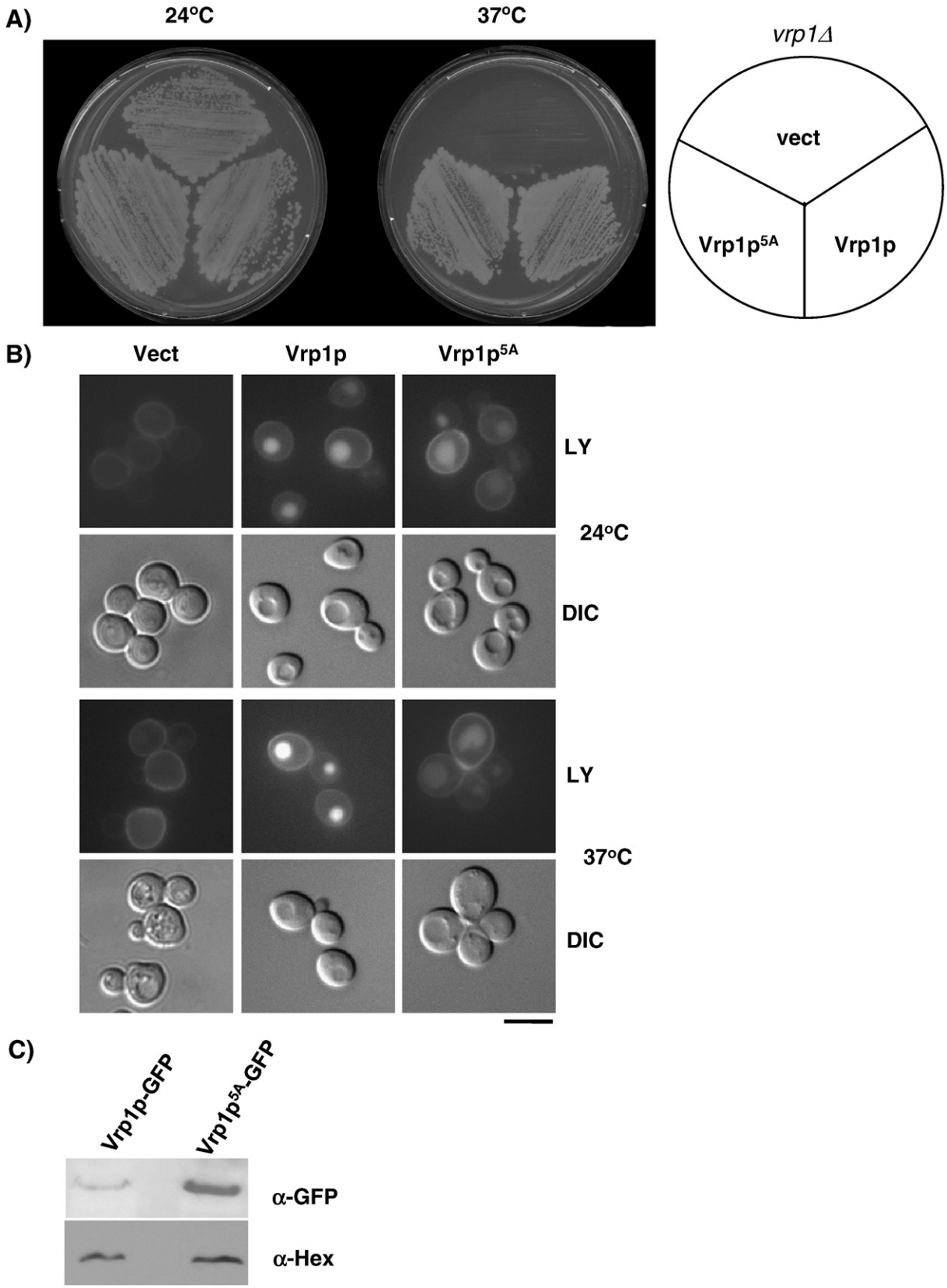


Fig. 6. Vrp1p–Las17p interaction is not critical for growth at 37 °C or endocytosis. (A) Growth of *vrp1Δ* cells harboring empty vector or plasmid expressing Vrp1p or Vrp1p^{5A} at 24 °C and 37 °C. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C and photographed after 3 days. (B) *vrp1Δ* cells harboring empty vector or plasmids expressing Vrp1p or Vrp1p^{5A} were grown in YPUAD to exponential phase at 24 °C and LY uptake was carried as described in materials and methods. *Upper panels*: LY. *Lower panels*: DIC. Bar, 5 μm. (C) *vrp1Δ* cells harboring plasmids expressing Vrp1p–GFP or Vrp1p^{5A}–GFP were grown in YPUAD to exponential phase at 24 °C. Protein extracts from the cells were analyzed by immunoblotting with anti-GFP (α-GFP) and anti-hexokinase (α-Hex) antibodies.

