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A Novel Role of Hrr25 in Cell Wall Integrity Maintenance and Functional Analysis of Hrr25 Domains

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A Novel Role of Hrr25 in Cell Wall Integrity Maintenance and Functional Analysis of Hrr25 Domains

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Dedication

I dedicate this thesis to my beloved father, Mr. Ravindra Bhattarai, who turned 50 years old while I was away here, in the United States, pursuing a master's degree in Biological Sciences.

Acknowledgements

I want to thank my advisor, Dr. Liu Zhengchang, for his guidance, patience and support. He helped me when I was struggling with experiments, pushed me when I was stagnating, and offered advice and was kind to me when I was going through personal hardships. I would also like acknowledge my committee members, Dr. Wendy Schluchter and Dr. Mary Clancy, for their professional advice and valuable comments on this thesis.

I would like to thank my labmate Manika Bhondeley for her help with making some of the plasmids and strains for the project.

I would like to thank my parents, Olga and Ravindra Bhattarai, and my little brother Yogesh for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

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Abstract

Hrr25 is a highly conserved serine/threonine protein kinase with diverse functions and it localizes to a number of intracellular sites. Hrr25 possesses an N-terminal kinase domain, a middle region, and a C-terminal proline/glutamine rich domain. In this thesis, we systematically characterized the roles of these three domains of Hrr25 on cell growth, cell morphology, and its cellular localizations. Additionally, we identified a novel target of Hrr25, Pin4. We show that Hrr25-dependent phosphorylation of Pin4 is required for cell wall integrity maintenance. We found that that Hrr25 and Pin4 physically interact *in vivo* and that the C-terminal domain of Hrr25 is required for this interaction and for maintaining cell wall integrity. Together, we identified a new target and a novel function for Hrr25 and provided insights into the structure-function relationship of Hrr25 domains.

Key words: Hrr25 domains, Pin4, cell wall integrity, DNA damage

Chapter I: General Introduction

Post-translational modification (PTM) of proteins is one of several methods that can be utilized by a cell to elicit a sophisticated response to changing internal and external stimuli. One of the most common types of PTM is protein phosphorylation, which is carried out by protein kinases. As the name implies, protein kinases phosphorylate target proteins by transferring the gamma phosphate group from adenosine triphosphate and covalently attaching it to specific amino acids. Depending on the amino acid residue that receives the phosphate, protein kinases can be divided into 4 distinct classes: serine/threonine kinases, tyrosine kinases, dual-specificity kinases, and kinases that phosphorylate other amino acids.

An important family of serine/threonine kinases is the Casein kinase I (CK1) family, which is highly evolutionarily conserved and plays a major role in cell cycle regulation, receptor-coupled signal transduction, transcription, translation, and the organization of the cytoskeleton. Malfunction of CK1 isoforms in humans has been linked to alteration of circadian rhythm and to colon, pancreatic, breast cancers and leukemia (Janovska et al., 2018; Knippschild et al., 2005; Schittek and Sinnberg, 2014). In *S. cerevisiae*, there are four CK1 family members: Yck1, Yck2, Yck3, and Hrr25, the latter being a homologue of mammalian CK1 isoform δ (Wang et al., 1996).

Hrr25 is a Functionally Diverse Kinase That Localizes to Multiple Cellular Compartments

Hrr25 regulates a diverse group of metabolic processes by phosphorylating different substrates. It has been reported to be implicated in ER-to-Golgi protein transport, calcineurin signaling (Kafadar et al., 2003; Lord et al., 2011; Murakami et al., 1999), ribosomal biogenesis

(Ray et al., 2008; Schafer et al., 2006), autophagy (Davis et al., 2016; Mochida et al., 2014; Pfaffenwimmer et al., 2014), Elongator function promotion and tRNA modification (Abdel-Fattah et al., 2015), weak acetic acid stress response (Collins et al., 2017), microtubule assembly and the spindle positioning (Peng et al., 2015b), clathrin-mediated endocytosis (Peng et al., 2015a), DNA damage response (Hoekstra et al., 1991), and meiosis (Arguello-Miranda et al., 2017; Corbett and Harrison, 2012; Katis et al., 2010; Petronczki et al., 2006; Ye et al., 2016).

Subcellular Localizations of Hrr25

Hrr25 has been shown to localize to different cellular compartments in a cell cycle dependent manner. During the mitotic cycle, Hrr25 is recruited to the nucleus at early G_1 stage (Lusk et al., 2007). As the cell cycle progresses, it localizes to spindle pole bodies (SPBs) and the bud neck (Kafadar et al., 2003). Hrr25 next travels to the bud cortex, while still localizing to the SPB. As cells enter the M stage, Hrr25 remains at the SPB sites, but no longer localizes to the bud cortex (Brockman et al., 1992; Lusk et al., 2007). When cells transition into the G_1 phase from the M phase, Hrr25 travels to the septin ring. Next, the cycle repeats.

Additionally, Hrr25 localizes to the preautophagosomal structures (PAS) under nitrogen starvation conditions to participate in macroautophagy (Wang et al., 2015). Under glucose deprivation, water treatment, and other environmental stresses, Hrr25 is recruited to cytoplasmic ribonucleoprotein (RNP) processing bodies (P-bodies). Localization of Hrr25 to P-bodies is crucial for completion of meiosis (Zhang et al., 2018; Zhang et al., 2016).

Domains of Hrr25 and Their Functions

Hrr25 consists of 3 domains: an N-terminal kinase domain, a middle region, and a C-terminal domain. Amino acids 1~295 comprise the N-terminal kinase domain, which is highly conserved among eukaryotic casein kinase I orthologs. The N-terminal kinase domain of Hrr25

shares 72% sequence similarity with Hhp1, an Hrr25 ortholog found in *S. pombe*, and 66% sequence identity with a mammalian CK1δ isolated from *M. musculus* (Ye et al., 2016). The kinase domain and the associated kinase activity of Hrr25 are crucial for its participation in most if not all processes mentioned above.

The N-terminal domain of Hrr25 is followed by the middle region (amino acids 296-394), that is only found in species closely related to *S. cerevisiae*. Nevertheless, it shares 21% homology with the centrosomal localization signal found at the noncatalytic C-terminus of human CK1δ (Peng et al., 2015b). The middle region of Hrr25, underappreciated before, has recently been shown to be important for sister chromatid mono-orientation in meiosis I and stress-induced P-body localizations of Hrr25 (Peng et al., 2015a; Peng et al., 2015b; Ye et al., 2016; Zhang et al., 2018). The middle region has also been suggested to be required for Hrr25's localization to endocytic sites and SPB. However, these possibilities have not been directly examined. The P/Q-rich C-terminal region of Hrr25 occupies amino acid positions 395-494 and its function is mostly unknown. A thorough analysis of the structure–function relationships of Hrr25 domains is needed for the elucidation of their respective functions.

The Cell Wall Integrity Pathway in Yeast

In the second part of my thesis, we report a novel function for Hrr25: the maintenance of cell wall integrity. In *S. cerevisiae*, the cell wall provides resistance to osmotic shock and mechanical stress, determines the cell shape, and serves as a surface for peripheral glycoproteins (Borovikova et al., 2016; Cid et al., 1995; Rodriguez-Pena et al., 2010; Schmidt et al., 2005). Thus, the cell wall integrity maintenance is crucial during normal cell growth and environmental stress conditions. The Cell Wall Integrity (CWI) signaling pathway is the central pathway that is

employed by yeast cells to respond to cell wall integrity perturbations. Figure 1 depicts a diagram of the CWI signaling pathway in the budding yeast.

