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## IDENTIFICATION OF A CONSERVED F-BOX PROTEIN 6 INTERACTOR ESSENTIAL FOR ENDOCYTOSIS AND CYTOKINESIS IN FISSION YEAST

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**Running head: A novel conserved F-box protein interactor essential for cytokinesis**

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### Abbreviations:

HEAT: Huntington, Elongation factor 3, the regulatory A subunit or protein phosphatase 2fA and Tor1

MudPIT, Multidimensional Protein Identification Technology

SCF, Skp1-Cdc53/Cullin 1-F-box protein

## SYNOPSIS

The F-box domain is a degenerated motif consisting of ~40 amino acid residues that specifically bind Skp1, a core component of the SCF (Skp1-Cdc53/Cullin 1-F-box protein) ubiquitin ligase. Recent work, mainly performed in budding yeast, indicates that certain F-box proteins form non-SCF complexes together with Skp1 in the absence of cullins and play various roles in cell cycle and signalling pathways. However it is not established whether these non-SCF complexes are unique to budding yeast or common in other eukaryotes. Here using TAP purification coupled to MudPIT analysis (Multidimensional Protein Identification Technology), we have identified a novel conserved protein, Sip1 in fission yeast, as an interacting partner of an essential F-box protein Pof6. Sip1 is a large HEAT-repeats containing protein (217 kDa) and forms a complex with Pof6 and Skp1. This complex does not contain cullins, indicating that it is a novel non-SCF complex. Like Pof6 and Skp1, Sip1 is essential for cell viability and temperature sensitive *sip1* mutants display cell division arrest as binucleate cells with septa. Sip1 localises to the nucleus and dynamic cytoplasmic dots, which turn out to be endocytic vesicles. Consistent with this, *sip1* mutants are defective in endocytosis. Furthermore towards the end of cytokinesis, constriction of the actomyosin ring and dissociation of type II myosin and septum materials are substantially delayed in the absence of functional Sip1. These results indicate that the conserved Sip1 protein comprises a novel non-SCF F-box complex that plays an essential role in endocytosis, cytokinesis and cell division.

**Keywords:** cytokinesis, endocytosis, F-box, fission yeast, HEAT repeats, MudPIT

## INTRODUCTION

Cytokinesis is a critical step in the cell life, as it couples the end of mitosis to the separation of the mother cell into two daughter cells. The main goal of cytokinesis is common to all organisms, and even if divergences exist, the fundamental sequences of events are universal [1]. By the end of anaphase, the fission yeasts, like animals, positions an actomyosin ring (CAR, Contractile Actomyosin Ring) in the medial cortex region and constricts it to bring about partitioning of the cytoplasm [2-5]. Concomitantly, the septum is deposited on the other side of the ring to separate the incipient daughter cells. Later a secondary septum is formed and the primary membrane is digested to allow the physical separation into two daughter cells. For these equatorial components including CAR, lipid rafts and septum to undergo assembly and dissolution in concert upon cytokinesis, membrane trafficking systems such as endocytosis, exocytosis and protein transport play vital roles. Accordingly not only actomyosin and glucans are essential in cytokinesis, but also a much larger set of conserved proteins are required for this process [2-5]. For example, the anillin-like protein Mid1, in *S. pombe* and other organisms, defines the position of the CAR, and the protein phosphatase Clp1/Flp1, related to budding yeast Cdc14, coordinates cytokinesis with cell cycle progression. Being simple entities, fission yeast provides the significant advantage of combining genetic and biochemistry techniques; such features make them a powerful model by which to study cytokinesis. Consistently, a precise timing of the process has been reported [6] and a plethora of mutants with defects in cytokinesis and septation have been isolated [3].

The F-box proteins were discovered for their essential role in cell cycle progression [7]. The F-box proteins are defined by the presence of a degenerated stretch of 40 amino acids, the F-box domain representing Skp1-binding motif, normally in their amino-terminal portion, and contain a substrates-interacting domain in their carboxy-terminal region. They play the crucial role in recognising in a timely and specific manner a variety of substrates as an adaptor subunit of SCF (Skp1-Cdc53/Cullin 1-F-box protein) ubiquitin ligases [8]. Beside this well-recognised function, an increasing number of F-box proteins has been described in partnership with Skp1 as non-SCF complexes [9]. For example, the budding yeast Rav2 F-box protein forms a complex with Skp1 and Rav1 and plays a role in vacuolar ATPase. It was recently shown that this complex appears to be conserved at least in fission yeast [10].

The *S. pombe* genome contains 18 genes encoding F-box proteins [9, 11]. Among the fission yeast F-box proteins, only two are essential, Pof1 and Pof6 [12, 13]. While Pof1 follows the traditional pattern as a component of SCF<sup>Pof1</sup>, a counterpart of the budding yeast SCF<sup>Met30</sup> ubiquitin ligase [13], Pof6 seems to be less canonical. Pof6 was first identified as a Skp1 interactor and no binding to cullins was reported, bringing up the idea of a non-SCF complex [12]. In this study we isolated by TAP purification and MudPIT analysis a novel interactor of Pof6, named Sip1 (see below). We report characterisation of this novel type of the non-SCF complex that plays an essential role in endocytosis and cytokinesis.

## EXPERIMENTAL

### Yeast strains and construction of *sip1* temperature sensitive alleles

Yeast strains used in this study are described in Table I. Cells were grown in standard culture media, and standard yeast genetic methods were used. DNA fragments containing error-prone PCR amplified *sip1*<sup>+</sup>-HA sequences with the G418 resistance

*kan<sup>r</sup>* gene were transformed into a wild type strain and 6 temperature sensitive (*ts*) alleles were isolated (see Supplementary methods for details).

### TAP purifications

The TAP purifications were mainly prepared as described previously [14]. For Pof6-TAP purification, 40 L of cells were grown in YE5S at 30°C and disrupted in liquid nitrogen. Proteins were solubilised in the lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 0.2 % NP-40, 1 mM NaF, 10 mM PMSF, adjusted to pH 8.0) and loaded for a two-step TAP purification. The eluted proteins were precipitated with TCA and subjected to MudPIT analysis (Multidimensional Protein Identification Technology, see Supplementary methods for experimental details). For Sip1-TAP purification, a total of 10 L of cells were cultured in YE5S at 30°C and disrupted in liquid nitrogen. The proteins were eluted in a single-step TAP purification and precipitated in TCA for further MudPIT analysis.

### Coimmunoprecipitation experiments

Protein extracts were prepared in lysis buffer by breaking cells at 4°C with glass beads (4 times for 40 s) in a FastPrep FP120 apparatus (Savant, Co, MN, USA). The protein extracts were collected after 15 min of centrifugation at 10,000 x g at 4°C. The coimmunoprecipitations were performed as described previously [15].