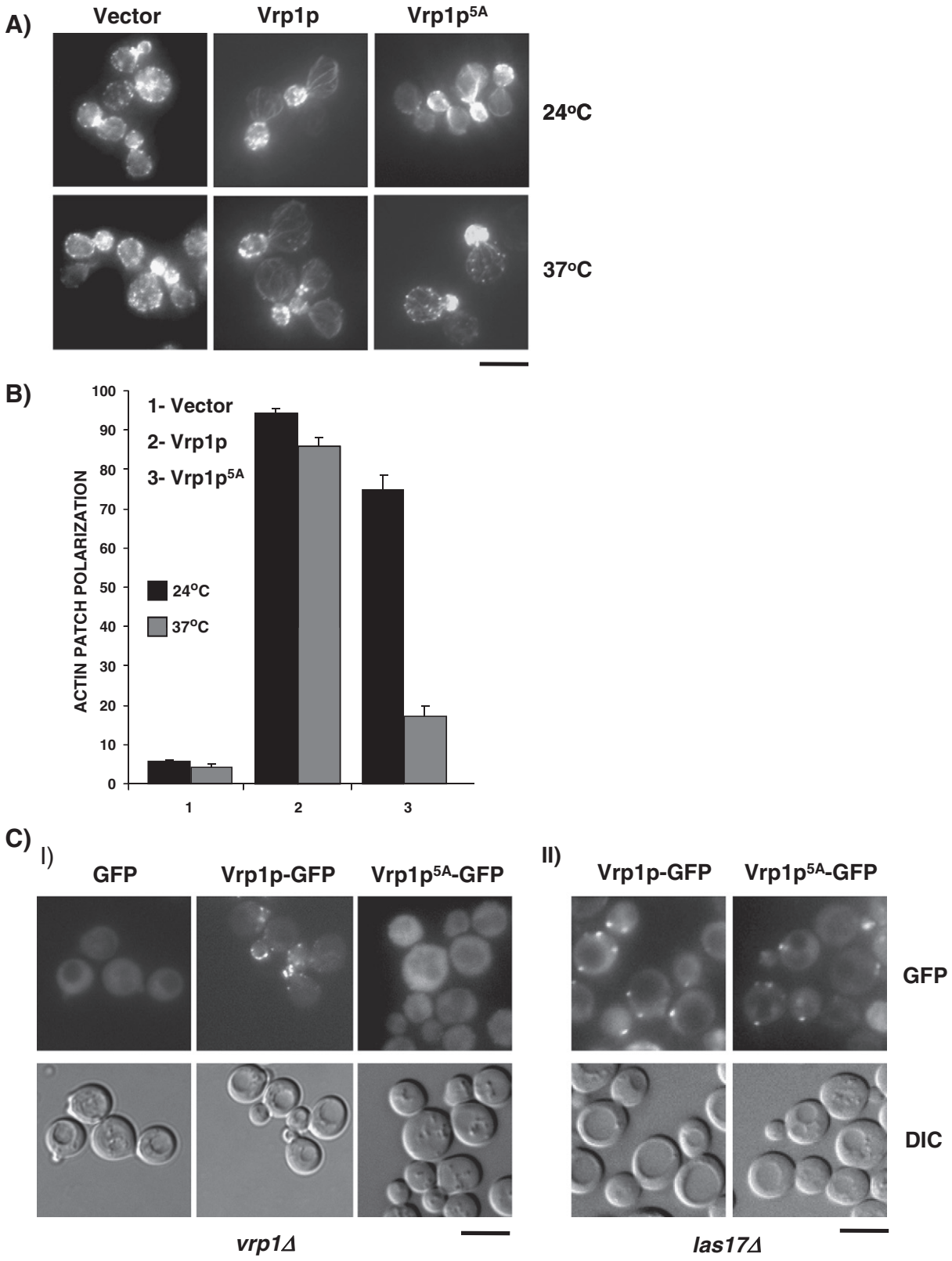


Fig. 7. Vrp1p–Las17p interaction is critical for actin patch polarization at 37 °C and localization to cortical patches. (A) *vrp1Δ* cells harboring empty vector or plasmids expressing Vrp1p or Vrp1p^{5A} were grown to exponential phase at 24 °C in YPUAD and either left at 24 °C or shifted to 37 °C for 2 h. The actin cytoskeleton was visualized as described in materials and methods. Bar, 5 μm. (B) The actin patch polarization of *vrp1Δ* cells expressing Vrp1p or Vrp1p^{5A} was quantified. Actin patches were analyzed as described in Fig. 3B. (C) *vrp1Δ* cells (I) or *las17Δ* cells (II) expressing Vrp1p–GFP or Vrp1p^{5A}–GFP were grown in YPUAD to exponential phase at 24 °C and GFP fluorescence was visualized in living cells by fluorescence microscopy. Bar, 5 μm. (D) *S. cerevisiae las17Δ* cells expressing Vrp1p–GFP, N–Vrp1p$_{1-364}$-GFP, C–Vrp1p$_{364-817}$–GFP or C–Vrp1p$^{5A}_{364-817}$-GFP in the presence or absence of N-Las17p$_{1-368}$ were grown in YPUAD to exponential phase at 24 °C. GFP fluorescence was visualized by fluorescence microscopy. *Upper panels*: GFP. *Lower panels*: DIC. Bar, 5 μm.