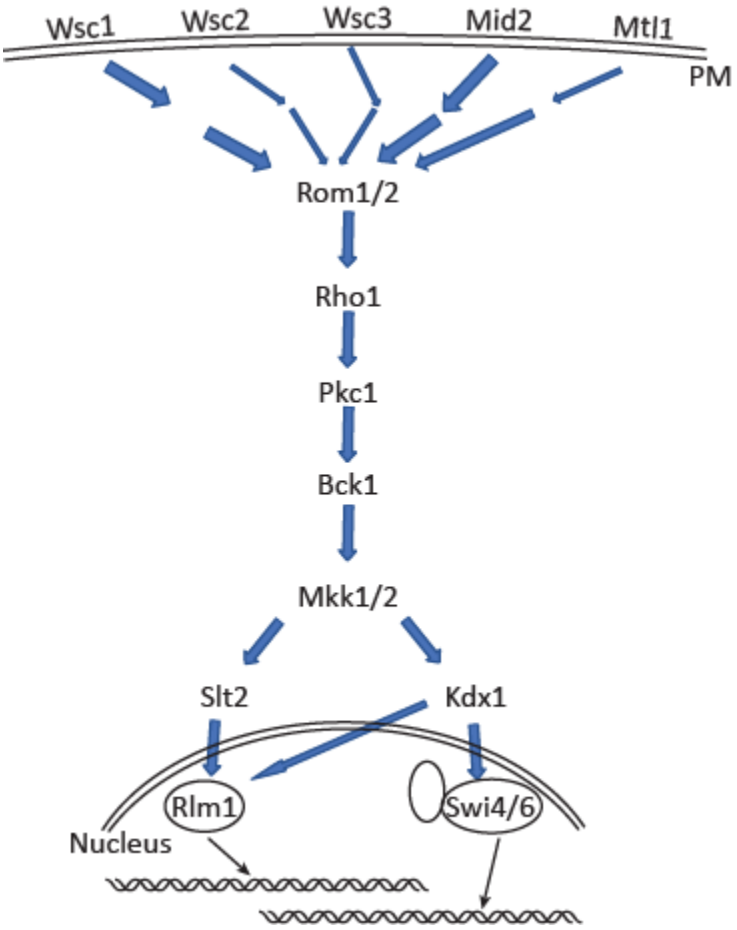

Figure 1. The cell wall integrity (CWI) signaling pathway in *S. cerevisiae* (Levin, 2005).

Overview of the CWI pathway

Cell wall stress conditions are recognized by the cell-surface mechanosensory receptors Wsc1/2/3, Mid2, and Mtl1, which are integral membrane proteins anchored in the plasma membrane (Jendretzki et al., 2011). Next, phosphatidylinositol (PI)-4,5-bisphosphate (PIP_2) binds guanosine nucleotide exchange factors (GEFs) Rom1 and Rom2 and recruits them to the plasma membrane (Audhya and Emr, 2002). PIP2-bound Rom1 and Rom2 receive cell wall stress signals from the sensors and bind the G protein Rho1 (Ozaki et al., 1996). Another GEF, Tus1, associates with Rho1 in response to intracellular signals in a cell-cycle dependent manner

(Kono et al., 2008; Schmelzle et al., 2002). A physical interaction of GEF with GDP-bound Rho1 allows the nucleotide exchange reaction to occur, resulting in an active GTP-bound Rho1. Rho1 activation leads to an array of cellular responses. Here, we focus on one of them: the activation of a MAPK cascade, eliciting a transcriptional response to cell wall integrity perturbations.

The CWI mitogen-activated protein (MAP) kinase cascade is a linear cell signaling pathway that is governed by protein kinase C, Pkc1 (Levin, 2011). GTP-bound Rho1 physically interacts with Pkc1 with the aid of phosphatidylserine (PS) to initiate a series of phosphorylation events (Levin, 2011; Nomura et al., 2017). The hierarchical CWI MAP kinase cascade consists of the MAP kinase kinase kinase Bck1, two homologous MAP kinase kinases, Mkk1 and Mkk2, and the MAP kinase Slt2, which activate one after another in a unidirectional consecutive manner (Levin, 2011). Once phosphorylated, Slt2 initiates the transcription of genes involved in cell wall biogenesis through the serum-response factor–like transcription factor Rlm1 and the Swi4/6 SBF transcription complex (Levin, 2011).

Activation of CWI signaling

The CWI signaling pathway is activated in response to a variety of factors: heat and osmotic stress, exposure to mating pheromones, cell wall stressors (Calcofluor white, Congo red, zymolyase, caffeine, bleomycin, sodium dodecyl sulfate (SDS)), actin depolarization, ER stress, and DNA damage (Buehrer and Errede, 1997; de Nobel et al., 2000; Krysan, 2009; Liu and Levin, 2018; Lottersberger et al., 2006; Rodriguez-Pena et al., 2010; Soriano-Carot et al., 2012). Recent findings have led to the proposal that “lateral” inputs of the stressors into the CWI MAP kinase cascade take place at different steps of the pathway, which helps to explain the

sophisticated cellular responses to a diverse group of CWI pathway activators (Harrison et al., 2004).

Cross-talk Between the CWI Pathway and DNA Damage response

The CWI pathway gets activated under DNA damage conditions. Slt2 phosphorylation increases in response to DNA damage through proteasomal degradation of its negative regulator, Msg5, independent of Slt2's upstream regulators Bck1 or Mkk1/2 (Liu and Levin, 2018; Soriano-Carot et al., 2012). Additionally, when grown in medium supplemented with caffeine, Slt2 undergoes Mec1/Tel1 and Rad53-dependent phosphorylation, which prevents it from binding the transcription factor Swi4 and activating SBF transcription complex Swi4/6 (Kim et al., 2010).

Research aims

Understanding the structure-function relationship of Hrr25 requires the elucidation of the functions of its three domains. In the first half of this thesis, I attempt to systematically characterize the roles of Hrr25 domains governing its localization to spindle pole bodies (SPBs), stress-induced mRNA processing bodies (P-bodies), endocytic sites, and cell morphology.

One of the first functions of Hrr25 discovered was its involvement in the DNA damage response pathway (Hoekstra et al., 1991). The transcription factor Swi6 has been proposed to be regulated by Hrr25 in the context of DNA damage repair, however the evidence was indirect and no mechanism was established (Ho et al., 1997). In the second half of this thesis, I aim to investigate whether the poorly studied protein Pin4 is a novel target of Hrr25 and whether their interaction is implicated in DNA damage response and cell wall integrity maintenance.

Chapter II: Characterization of Hrr25 Domain Functions in *S. cerevisiae*

Materials and methods

Growth Media and Growth Conditions

Yeast cells were grown at 30°C in YPD (2% peptone, 1% Bacto-yeast extract, and 2% D-glucose), SD medium (0.67% yeast nitrogen base and 2% D-glucose), and complete supplement mixture (CSM) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.6 g/liter CSM minus histidine, leucine, and tryptophan). When needed, L-leucine, L-lysine, L-histidine, L-methionine, L-tryptophan, and/or uracil were added to selective growth media to meet auxotrophic requirements (Amberg, 2005). Plate media contained 2% agar in addition to the components described above.

Yeast Transformations

Plasmids were transformed into yeast strains using the high efficiency lithium acetate-PEG method as described (Amberg, 2005).

Mating and Tetrad Dissection

MATa and *MATα* haploid yeast strains were mated on YPD plate media for four hours to overnight. Diploids were selected on SD plate media supplemented with amino acids to cover auxotrophic markers. Diploid cells were induced to undergo sporulation by growing in sporulation media (1% potassium acetate, 0.1% Bacto-yeast extract, 0.02% raffinose) supplemented with required arm analysis and/or uracil for 3-5 days. Tetrads were dissected on YPD or YNBcasD plates using a micromanipulator.