### Synchronisation by centrifugal elutriation and FACS analysis

3 L of YE5S media were inoculated with a preculture overnight. Elutriation was performed with Avanti J-20 XP (Beckman Coulter, MN, USA) by the standard procedures. Concentrations and sizes of cells were measured with a Cell Counter (Sysmex KX-21N, Kobe, Japan). Septa were stained with calcofluor and the percentage of septated cells were calculated by counting over a total of 100 cells. The cell survivals were estimated by spreading a calculated number of cells on YE5S plates and incubating them at 30°C for 3-4 days. Standard methods for FACS analysis were followed using a FACScan machine (Becton Dickinson, CA, USA).

### Fluorescence Microscopy

For all microscopy experiments, cells were grown and observed in minimum medium at 27°C, unless otherwise stated. Signals of GFP-Sip1 (expressed under the control of the thiamine-repressible *nmt1* promoter) are essentially the same between in the presence (repressed) and absence (derepressed) of thiamine, though those in the absence of thiamine are stronger. For time-lapse analysis, therefore, derepressed conditions (-thiamine) were used as stronger signals were required for recording. Live cell imaging was performed in an imaging chamber (CoverWell, 20 mm diameter, 0.5 mm deep) (Molecular Probes) filled with 800 µl of 2% agarose in minimum medium and sealed with a 22x22 mm glass coverslip. FM4-64 (Molecular Probes Inc.) was dissolved in DMSO at a concentration of 1.64 mM and added to the cells at a final concentration of 1.89 µM. Cells were then incubated in the dark before being applied to the imaging chamber. Cells were imaged using an Olympus IX71 microscope with a 63X oil immersion lens, NA=1.4. Images were captured with a Coolsnap HQ digital CCD camera (Roper Scientific). Counts and measurements were made using Metamorph (Molecular Devices Corporation) and downloaded to Microsoft Excel for analysis.

## RESULTS

### Identification of a novel interactor of the Pof6 F-box protein

To identify Pof6 interactors, we have performed a non-stringent Pof6-TAP purification in two steps from a large-scale culture (40 L) and coupled it to MudPIT analysis. A protein migrating at the size of Pof6 is specifically detected in the silver stain of Pof6-TAP elution (Figure 1A). Indeed the MudPIT data reveals a high coverage of Pof6 (19 unique peptides covered 56.2% the sequence) considering the size of the protein, 99.9 kDa (Supple. Figure S1A). As predicted, Skp1 appears in the elution with 58.4% coverage and a total of 30 peptides. The specificity of the interaction between Pof6 and Skp1 was confirmed by coimmunoprecipitations with both proteins (Figure 1B) as reported previously [12].

An uncharacterised ORF (SPBC27B12.08) was identified as one of the most abundant proteins pulled down in Pof6-TAP purification. Although the corresponding protein has the large size of 217 kDa, the peptide coverage reaches up to 60.6% of the sequence and a total of 792 peptides were isolated (Supple. Figure S1A). The specificity of the interaction between Pof6 and this novel protein was confirmed by coimmunoprecipitation (Figure 1C). We, consequently, named the protein according to that specific feature, Sip1 (pofSix interactor protein 1).

In a reciprocal experiment, we performed a single step Sip1-TAP purification from a 10 L culture to identify its binding partners (Figure 1D). One of the most abundant proteins identified by MudPIT analysis is Skp1 (Supple. Figure S1B). The peptides cover 66.5% of Skp1 sequence and a total of 35 peptides are present. The F-box protein Pof6 is also detected as an interactor of Sip1 albeit with a much restricted coverage (6.1%). The difference between Pof6 coverage in Sip1-TAP elution and Sip1 coverage in Pof6-TAP elution may be explained by the amount of cells used for the purifications, the differences in the TAP purification protocols and the abundance/instability of each protein. To confirm the interactions observed by Sip1-TAP MudPIT analysis, Sip1-HA was immunoprecipitated. As shown in Figure 1E, Pof6 and Skp1 were specifically pulled down with Sip1-HA.

We then attempted to examine whether Pof6 and Sip1 could interact with each other in the absence of Skp1. For this purpose, an Skp1 shut-off strain was used. In this strain, the thiamine-repressible *nmt1* promoter was inserted in front of the initiation codon of the genomic *skp1*<sup>+</sup> gene together with HA epitope, by which the expression of HA-Skp1 could be shut off by adding thiamine to the medium [16]. Rather unexpectedly we found that when Skp1 levels were downregulated, both Sip1 and Pof6 became unstable, and accordingly we could barely detect either protein by immunoblotting (Figure 1F). Thus despite being not conclusive, this result indicates that Pof6 and Sip1 requires Skp1 for their stability, supporting the notion that these three proteins form a ternary complex in the cell.

It is of note that none of the two MudPIT datasets contains peptides for cullins (Supple. Figure S1) confirming the absence of interaction between Pof6 and the cullins as previously reported [12]. We made a careful visual inspection of the sequence corresponding to the Pof6 F-box domain and aligned it with different F-box domains present in SCF complexes [17, 18] (Supple. Figure 2A). The alignment shows that several residues important for the Skp1/F-box domain interface are present in Pof6 but the two residues involved in the Cull1/F-box domain core interface are not conserved; a highly conserved proline residue is replaced by threonine in Pof6 and an acidic residue is exchanged with asparagine. These residues have been shown to be specifically responsible for the interaction between Skp2 F-box protein and Cull1 [18],

substantiating our and previous results [12] showing that Pof6 does not interact with cullins.

The analysis of the Pof6 protein also reveals the presence of unusual domains in its carboxy-terminal region: two Sec10 domains and a CAAX sequence are present in the substrate-binding portion (Supple. Figure 2B). Such features suggest that there may be potential functional similarities between Pof6 and Rcy1 from budding yeast. Like Pof6, Rcy1 interacts with Skp1 and contains two Sec10 domains and a CAAX sequence in its carboxy-terminal region. In addition, it has been suggested that Rcy1 is part of a non-SCF complex [19]. These results collectively suggest that Pof6 does not belong to a conventional SCF complex but instead forms a novel complex with Sip1 and Skp1.

### **The *sip1*<sup>+</sup> gene is essential in *S. pombe* and conserved amongst eukaryotes**

To investigate Sip1 function, we asked whether the *sip1*<sup>+</sup> gene is essential in *S. pombe*. We deleted by PCR one copy of the genes with a G418-resistant cassette in a diploid strain. This *sip1::kan<sup>r</sup>/sip1<sup>+</sup>* strain was left to sporulate and the resulting tetrads were dissected to examine the viability of each haploid progeny. Only two out of four haploids grew into colonies after germination, and these viable colonies were sensitive to G418 (Figure 2A, left panel). We concluded that *sip1*<sup>+</sup>, like *pof6*<sup>+</sup> and *skp1*<sup>+</sup>, is essential for cell viability. A close observation of the non-viable spores showed that germinations occur but not cell division (Figure 2A, right panel).