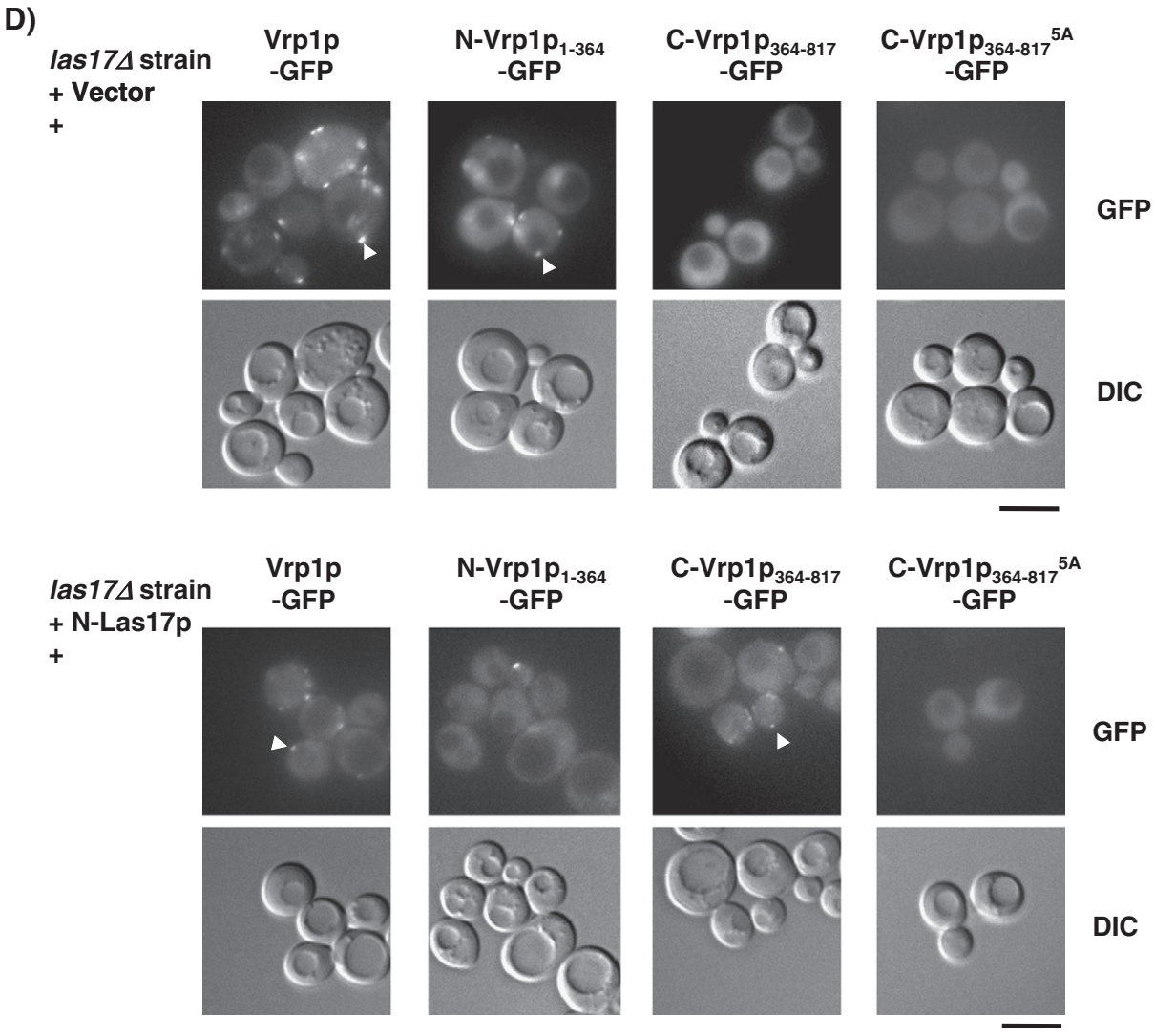


Fig. 7 (*continued*).

vrp1Δ strain. Unlike N–Vrp1p$_{1–364}$, C–Vrp1p$_{364–817}$ can form a complex with Las17p [20]. We have recently identified a mutant of Las17p which complements all the defects of *las17Δ* strain except actin patch polarization at 37 °C. The mutation L80T and H94L of Las17p abolished interaction with Vrp1p, however we cannot rule out the possibility that the mutation has abolished interaction with other uncharacterized protein.

We have identified the motif ($_{788}$MPKPR$_{792}$) essential for Vrp1p–Las17p interaction. These residues correspond to epitope 2 in human WIP [34] and mutation of the corresponding residues in WIRE abolished interaction with WASP [42]. Mutating these amino acids in C–Vrp1p$_{364–817}$ results in a peptide C–Vrp1p$^{5A}_{364–817}$ unable to complement any of the defects of *vrp1Δ* strain and this is due to lack of localization of the mutant protein and not due to poor expression (Figs. 4C and 5C). Adding a CAAX motif to C–Vrp1p$^{5A}_{364–817}$ restored the ability of C–Vrp1p$^{5A}_{364–817}$-CAAX to rescue both the endocytic and growth defects of *vrp1Δ* strain (Fig. 4A, B) suggesting that membrane localization is sufficient to rescue these defects. However, C–Vrp1p$^{5A}_{364–817}$–CAAX did not restore the actin patch polarization defect of the