Fluorescence microscopy

Cells expressing various GFP or RFP (mRFP1, mCherry) fusion proteins as indicated in the text and in the figures were grown in the indicated media to mid-logarithmic phase. 3µl of

cell cultures were put onto the slide and let settle down for 3 min before coverslips were applied. If indicated, cells were subjected to water stress: 1ml of cell culture at mid-logarithmic growth phase was centrifuged, supernatant was removed and 1ml of water was added to incubate for 2 hours. GFP fluorescence, RFP fluorescence and differential interference contrast (DIC) images of live cells were immediately captured using a Nikon Eclipse E800 microscope equipped with an HBO 100 W/2 mercury arc lamp, a Nikon Plan Fluor 100# objective lens, and Nikon filter sets for capturing GFP and RFP fluorescence images. Images were acquired with a Photometrics CoolSNAP DYNO camera and the Nikon NIS-Elements imaging software and processed using ImageJ (National Institutes of Health) and Adobe Photoshop (Mountain View, CA) software.

FM4-64 treatment of cells

Yeast cells as indicate in the figure legends were grown in YPD medium to mid-logarithmic phase and precipitated by centrifugation. The supernatant was removed, and cells were resuspended in fresh YPD medium. FM4-64 dye was added at the final concentration of 0.1mM and cells were incubated with the dye for 30 minutes in the dark. After incubation, cells were centrifuged, washed once with fresh YPD medium and chased with YPD for 30 minutes at room temperature. Cells were then washed once with YPD medium and once with PBS buffer (Phosphate-buffered saline). Samples were centrifuged, most of the supernatant was removed, and cells were resuspended in the remaining supernatant to be subjected to fluorescence microcopy analysis.

Results

Main constructs used for GFP microscopy analysis

Hrr25 protein consists of 3 distinct domains: an N-terminal kinase domain (1-295), a middle region (amino acid residues 296-394), and a C-terminal region (amino acid residues 395-

494). Hrr25 can localize to many cellular foci (Brockman et al., 1992; Kafadar et al., 2003; Vancura et al., 1994; Wang et al., 2015; Zhang et al., 2018; Zhang et al., 2016; Zientara-Rytter et al., 2018). The middle region has been reported to be required for the localization of Hrr25 to the spindle pole body and P bodies (Ye et al., 2016; Zhang et al., 2016). In this study, we aim to systematically identify the domains of Hrr25 that are responsible for its localization to various cellular compartments and are required for its involvement in vital cellular processes.

The N-terminal kinase domain of Hrr25 is approximately 295 amino acids long. The kinase activity of Hrr25 is mediated by a DFG (Asp-Phe-Gly) motif at positions 149-151 and by a number of charged residues outside that region, including lysine (K) at position 38 and glutamic acid (E) at position 52 (Ye et al., 2016). In order to evaluate the importance of kinase activity for Hrr25-substrate interactions and for cell morphology, we generated a kinase loss-of-function mutant by mutating lysine 38 to alanine. The *hrr25(K38A)* mutation has been reported to lead to severe growth impairment and the abolishment of kinase activity (Murakami et al., 1999).

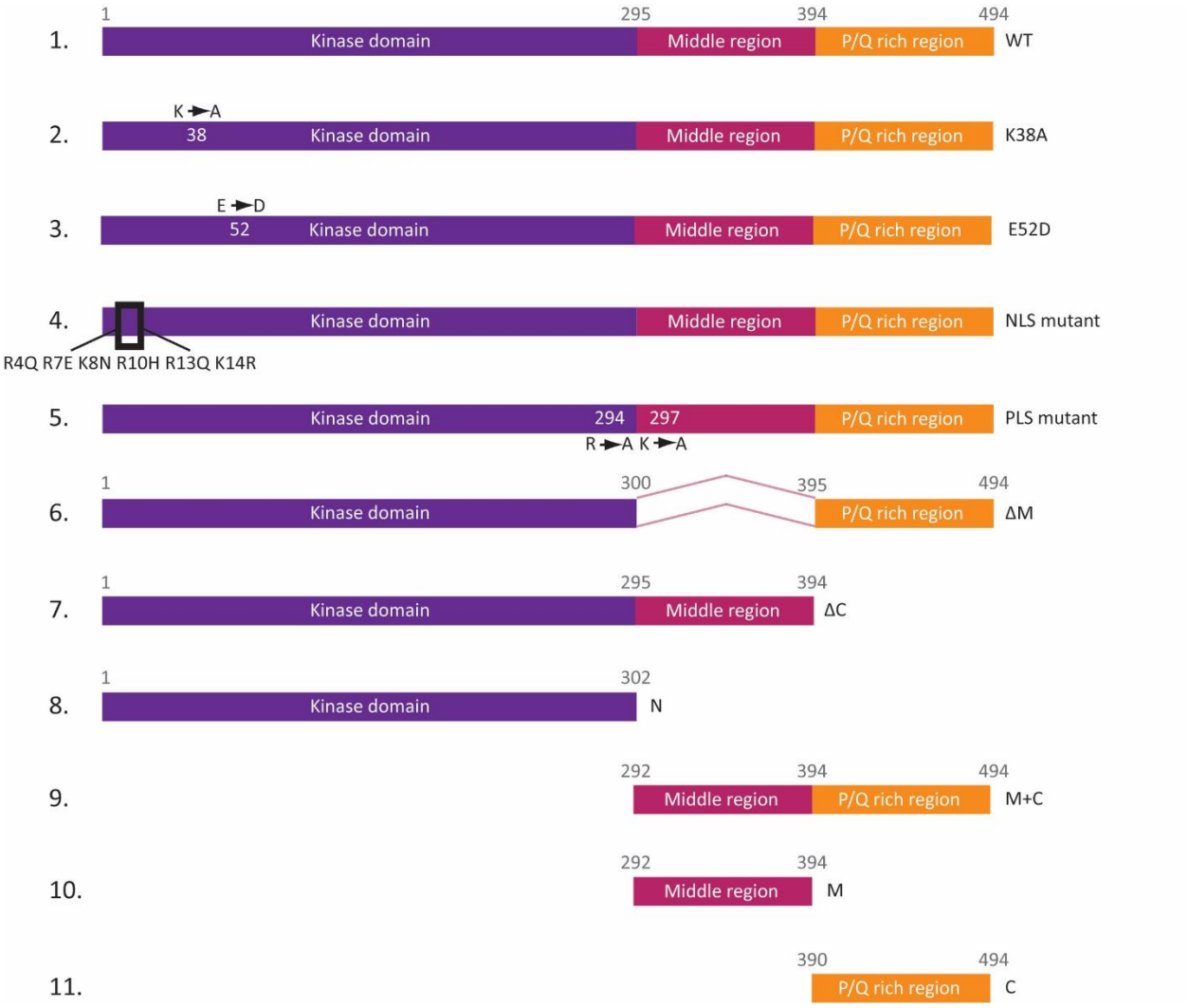

Figure 2. The main constructs used in this study: (1) Wild-type Hrr25; (2) Hrr25(K38A)*;* (3) Hrr25(E52D)*;* (4) Putative nuclear localization signal mutant Hrr25(NLSm); (5) P-body localization signal mutant Hrr25(PLSm); (6) the middle region truncation mutant Hrr25(ΔM), (7) the C-terminal region truncation mutant Hrr25(ΔC); (8) the kinase domain only construct Hrr25(N); (9) the kinase domain truncation mutant Hrr25(M+C), (10) middle region only Hrr25(M), (11) the P/Q rich C-terminal domain construct Hrr25(C).