Homology search reveals that Sip1 contains several HEAT repeats (closed boxes in Figure 2B), which are implicated in protein-protein interactions and often found in subunits of multi-protein complexes [20]. These HEAT repeats are distributed widely along the whole Sip1 protein (Figure 2B). Interestingly, Sip1 protein appears to be conserved throughout the evolution from yeasts to human beings (Figure 2C). Homologies are observed in not only HEAT repeats but also other regions as well.

### **Sip1 plays an essential role in cytokinesis**

To examine the function of Sip1, we produced ts alleles of *sip1*<sup>+</sup> by mutagenic PCR (*sip1-62*, see Supplementary methods for experimental details). Centrifugal elutriation was performed to synchronise cell cultures in early G2. Wild type and the *sip1-62* ts cells were grown at 25°C, elutriated and then shifted to 36°C. *sip1-62* ts cells ceased division almost immediately and their viability decreased concomitantly with the increase of septated cells upon mitotic exit (40-60 min, Figure 3A). In addition unlike wild type cells, the percentage of septated cells did not decline afterwards once it reached a plateau. Consistent with this, the population of the binucleate cells closely followed the profile of the septated cells. These observations suggest that *sip1* ts cells proceed through the G2 phase and enter mitosis to lose viability during cytokinesis. The DNA content of the synchronised cells showed that *sip1-62* ts cells arrest with a 4C DNA content, indicating a completion of DNA replication in each separated nucleus (Figure 3B).

We further investigated the role of Sip1 in cell division by following the fate of the CAR and septum in *sip1-62*. The CAR was visualised using the type II myosin heavy chain protein, Myo2, tagged with GFP [6, 21]. Cells were grown at the permissive temperature, shifted up to the restrictive temperature for 1 hr, stained with calcofluor and observed at 36°C over-time (Figure 4 and Supp Movies S1 and S2). In the *sip1* mutant, the initiation timing of CAR and septum formation appeared very similar to that of wild type cells (Figure 4A and B). However, the CAR took nearly

twice as much time to constrict. Although in the *sip1* mutant, much variability was observed from one cell to the other in the timing of Myo2-GFP detachment, it was delayed ascribable to a prolonged CAR constriction process at the medial region (Figure 4B). In addition, septum materials remained at the equator. We could not even observe the execution of this process within the timeframe of experiments. Therefore Sip1, a newly identified conserved protein, plays an essential role in cytokinesis, in which it is required for CAR constriction and septum dissolution.

### **Sip1 localises to the nucleus and endocytic vesicles**

To investigate the localisation of Sip1, we constructed a strain in which Sip1 was chromosomally tagged with GFP at its N-terminus and expressed under the *nmt1* promoter. GFP-Sip1 fluorescence was detected in the nucleus as well as in cytoplasmic punctuate structures of various sizes and shapes (Figure 5). Time-lapse imaging showed that these dots were capable of fusing and fragmenting and were also highly motile (Figure 5A and Supple. Movie S3). Tracking of individual patches over time revealed two types of motile behaviour: a local, possibly, Brownian movement, and a long-range linear displacement (Figure 5A and B). Both movements were observed independently of the stage of the cell cycle (not shown). It is clear however that the GFP-Sip1 dots used the long-range movement to accumulate during nuclear separation at the position of the future division plane (Figure 5A-C). Three-dimensional reconstructions of wide-field microscopy images showed that the GFP-Sip1 fluorescence lined up on both sides of the primary, calcofluor-stained septum. They did not comprise rings or plates, but instead remained as the form of dots (Supple. Movie S4).

To evaluate the possibility that the Sip1 patches represent Golgi vesicles, we constructed a strain containing GFP-Sip1 and mCherry-tagged Anp1 or Sec72, a *cis*-Golgi or *trans*-Golgi marker protein, respectively (kindly provided by A. Vjestica and S. Olifelenko) [22]. GFP-Sip1 did not colocalise with Anp1-mCherry, and only very partially did so with Sec72-mCherry (Supple. Figure S3), indicating that Sip1 dots do not represent Golgi organelles. We next set out to examine whether GFP-Sip1 patches were stained with the amphiphilic dye FM4-64 [23, 24]. In fission yeast, a 5-30 min incubation of the cells with this dye allows the visualization of endocytic vesicles that are being transported from the plasma membrane to the vacuolar membrane [25]. Under these conditions, we found that FM4-64 clearly stained GFP-Sip1-containing structures (Figure 6A). To test if Sip1 has any role in endocytosis, we incubated wild type and *sip1-62* cells with FM4-64 for up to 90 min. Contrary to the situation in wild type cells where the dye was delivered to vacuoles by endocytosis, in the *sip1-62* mutant, FM4-64 remained localising to the tips in growing cells, or to the equator in dividing cells, that is the sites where endocytosis should have occurred (Figure 6B). It is worth pointing out that the endocytosis defect was observed even at the permissive temperature, but was most strikingly obvious at the restrictive temperature. Taking all together, we conclude that Sip1 is a novel component of endocytic vesicles and essential for endocytosis and the completion of cytokinesis.

### **DISCUSSION**

In this report, we identify a novel protein, Sip1, as a component of the Pof6/Skp1 complex in *S. pombe*. Immunoprecipitation experiments confirm physical interactions between Sip1 and Pof6-Skp1. These three proteins appear to form a functional ternary complex, as in addition to coimmunoprecipitation data, we have found that protein



stability of Sip1 and Pof6 is greatly compromised in the absence of Skp1, the phenomenon often observed in multi-subunit complexes. Given that a canonical SCF is a ubiquitin ligase in which stability of F-box proteins is often dependent upon structural integrity of a holo-SCF complex [9], this instability might imply that the Skp1-Pof6-Sip1 complex is involved in ubiquitin-mediated reactions. Sip1 is essential and conserved amongst eukaryotes. This protein consists of a number of HEAT repeats, protein-protein interaction motifs, implying that Sip1 plays a scaffolding role in this ternary complex. As none of cullins have been identified in TAP-purification (this study and [12]), it is likely that Skp1, Pof6 and Sip1 constitute an essential non-SCF F-box complex.