strain (Fig. 5A), this is probably not due to global change in the conformation of the protein. Mutation of the five amino acids in the full-length Vrp1p results in a protein, Vrp1p^{5A} which rescued the growth defect, endocytosis defect (24 °C and 37 °C) and actin patch polarization defect at 24 °C but not at 37 °C (Figs. 6A, B, and 7A, B). This is because C–Vrp1p$_{364–817}$ requires interaction with Las17p for localization to cortical patches while Vrp1p can localize to cortical patches even in the absence of Las17p [20]. In the absence of Las17p, Vrp1p, Vrp1p^{5A} and N–Vrp1p$_{1–364}$ localize efficiently to cortical patches while C–Vrp1p$_{364–817}$ and C-Vrp1p$^{5A}_{364–817}$ does not (Fig. 7C, D). In the presence of Las17p, Vrp1p and C–Vrp1p$_{364–817}$ localizes efficiently to cortical patches by binding to Las17p while Vrp1p^{5A} and N–Vrp1p$_{1–364}$ localizes poorly to cortical patches but C–Vrp1p$^{5A}_{364–817}$ does not localize to cortical patches. The localization of Vrp1p^{5A} to cortical patches is similar to N–Vrp1p$_{1–364}$ [20] and is below the detection limit. Though Vrp1p^{5A} does not localize as effectively as Vrp1p the localization to cortical patches is sufficient to rescue the growth defect at 37 °C, endocytic defect (24 °C and 37 °C) and actin patch polarization defect at 24 °C of *vrp1Δ* cells but not at 37 °C.