The extremely slow growth of *hrr25(K38A)* mutant cells could potentially impact cellular localization of proteins. Therefore, we sought to obtain an alternative mutant allele, the substitution of glutamic acid residue 52 for aspartic acid, *hrr25(E52D)*, which has been shown to dramatically reduce the kinase activity of Hrr25 without substantially compromising cell growth (Mehlgarten et al., 2009).

Hrr25 localizes to the nucleus and the targeting signal has not been mapped. The archetypal nuclear localization signal of a protein typically includes a cluster of positively charged residues. Hrr25 possesses a putative nuclear localization signal (NLS) at the extreme N-terminal end, which consists of 6 positively charged residues: four arginine residues at positions 4, 7, 10, 13 and two lysine residues at positions 8 and 14 (Boulikas, 1997). In order to elucidate the role of this putative NLS in the cellular localization and functionality of Hrr25, we introduced six point mutations, namely, R4Q, R7E, K8N, R10H, R13Q, and K14R into the *HRR25* gene to obtain the mutant allele *hrr25(NLSm)*. The Hrr25(NLSm) was fused with a C-terminal GFP tag and the fusion construct was tested for cellular localization using fluorescence microscopy.

To determine the importance of the kinase domain alone for the cellular localization of Hrr25, we generated a C-terminal GFP-tagged Hrr25 kinase domain comprising of amino acid residues 1-302. To determine the role of the middle region and the C-terminal P/Q rich region to the cellular localization of Hrr25, we generated C-terminal GFP tagged Hrr25 middle region (Hrr25(M)), the C-terminal P/Q rich region (Hrr25(C)), and the combined middle and C-terminal regions (Hrr25(M+C)). We also generated GFP-tagged Hrr25 construct lacking the middle region (Hrr25(ΔM)) and the C-terminal region (Hrr25(ΔC)) for fluorescence microscopy analysis (Figure 2)

A paper published in 2016 identified amino acid residues that are required for localization of Hrr25 to P-bodies (Zhang et al., 2016). In that study, the group created a P-body localization signal 1 mutant Hrr25(PLM1m) by introducing a double mutation of Arg294 and Lys297 to Ala, which resulted in abolishment of Hrr25 localization to P-bodies. We created a GFP-tagged PLS1 mutant of Hrr25 (Hrr25(PLSm)) to evaluate the possible effect of these

mutations on the localization of Hrr25 to other sites and to use it a control for P-body localization.

Wild-type *S. cerevisiae* cells divide every 2-2.2 hours in rich growth medium. When studying the cellular localization of a protein, it is important that the cells maintain the generation time as close to the wild type as possible, because slow growth might have an unforeseen effect on the cellular localization of the protein. In this regard, studying *hrr25* mutant cells has been challenging: an *hrr25* deletion mutant is considered almost inviable. However, recent studies have shown that the deletion of a ribosome-assembly factor Ltv1 was able to partially suppress the growth defect of *hrr25Δ* mutant cells (Ghalei et al., 2015). We used this knowledge to improve the growth of not only an *hrr25(K38A)* mutant, but also some of the truncation mutant constructs, by introducing an *ltv1Δ* mutation into the mutant strains for cellular localization of some of the above-mentioned Hrr25 constructs.

Truncation from the C-terminal end of Hrr25 leads to a progressive reduction in cell fitness

We sought to generate *hrr25Δ* mutant strains carrying full-length *HRR25*, *hrr25(ΔC)*, *hrr25(ΔM)*, *hrr25(N)*, or *hrr25(K38A)* to analyze cell growth relative to wild type. We thus transformed a heterozygous *hrr25* mutant diploid strain with a centromeric plasmid encoding wild-type or various *hrr25* mutant constructs, each carrying a C-terminal 3xmyc epitope tag. Transformants were grown in liquid sporulation medium for 3 days and tetrads were then dissected onto a YPD plate.

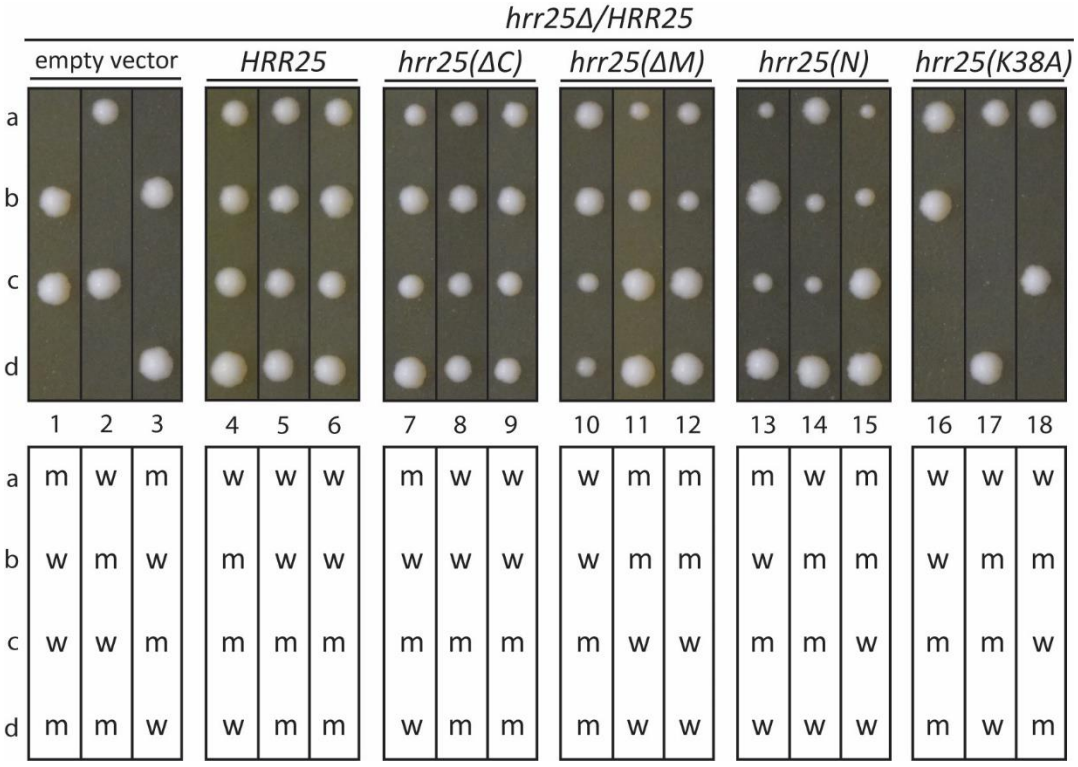

Figure 3. The effect of *hrr25* mutations on cell growth. An *hrr25* heterozygous mutant diploid strain carrying a centromeric plasmid encoding myc-tagged wild-type, mutant, or truncation *HRR25* construct as indicated were sporulated and tetrads were dissected onto YPD plate. Plasmids used in the analysis: empty vector (rows 1-3), *HRR25* (rows 4-6), *hrr25(ΔC)* (rows 7-9), *hrr25(ΔM)* (rows 10-12), *hrr25(N)* (rows 13-15), *hrr25(K38A)* (rows 16-18). Colonies labelled "w" and "m" are wild-type and mutant, respectively.