Creation and analysis of a *sip1-62* ts strain have unravelled an essential role for Sip1 in cytokinesis and endocytosis. It is worth mentioning that a ts *skp1-3* allele and *pof6*-deleted haploid cells germinating from heterozygous diploids exhibited cytokinetic defects [12], reminiscent of the terminal phenotype of *sip1-62* cells reported in this study. Sip1 localises to the nucleus and endocytic vesicles. Consistent with an interaction, Pof6 localises to the nucleus [12]. However, their cellular localisation is not completely parallel. In addition to the nucleus, Pof6 localises to cell tips during interphase and the CAR upon cytokinesis [12, 26], not to endocytic vesicles. It should, however, be noted that GFP-Sip1 vesicles appear to constantly gather around the CAR during CAR formation, which might reflect a physical interaction between these two proteins.

One of the most important results presented in this study is the identification of Sip1 as an essential factor for endocytosis. Cytokinesis is a complex process, in which a number of intra- and extra-cellular signalling pathways are involved and converged [27] including membrane trafficking [28]. Despite its importance, compared to our understanding of CAR formation and function, knowledge with regards to roles and mechanics of endocytosis in cytokinesis is still limited. Our study uncovers a novel component for endocytic vesicles, which will provide a tool by which to pursue the molecular pathways underlying endocytosis-mediated cytokinesis and cell separation.

Analysis of orthologues for Pof6 and Sip1 has been reported only in budding yeast. Like Pof6, F-box protein Rcy1 binds Skp1 and interestingly forms a non-SCF complex. Rcy1 participates in protein recycling from the endosomes to the Golgi and the plasma membrane [19, 29]. However, unlike Pof6, Rcy1 is non-essential and these two proteins may not perform identical molecular functions [9, 12]. So far we are not able to detect Pof6 orthologues by homology search in higher eukaryotes. The identification of functional counterparts of Pof6 in higher eukaryotes would be crucial to delineate their conserved roles. In the case of Sip1, its budding yeast orthologue is called Laa1. Like Rcy1 and unlike Sip1, Laa1 is non-essential [30]. Although no physical interaction between Rcy1 and Laa1 is known, similar to Rcy1, Laa1 is reportedly involved in clathrin-mediated transport between *trans*-Golgi network and endosomes, which displays some analogy to Sip1 functions.

In conclusion, we describe the first non-SCF complex in *S. pombe*. We show that the Skp1-Pof6-Sip1 complex plays an essential role in cell division. Further analyses point towards a precise role for this complex in endocytosis and cytokinesis. It would be now vital to explore a role of Sip1 in membrane trafficking and identify functional pathways in which the Sip1-Pof6-Skp1 complex is involved. Investigation of roles for Sip1 orthologues in higher eukaryotes would be the next phase of our research. Finally, besides Pof6, there are a number of F-box proteins in both fission yeast and other organisms, in which two critical proline and acidic residues are not

conserved [7]. We envision that non-SCF F-box complexes are more general than currently recognised and the elucidation of the biological functions of these novel complexes is a key challenge in the future.

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## FIGURE LEGENDS

### Figure 1: Identification of a novel interactor of Pof6

(A) TAP purifications of a non-tagged control strain (Table 1) and Pof6-TAP. Fractions corresponding to 1/100 of each elution (E) were loaded in parallel to 100  $\mu$ g of total extract (TE) and silver-stained. Two bands migrating around 220 kDa and 100 kDa (arrows) may correspond to Sip1 and Pof6 respectively. (B) Coimmunoprecipitation of Pof6-TAP and Skp1. Proteins were immunoprecipitated (IP) with anti-TAP, anti-Skp1 or no antibodies (Ab). 100  $\mu$ g of whole cell extract (WCE) were loaded and 2 mg of proteins were used for each IP. Pof6-TAP was immunodetected with anti-Pof6 antibodies and Skp1 with anti-Skp1 antibodies. (C) Coimmunoprecipitation of Pof6/Pof6-TAP and Sip1-HA. Extracts were immunoprecipitated with IgG Sepharose beads or anti-HA antibodies. Immunoprecipitates were blotted with anti-HA and anti-Pof6 antibodies. The asterisk indicates a non-specific band of the IgG. (D) Single step TAP purification of Sip1-TAP. 1/100 of the final elution (E) was loaded on the gel in parallel to 100  $\mu$ g of total extract (TE) and silver-stained. A band around 75 kDa probably represents mixtures of heat shock 70 chaperone family (the coverage percentages of Ssa2, Ssc1 and Ssa1 are 80.2%, 68.8 % and 51.7 % respectively). (E) Coimmunoprecipitation of Sip1-HA, Pof6 and Skp1. Sip1-HA was immunoprecipitated with anti-HA antibodies and immuno-complex was blotted with individual antibodies. (F) Instability of Sip1 and Pof6 in the absence of Skp1. Whole cell extracts were prepared from a strain containing *nmt1*-HA-Skp1, Sip1-HA and Pof6-TAP in the absence (left, derepressed, + for Skp1) or presence (right, repressed, - for Skp1) of thiamine. Skp1 and Sip1 were immunodetected with anti-HA antibody, whilst Pof6-TAP was detected with anti-Pof6 antibody.

### Figure 2: *sip1*<sup>+</sup> is an essential gene present amongst eukaryotes

(A) Tetrad dissection of asci produced by a *sip1::kan<sup>r</sup>/sip1*<sup>+</sup> diploid strain. Spores were grown for 3 days at 30°C on rich media (left). Phase contrast micrographs showing the terminal morphology of germinating *sip1*-deleted spores (right). Bar indicates 5  $\mu$ m. (B) Schematics of Sip1 protein domains. Sip1 protein is 1920 amino acids long and contains HEAT repeats (black). (C) Dendrogram of Sip1 orthologues identified by YOGY search. Proteins are labelled with the UniProtKB nomenclature. The ClustalW alignment default parameters of MacVector 9.0 were used to create the tree. The evolutionary distance is indicated on each branch. S.p: *Schizosaccharomyces pombe*; D.m: *Drosophila melanogaster*; H.s: Homo sapiens; C.e: *Caenorhabditis elegans*; S.c: *Saccharomyces cerevisiae*.

### Figure 3: Characterisation of the *sip1-62* mutant

(A) Centrifugal elutriation. Wild type (top) or *sip1-62* cells (bottom) were synchronised by elutriation at 25°C, shifted to 36°C (T0) and grown for 4 h. Samples were taken every 20 min to measure cell viability, septation index and cell numbers. (B) DNA content analysis of wild type and *sip1-62*. Each strain was grown and treated as described in (A) and samples were taken every 20 min interval, followed by FACS analysis.