In *S. cerevisiae*, Vrp1p and Las17p form a complex which is localized to cortical actin patches. Las17p is recruited first to the cortical patches which in turn recruit Vrp1p and Type I myosins [25]. Though Vrp1p–Las17p interaction is not critical for growth at 37 °C and fluid phase endocytosis [33], the Vrp1p–Las17p interaction is critical for actin patch polarization at 37 °C. The cells have evolved a mechanism to ensure that both the proteins are localized efficiently to the cortical patches together. Vrp1p has a Las17p independent localization motif

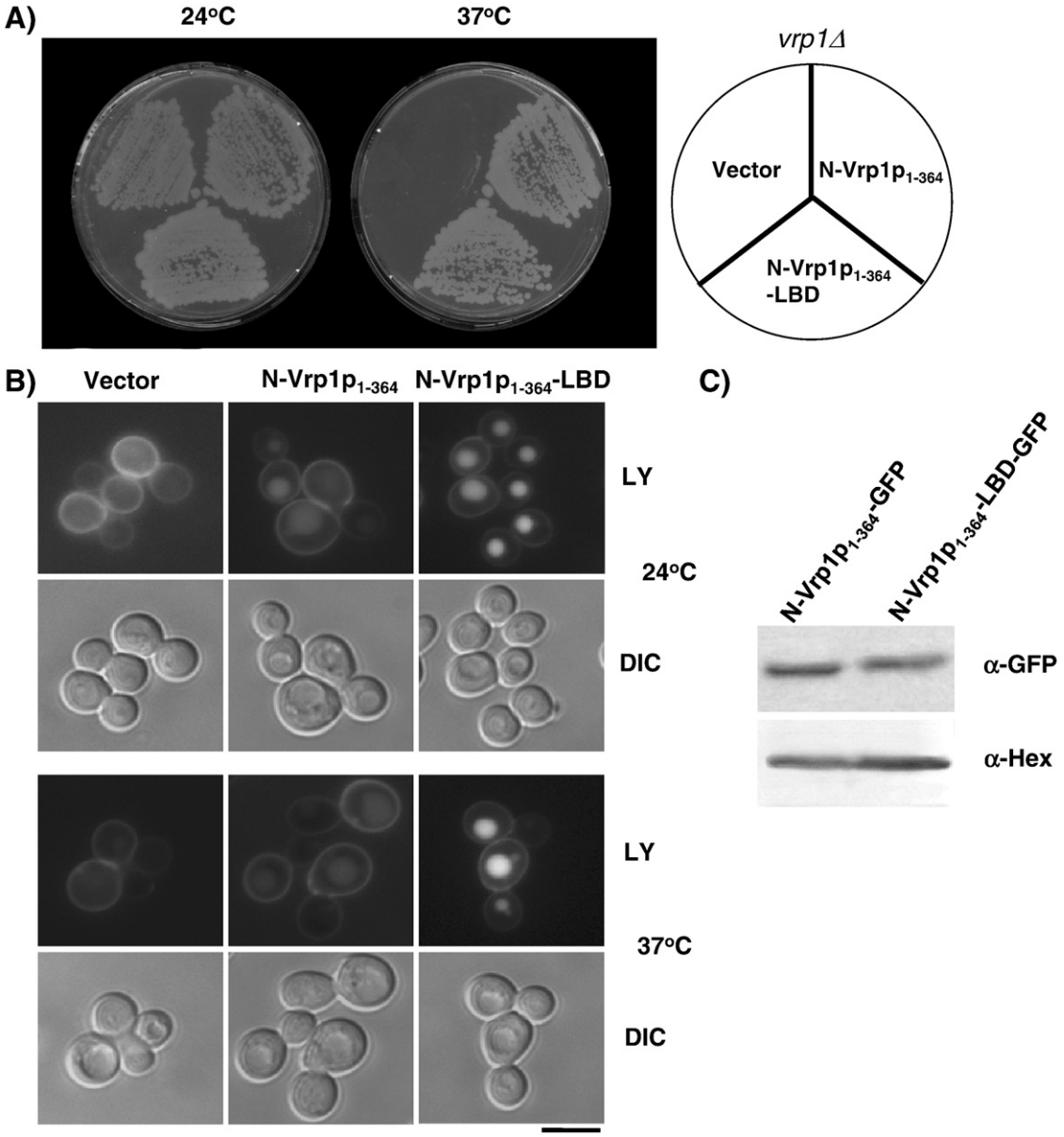


Fig. 8. N–Vrp1p$_{1-364}$ and N–Vrp1p$_{1-364}$–LBD complement the growth and endocytosis defect of *vrp1Δ* strain. (A) Growth of *vrp1Δ* cells harboring empty vector or plasmid expressing N–Vrp1p$_{1-364}$ or N–Vrp1p$_{1-364}$–LBD at 24 °C and 37 °C. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C and photographed after 3 days. (B) *vrp1Δ* cells harboring empty vector or plasmids expressing N–Vrp1p$_{1-364}$ or N–Vrp1p$_{1-364}$–LBD were grown in YPUAD to exponential phase at 24 °C and LY uptake was carried as described in materials and methods. *Upper panels*: LY. *Lower panels*: DIC. Bar, 5 μm. (C) *vrp1Δ* cells harboring plasmids expressing N–Vrp1p$_{1-364}$–GFP or N–Vrp1p$_{1-364}$–LBD–GFP were grown in YPUAD to exponential phase at 24 °C. Proteins extracts from the cells were analyzed by immunoblotting with α-GFP and α-Hex antibodies.

in the N-terminal which mediates efficient localization of Vrp1p to cortical patches in the absence of Las17p and thereby accounting for the viability of Las17p deficient (*las17Δ*) cells. In the presence of Las17p, the localization of Vrp1p is predominantly through binding with Las17p [25]. In contrast to Vrp1p, Las17p can localize efficiently to cortical patches both in the presence and absence of Vrp1p. Both Las17p and Vrp1p interact with type I myosins though Vrp1p is predicted to have more type I myosin binding sites [21]. Las17p has been shown to have an actin binding site at the C-terminus in the VCA region while we and others have shown that Vrp1p has three actin binding motifs [13,26]. The C-terminal of type I myosin contains an SH3 domain which mediates interaction with proline rich proteins such as Vrp1p and Las17p while the adjacent acidic domain can activate the Arp2/3 complex just like Las17p [21]. Thus in wild types cells, Las17p localizes to cortical patches and mediates efficient recruitment of Vrp1p to cortical patches and this ensures the ordered assembly of Vrp1p–Las17p complex and subsequent recruitment of type I myosins which promotes actin polymerization by activating Arp2/3 complex and actin patch polarization.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2010.08.013.