As shown in Figure 3, rows 1-3, *hrr25Δ* resulted in severe growth defect and small colonies were visible only after approximately 4 days of growth (data not shown). The growth defect caused by *hrr25Δ* was comparable to an *hrr25(K38A)* mutation (Fig. 3, rows 16-18), indicating that the kinase activity of Hrr25 is strictly required for cell growth. The loss of the C-terminal region led to a very subtle growth defect (Fig. 3, rows 7-9), while the loss of the middle region resulted in a small but clearly visible growth defect (Fig. 3, rows 10-12). The loss of both the middle and C-terminal region further exacerbated the growth defect (Fig. 3, rows 13-15). Using C-terminal GFP-tagged contracts, we found that NLSm and PLSm exhibited no growth defect and that the expression of Hrr25(M), Hrr25(C) or Hrr25(M+C) did not suppress the growth defect caused by *hrr25Δ* (data not shown). Together, our data indicate that the kinase

activity of Hrr25 is strictly required for cell growth and that the middle and C-terminal regions of Hrr25 are only partially required for cell growth.

The N-terminal kinase domain of Hrr25 is sufficient to confer normal cell morphology

We performed a differential interference contrast microscopy analysis to determine the effect of *hrr25* mutations mentioned above on cell morphology. Cells were grown overnight in liquid selective growth medium to maintain the centromeric plasmid encoding various *HRR25* contracts to mid-logarithmic phase and observed under a Nikon Eclipse E800 microscope. Pictures were taken using a Photometrics CoolSNAP DYNO camera and analyzed in ImageJ.

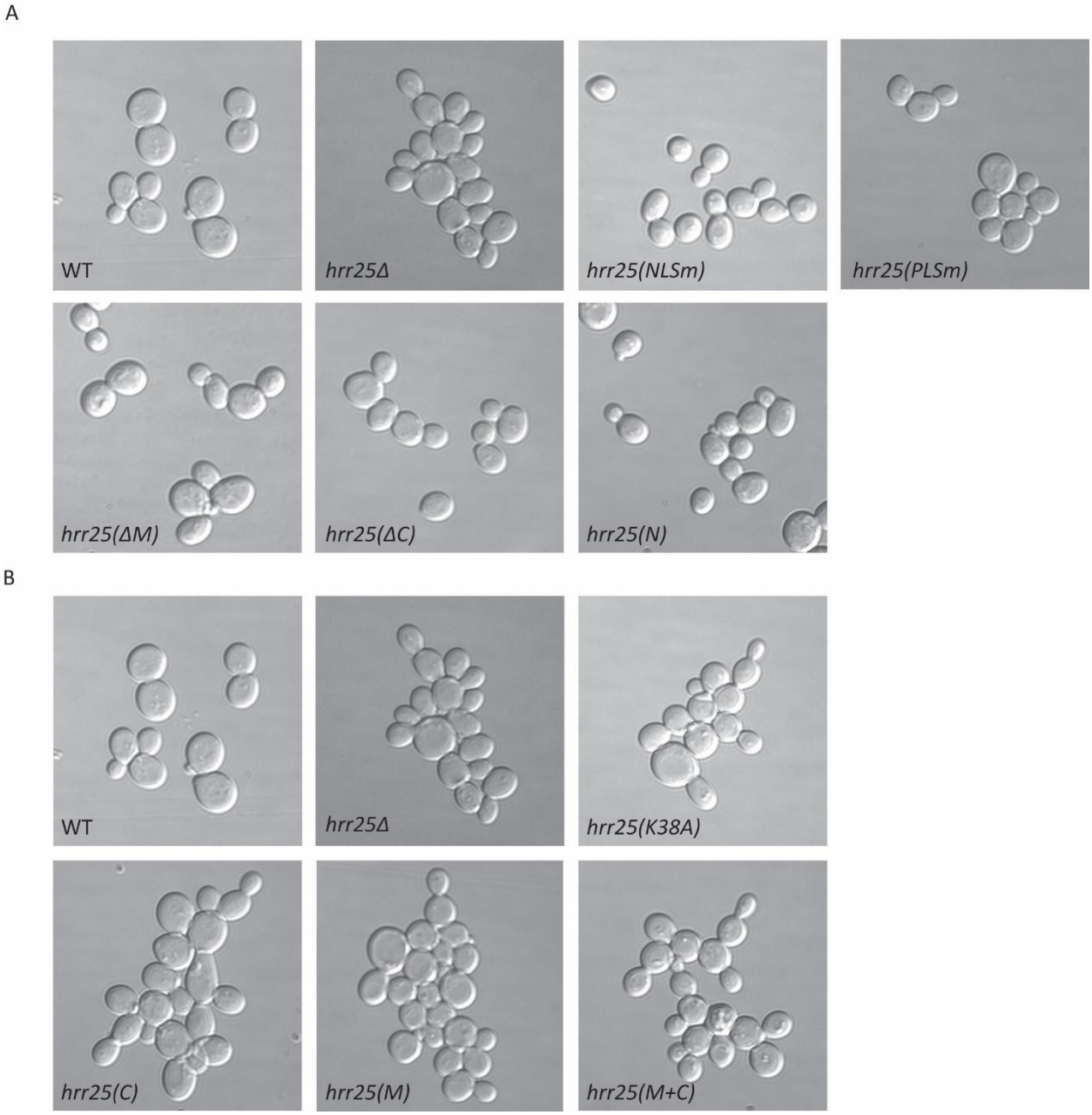

Figure 4. The effect of *hrr25* mutations on cell morphology using DIC microscopy. (A) *hrr25Δ* cells carrying *HRR25* constructs including the kinase domain have wild-type cell morphology. (B) *hrr25Δ* cells carrying the kinase-dead mutant *hrr25(K38A)*, the middle and/or C-terminal region of Hrr25, have the same cell morphology as *hrr25Δ* mutant cells.

Wild-type cells in mid-log phase are oval-shaped and often form clusters of 2-4 cells when grown in dextrose medium. In contrast, *hrr25Δ* mutant cells failed to separate from each other and often formed larger clusters of cells. Truncation of the middle region and/or the C-terminal region from Hrr25 did not result in any apparent morphological defect (Fig. 4A,

hrr25(ΔM), *hrr25(ΔC)*, *hrr25(N)*), suggesting that these two domains are not required for determining the cell morphology. *hrr25(NLSm)* and *hrr25(PLSm)* mutations did not seem to alter the appearance of cells either (data not shown). In contrast, *hrr25Δ* cells expressing the middle and/or C-terminal region of Hrr25 (*hrr25(M)*, *hrr25(C)*, and *hrr25(M+C)*) had the same cell morphology as *hrr25Δ* mutant cells (Fig. 4B)). Furthermore, the kinase-dead mutant *hrr25(K38A)* phenocopied *hrr25Δ*. Together, our data indicate that the kinase domain of Hrr25 alone is sufficient to confer normal cell morphology. Although it is an appealing model that the middle and C-terminal region of Hrr25 are required for the interaction of Hrr25 with certain substrate(s), it is clear that in the case of cell morphology, these two regions are not required for Hrr25 to perform its function in determining cell morphology.

Hrr25(E52D) localizes to the nucleus and the cytoplasm

Hrr25 has been reported to localize to the cytoplasm, the nucleus, the cell periphery, the bud neck, P-bodies, and the spindle pole bodies (Kafadar et al., 2003; Lusk et al., 2007; Peng et al., 2015a; Vancura et al., 1994; Zhang et al., 2016). Our data on the cellular localization of a functional C-terminal GFP-tagged Hrr25 are consistent with published results (data not shown).

GFP-tagged Hrr25(M), Hrr25(C), and Hrr25(M+C) localize diffusely in the cytoplasm (data not shown), indicating that the middle and the C-terminal regions alone or in combination are not sufficient to target Hrr25 to distinct cellular sites other than the cytoplasm. These results emphasize the importance of the kinase activity of Hrr25, mediated by the N-terminal kinase domain, for its involvement in diverse cellular processes. Supporting that conclusion, the GFP-tagged Hrr25 kinase activity mutant Hrr25(E52D) did not colocalize with RFP-tagged Spc42 or Syp1, or Edc3-mCherry and localized to cytoplasm and nucleus only (Fig. 5B). Similarly, Hrr25(K38A) only localize to the cytoplasm and the nucleus (data not shown).