### Figure 4: Cytokinesis is not completed in the *sip1-62* mutant

(A) Time-lapse imaging of Myo2-GFP. *sip1*<sup>+</sup> and *sip1-62* cells containing Myo2-GFP (green) were stained with calcofluor (blue) in liquid cultures. These cells were shifted from 25°C to 36°C for 1 h and live imaging started. Pictures were acquired at 2 min intervals for up to 2 h. Every second picture of the cell medial plane is shown. Asterisks show the time when the septum appeared. Bar, 3µm. See also Supple. Movies S1 and S2. (B) Graphics showing the timing of cytokinetic events as they occur in *sip1*<sup>+</sup> (blue) and *sip1-62* (orange) cells. Cells were prepared as in (A). For each strain, results represent the mean of two independent experiments and error bars are SD of the average. A total of 39 cells for the wild type, and 31 cells for *sip1-62* were counted. The time of septum dissolution in *sip1-62* could not be accurately determined under our experimental conditions and is shown as an open diamond. Sept., septum; CAR, Cytokinetic Actomyosin Ring.

### Figure 5: Localisation tracking analysis of GFP-Sip1

(A) Time-lapse imaging of cells expressing GFP-Sip1. The GFP fluorescence is detected in the nucleus and in punctuated structures, which accumulate at the medial plate of the cell. The time is indicated in min:sec and matches the time used in Supple. Movie S3. Red arrowheads, a patch being rapidly displaced towards the division plane. Note that this patch resulted from the fusion of at least 3 patches (e.g. compare times 12:30 and 14:00) (B) Trajectories of individual GFP-Sip1 patches movement. Each color represents a patch and each pattern represents the path followed by the patch over time. Open symbols (circles) indicate patches starting points. The path of the dot shown in (A) with the red arrowhead is shown in red in (B). Only patches that could unambiguously be followed for at least 4 min are shown. (C) Kymographs of a GFP-Sip1 cell stained with calcofluor. GFP-Sip1 gathers at the site of the division plane when the nuclei separate. As soon as the septum forms (blue arrowhead), patches line lateral to it. See also Supp Movie S4. N, Nucleus. Bar, 3µm.

### Figure 6. Sip1 localises to endocytic vesicles and is required for endocytosis

(A) Localisation of GFP-Sip1 to endocytic vesicles. *nmt1*-GFP-Sip1 cells were stained for 5 min with FM4-64 and observed immediately before the dye was transported to the vacuoles. The enlargement (right panel) shows that both signals perfectly overlap. (B) Endocytosis defects in the *sip1* mutant. Wild type (*sip1*<sup>+</sup>) and *sip1-62* cells were stained for an extended time with FM4-64. Whilst in wild type, the dye localises to peripheries of individual vacuoles, in the mutant, this dye is not incorporated inside the cell and instead accumulates at the tips (plain arrowhead) and septum (open arrowhead). Bars, 5µm. Enlargement bar, 1 µm.

**Table I: Strain list**

Strain name	Genotypes	Derivations
513	<i>h<sup>-</sup> leu1 ura4</i>	Our stock
972	<i>h<sup>-</sup></i>	Our stock
NSY001	<i>h<sup>-</sup> pof6<sup>+</sup>-2TAP-kan<sup>r</sup></i>	This study
NSY065	<i>h<sup>-</sup> leu1 ura4 sip1<sup>+</sup>-3HA-kan<sup>r</sup></i>	This study
NSY069	<i>h<sup>-or+</sup> pof6<sup>+</sup>-2TAP-hph<sup>r</sup> sip1<sup>+</sup>-3HA-kan<sup>r</sup></i>	This study
NSY110	<i>h<sup>-</sup>/h<sup>+</sup> leu1/leu1 ura4/ura4 his7/his7 ade6-210/ade6-216 sip1::kan<sup>r</sup>/+</i>	This study
NSY127	<i>h<sup>-</sup> leu1 ura4 sip1<sup>+</sup>-2TAP-kan<sup>r</sup></i>	This study
NSY165	<i>h<sup>-</sup> leu1 ura4 sip1-62-HA-kan<sup>r</sup></i>	This study
NSY303	<i>h<sup>-</sup> leu1 ura4 pof6<sup>+</sup>-2TAP-hph sip1<sup>+</sup>-3HA-kan<sup>r</sup> hph<sup>r</sup>-nmt-HA-skp1<sup>+</sup></i>	This study
NSY178	<i>h<sup>-</sup> leu1 ura4 myo2<sup>+</sup>-GFP-kan<sup>r</sup></i>	Our stock
NSY191	<i>h<sup>-or+</sup> sip1-62-HA-kan<sup>r</sup> myo2<sup>+</sup>-GFP-kan<sup>r</sup></i>	This study
IJ449	<i>h<sup>-or+</sup> ade<sup>-</sup> leu1 kan<sup>r</sup>::nmt1-GFP-sip1<sup>+</sup> anp1<sup>+</sup>-linker-mCherry::ura4</i>	This study
IJ450	<i>h<sup>-or+</sup> ade<sup>-</sup> leu1 kan<sup>r</sup>::nmt1-GFP-sip1<sup>+</sup> sec72<sup>+</sup>-linker-mCherry::ura4<sup>+</sup></i>	This study