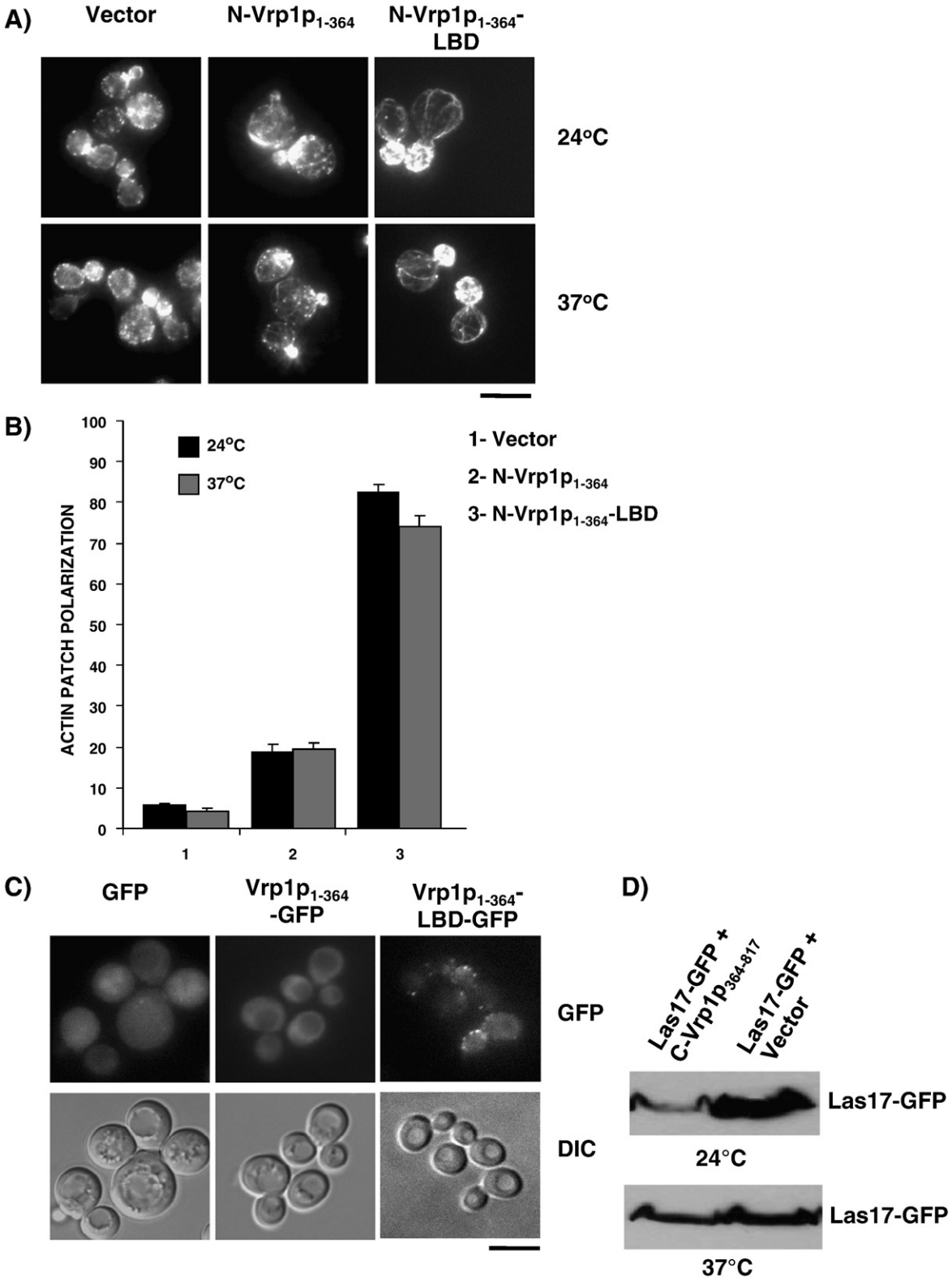

Fig. 9. N–Vrp1p$_{1-364}$–LBD but not N–Vrp1p$_{1-364}$ complemented the actin patch polarization defect of *vrp1Δ* strain. (A) *vrp1Δ* cells harboring empty centromeric plasmid or plasmids expressing N–Vrp1p$_{1-364}$ or N–Vrp1p$_{1-364}$–LBD were grown to exponential phase at 24 °C in YPUAD and either left at 24 °C or shifted to 37 °C for 2 h. The actin cytoskeleton was visualized as described in materials and methods. Bar, 5 μm. (B) The actin patch polarization of *vrp1Δ* cells expressing N–Vrp1p$_{1-364}$ or N–Vrp1p$_{1-364}$–LBD was quantified. Actin patches were analyzed as described in Fig. 3B. (C) *vrp1Δ* cells expressing GFP, N–Vrp1p$_{1-364}$–GFP or N–Vrp1p$_{1-364}$–LBD–GFP were grown in YPUAD to exponential phase at 24 °C. GFP was visualized in living cells by fluorescence microscopy. Bar, 5 μm. (D) *vrp1Δ* cells harboring centromeric plasmids expressing Las17p-GFP with either empty centromeric plasmid or centromeric plasmid expressing C–Vrp1p$_{364-817}$ were grown to exponential phase at 24 °C in YPUAD. The culture was split into two and one maintained at 24 °C while the other was shifted to 37 °C. Proteins extracts were analyzed by immunoblotting with α-GFP and α-Hex antibodies.

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