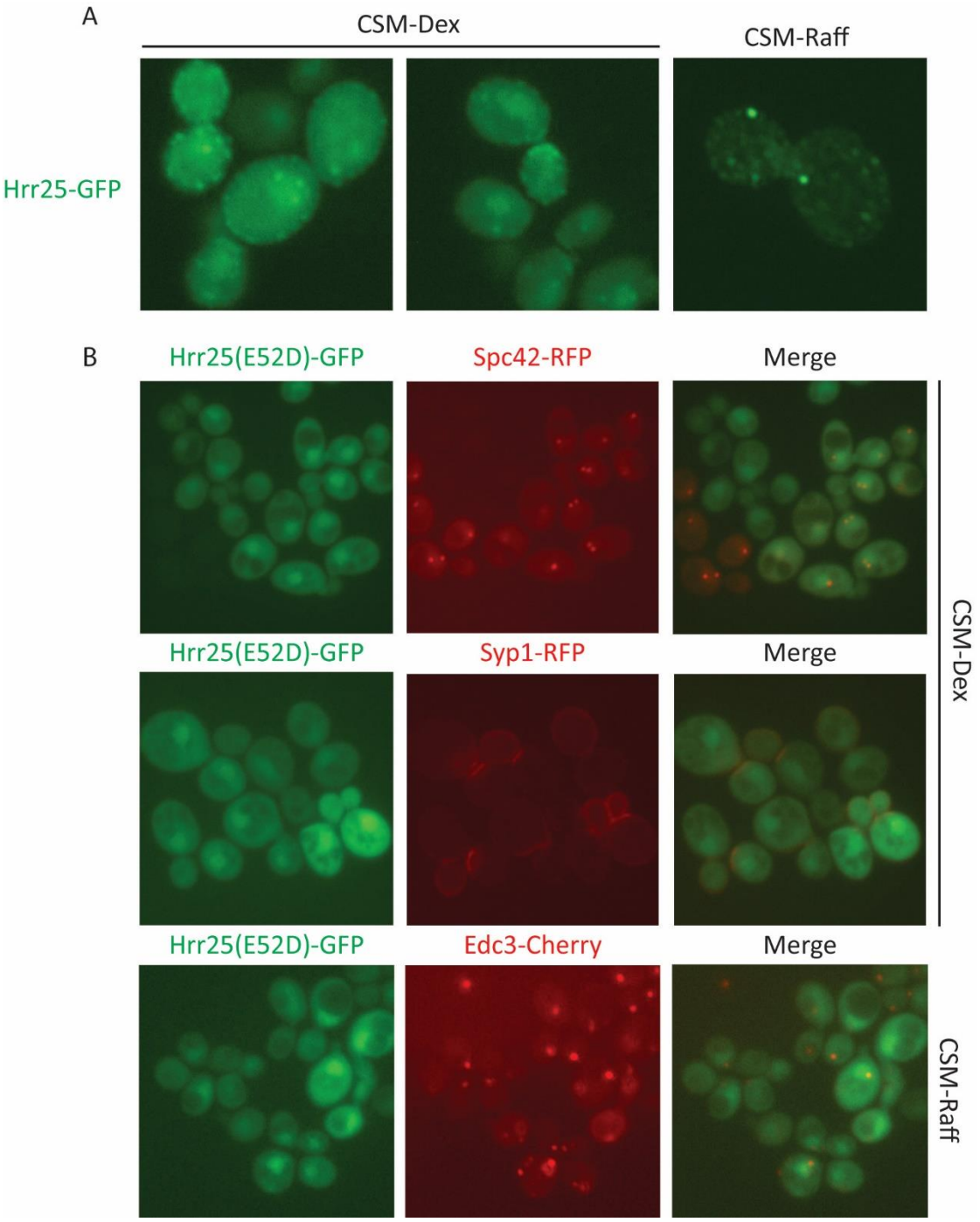

Figure 5. A fluorescence microscopy analysis of the cellular localization of GFP-tagged wild type Hrr25 and GFP-tagged Hrr25(E52D). Cells were grown in CSM-dextrose or CSM-raffinose. Colocalization analysis of mRFP1-tagged SPB marker Spc42, mCherry-tagged P-body marker Edc3, mRFP-tagged endocytic site marker Syp1, with GFP-tagged Hrr25(E52D). Cells co-expressing mRFP1-tagged Syp1 and Spc42 were grown in CSM-dextrose medium before pictures were taken. Cells co-expressing Edc3-mCherry were grown CSM-raffinose medium and subjected to water treatment before images were captured.

In this thesis, we focus on the analysis of the cellular localization of GFP-tagged Hrr25, Hrr25(ΔM), Hrr25(ΔC), and Hrr25(N) by examining their cellular localization together with mRFP1-tagged Spc42, a spindle pole body protein, or Syp1, an endocytic site marker, or mCherry-tagged Edc3, a protein localized to the P-bodies (Donaldson and Kilmartin, 1996; Dong et al., 2010; Reider et al., 2009).

The middle region of Hrr25 is required for Hrr25's localization to SPBs

Hrr25 has been shown to regulate spindle pole body positioning via its N-terminal domain and the middle region (Peng et al., 2015b). The group truncated the C-terminal domain of Hrr25 and observed no change in Hrr25 localization to SPBs, whereas simultaneous truncation of the C-terminal domain and the middle region resulted in abolishment of Hrr25 localization to those foci (Peng et al., 2015b). However, the consequence of truncation of the middle region of Hrr25 alone on its SPB localization has not been reported.

To investigate the contribution of individual domains of Hrr25 to its SPB localization, we sought to perform a colocalization fluorescence microscopy analysis of Hrr25 truncation mutants with Spc42, a protein localized to the spindle pole body (Donaldson and Kilmartin, 1996). Accordingly, a centromeric plasmid encoding C-terminal *GFP*-tagged full-length *HRR25, hrr25(ΔM), hrr25(ΔC),* or *hrr25(N)* truncation construct was co-transformed with a plasmid encoding *mRFP1*-tagged *SPC42* into *hrr25Δ* yeast cells. Cells were cultured in liquid CSM medium with 2% raffinose as the sole carbon source to mid-logarithmic phase and observed under the Nikon Eclipse E800 microscope. Images of cells were taken with CoolSNAP Photometrics DYNO camera using both red and green channels. The corresponding GFP and RFP pictures were processed and merged in ImageJ.