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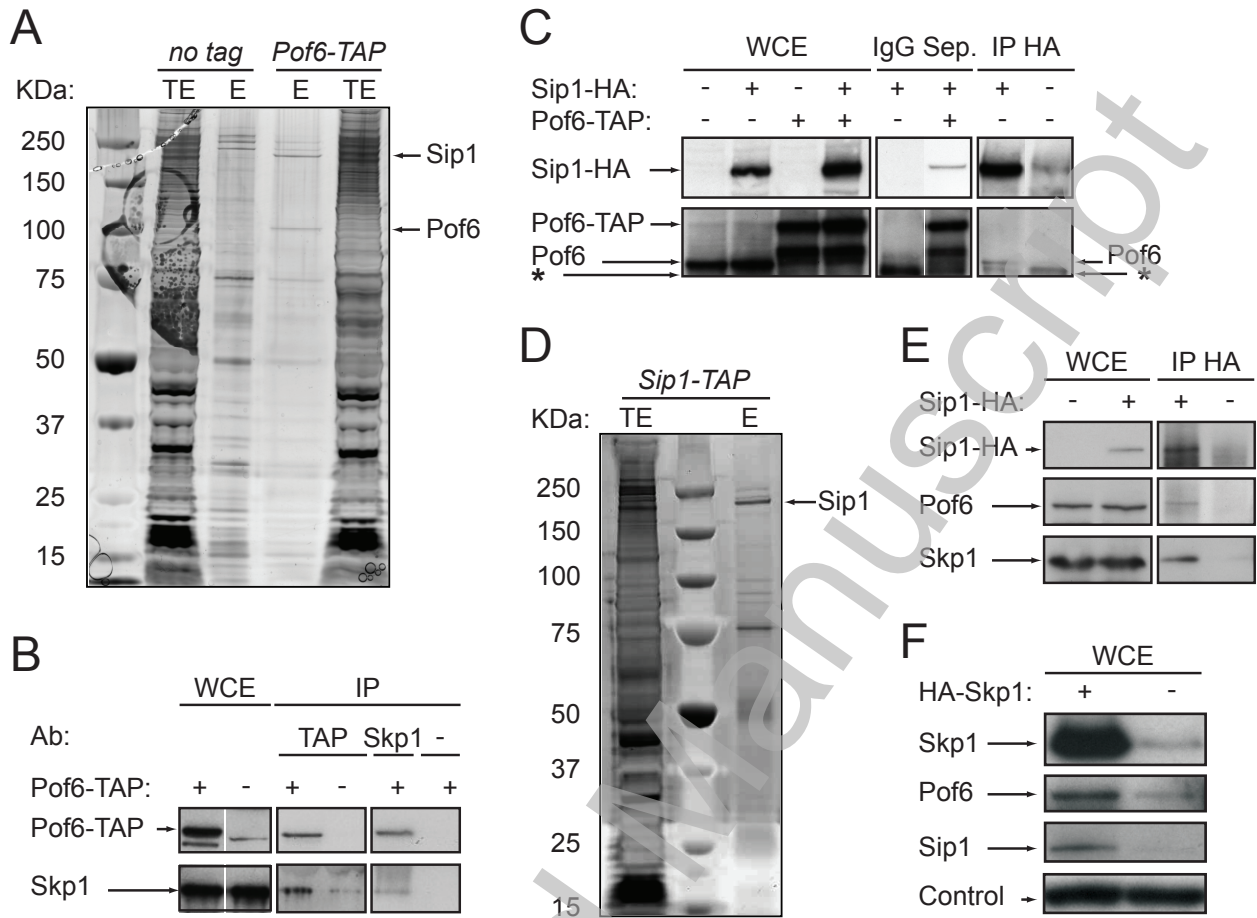


Figure 1: Jourdain *et al.*



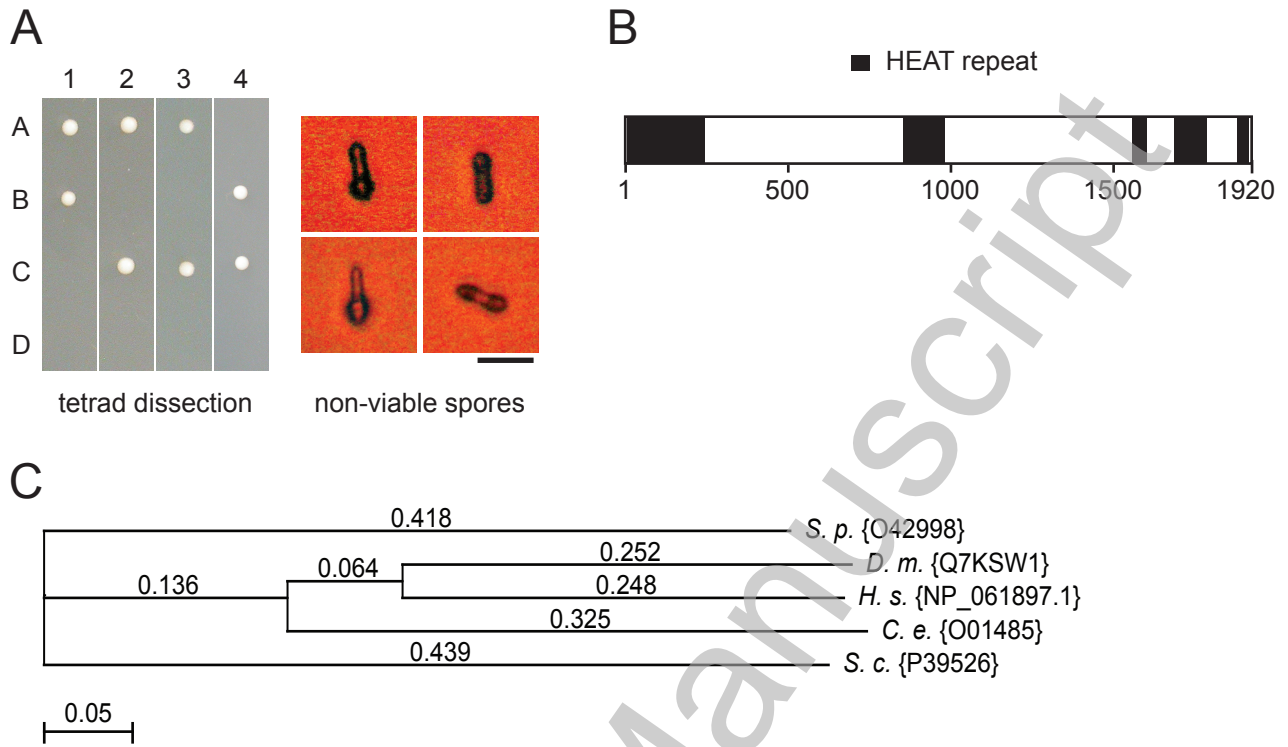


Figure 2: Jourdain *et al.*

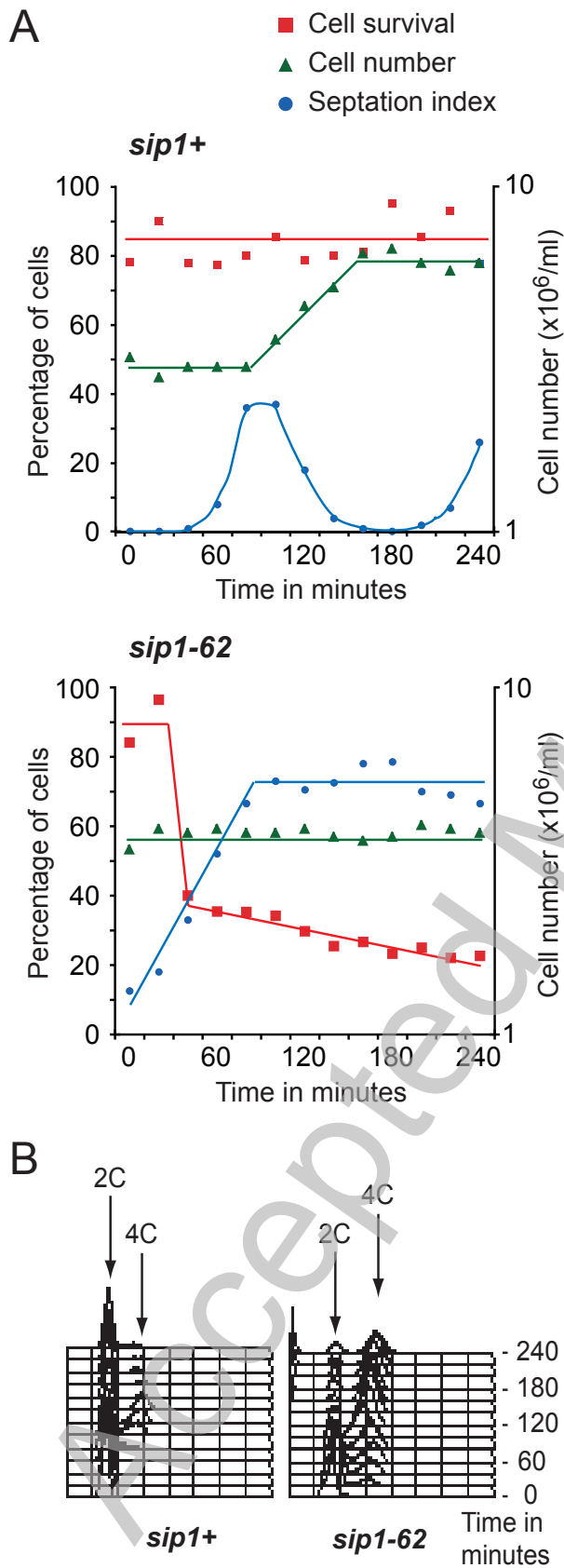
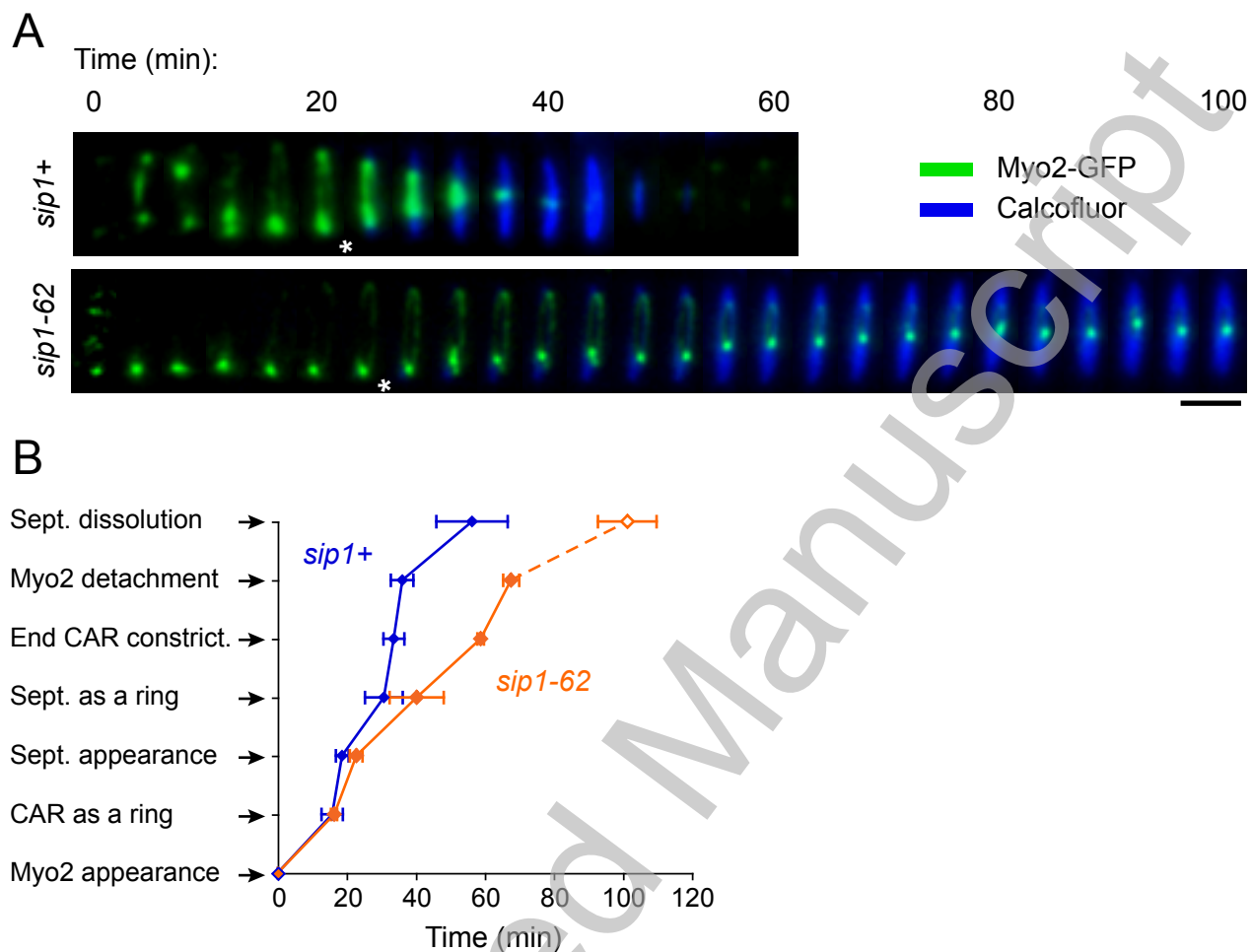