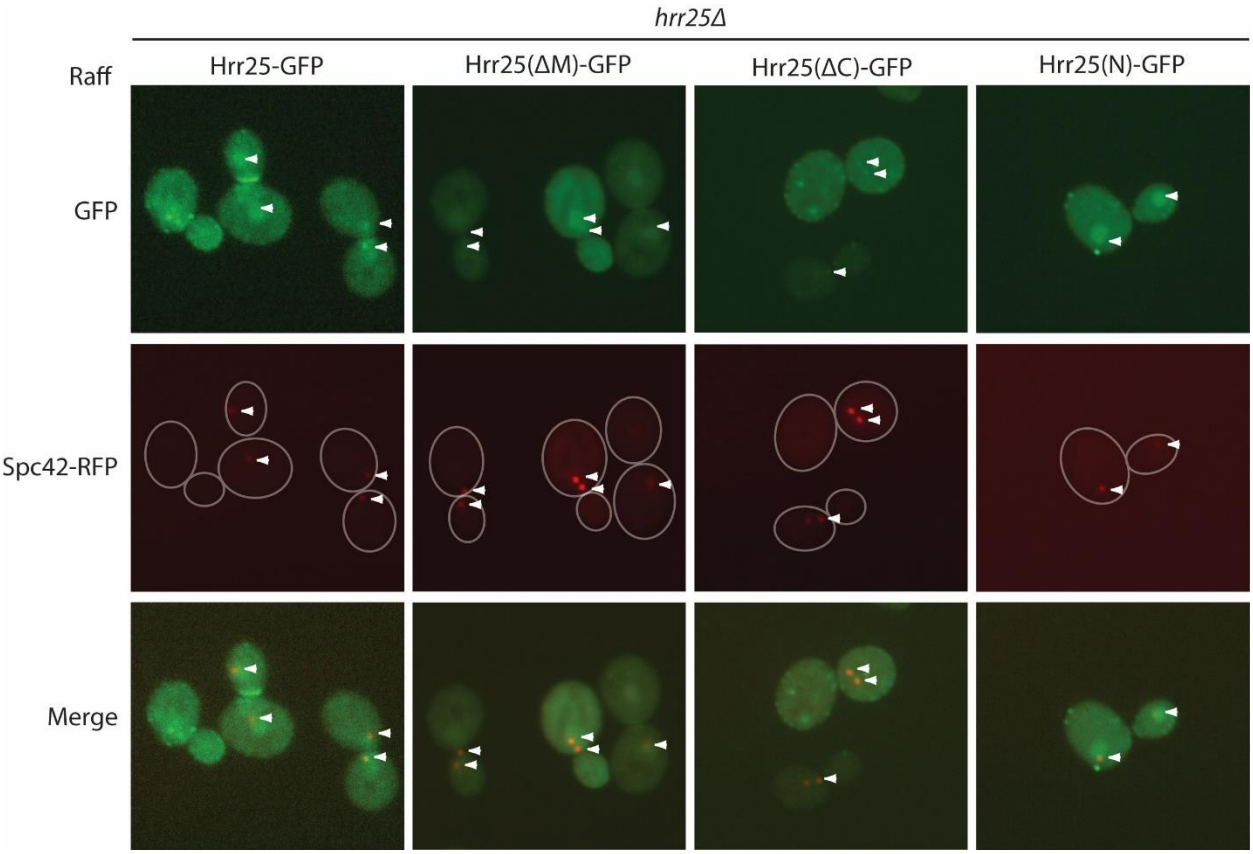

Figure 6. Colocalization analysis of mRFP1-tagged SPB marker Spc42 with GFP-tagged Hrr25 constructs using fluorescence microscopy.

The dotted foci formed by full-length Hrr25 and the C-terminal truncation mutant Hrr25(ΔC) colocalized with the SPB marker Spc42, indicating that the C-terminus of Hrr25 is disposable for Hrr25's SPB localization (Fig. 6). The two mutants lacking the middle region, Hrr25(ΔM) and Hrr25(N), had very few punctate structures that colocalized with Spc42. Consistent with findings of Peng *et al.*, these data indicate that the middle region of Hrr25 plays a major role in the localization of Hrr25 to SPBs (Peng et al., 2015b).

To quantify the fluorescence microscopy data, we calculated the frequency of the colocalization of Spc42 with the four Hrr25 constructs tested. Only cells that simultaneously expressed RFP and GFP were selected for the analysis.

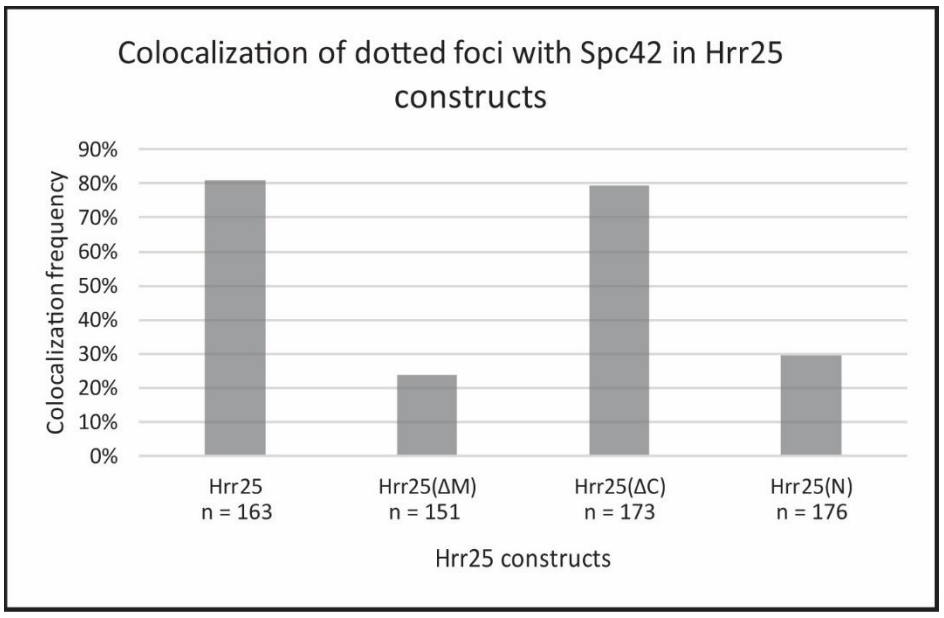

Figure 7. Quantitative colocalization analysis of Spc42 with dotted intracellular foci formed by full-length Hrr25 and the truncation mutants, Hrr25(ΔC), Hrr25(ΔM), and Hrr25(N). The X axis: Hrr25 constructs analyzed; the Y axis: the colocalization percentage of Hrr25 with Spc42. The total number of cells (n) analyzed for each Hrr25 construct is indicated under the bars.

As seen in Figure 7, the dotted cellular structures, found in Hrr25 and Hrr25(ΔC), colocalized with Spc42-GFP in 81% and 79% of the cases, respectively. This supported the conclusion that the C-terminal region of Hrr25 is not required for the localization of Hrr25 to SPBs. The deletion of the middle region reduced the colocalization frequency by 57%, bringing it down from 81% in Hrr25 to 24% observed in Hrr25(ΔM). The additional truncation of the C-terminus in Hrr25(N) did not further reduce the colocalization frequency. These results highlight the importance of the middle region of Hrr25 for its localization of to SPB.

The middle region of Hrr25 is required for Hrr25's localization to endocytic sites

Hrr25 is required for clathrin-mediated endocytosis by phosphorylating Ede1 (Peng et al., 2015a). The kinase activity of Hrr25 is thus important for this process. Indeed, cells carrying an *hrr25(K38A)* mutation formed much fewer endocyclic sites compared to wild type (data not shown) (Peng et al., 2015a). Although the importance of kinase activity for the localization of

Hrr25 to endocytic sites has been established, the contribution of individual domains of Hrr25 to the initiation of endocytosis has not been thoroughly assessed.

To evaluate the role of the middle and C-terminal regions of Hrr25 in Hrr25's targeting to endocytic sites, a fluorescence colocalization microscopy assay was performed. Yeast strains carrying an *hrr25Δ* mutation were co-transformed with a plasmid encoding GFP-tagged full-length *HRR25* or its truncation mutants and a plasmid encoding an mRFP1-tagged endocytic site marker Syp1 (Reider et al., 2009). Cells were grown overnight in CSM medium with raffinose as the sole carbon source and fluorescence microscopy analysis was conducted.