Figure 3: Jourdain *et al.*



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Figure 4: Jourdain *et al.*

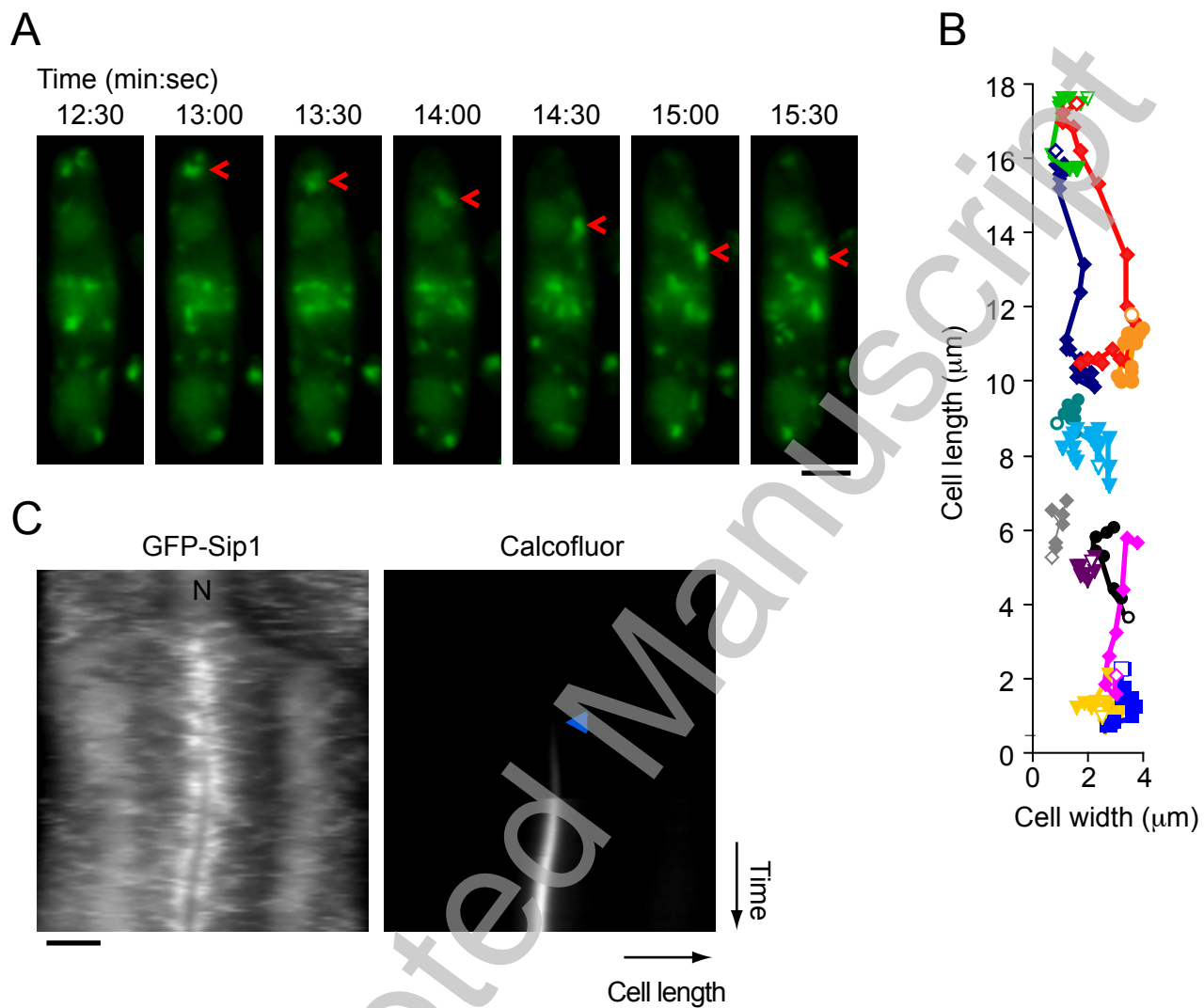
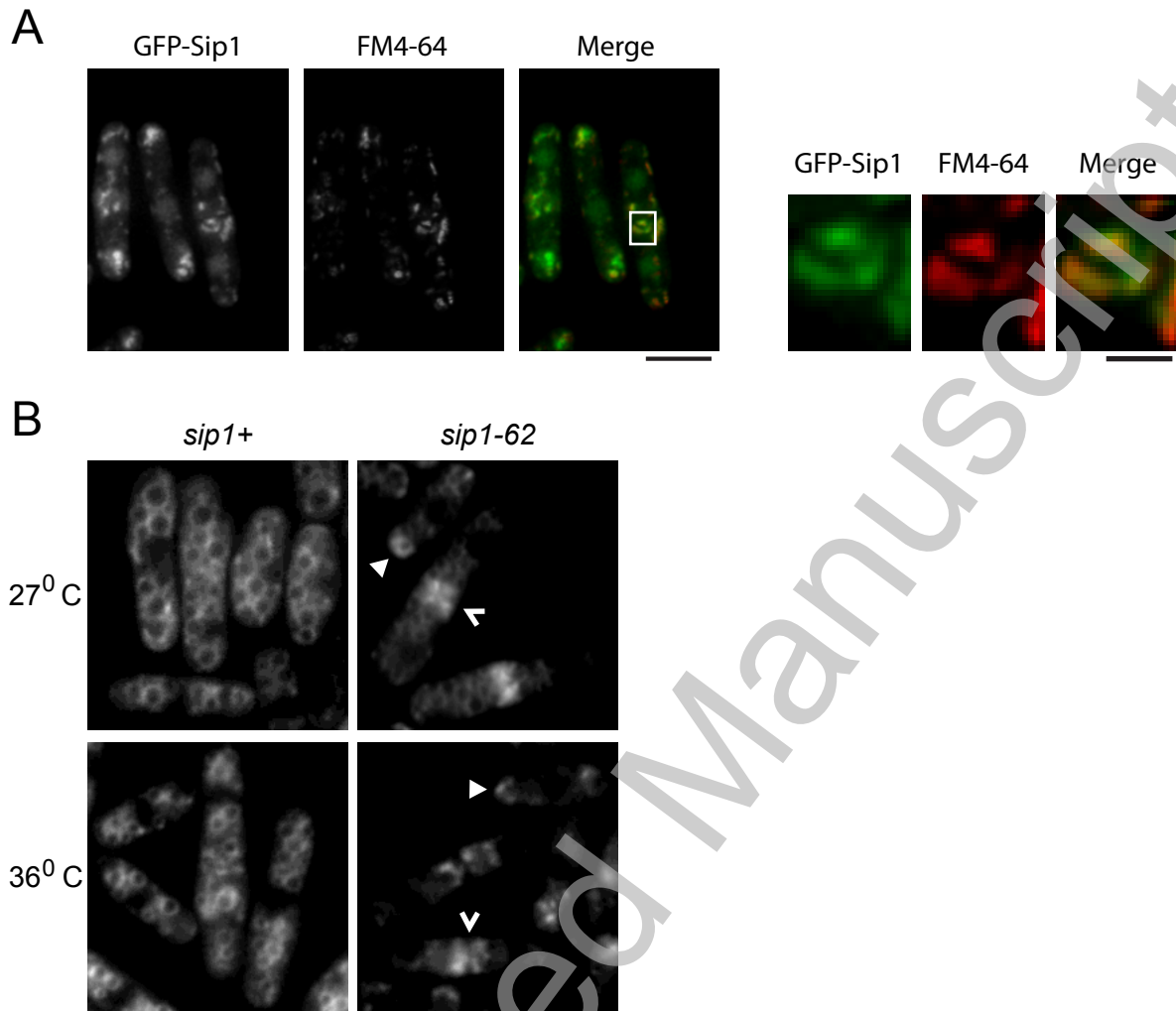


Figure 5: Jourdain *et al.*



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Figure 6: Jourdain *et al.*

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