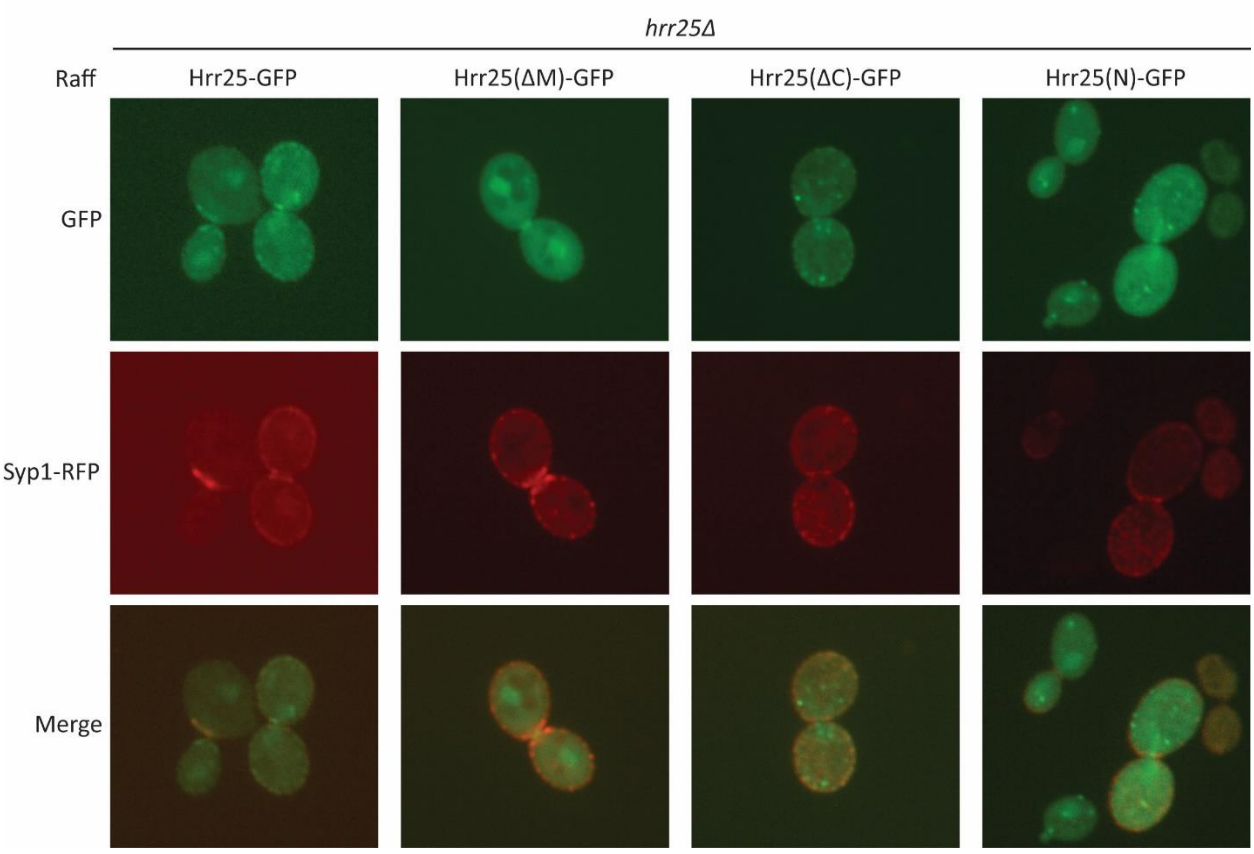

Figure 8. Colocalization analysis of GFP-tagged full-length Hrr25, Hrr25(ΔM), Hrr25(ΔC) or Hrr25(N) with mRFP1-tagged endocytic site marker Syp1.

Figure 8 shows that full-length Hrr25 and Hrr25(ΔC) colocalized with Syp1. In contrast, Hrr25(ΔM) did not colocalize with Syp1 and thus was not recruited to endocytosis sites.

Hrr25(N), lacking both the middle and the C-terminal regions, failed to colocalize with Syp1. The nature of the prominent punctate structures on the cell periphery formed by Hrr25(N)-GFP was not known. Together, our data indicate that the middle region of Hrr25 is required for its targeting to endocytic sites.

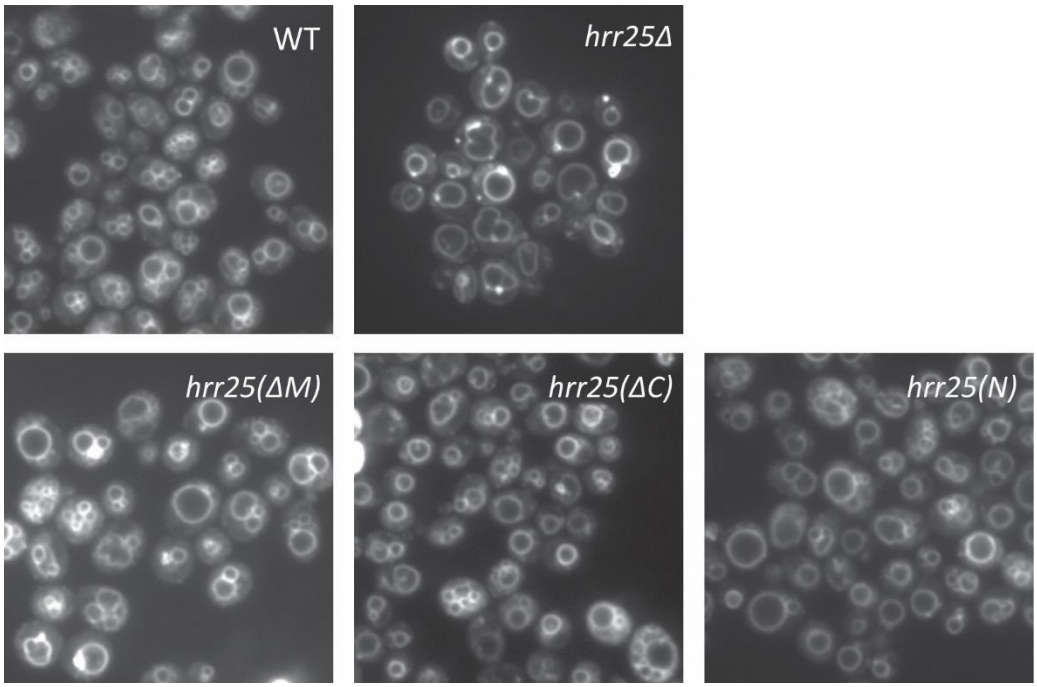

Figure 9. Fluorescence microscopy analysis of endocytic uptake of the lipophilic dye FM4-64 in wild type, *hrr25(ΔM), hrr25(ΔC), hrr25(N),* and *hrr25Δ* mutant cells. Wild-type and mutant cells were grown in YPD medium for at least six generations, treated with FM4-64 as described in materials and methods and subjected to fluorescence microscopy analysis.

To determine the significance of Hrr25's localization to endocytic sites on clathrin mediated endocytosis, we next treated the cells with a fluorescent lipophilic styryl dye FM4-64 to visualize its uptake and transport to the vacuole in wild type, *hrr25Δ* and *hrr25* truncation mutants (Vida and Emr, 1995). As seen in Figure 9, *hrr25Δ* failed to internalize FM4-64 efficiently, which was indicated by faint fluorescence at the cell periphery. All three *hrr25* truncation mutants showed normal uptake of FM4-64, suggesting that Hrr25's localization to endocytic sites is not essential for endocytosis. Despite the lack of a qualitative defect in

endocytosis, *hrr25(N)* and *hrr25(ΔM)* mutants, but not the *hrr25(ΔC)* mutant, had a significant increase in the percentage of cells that had a large vacuole, which was also observed in *hrr25Δ* mutant cells. It is still possible that *hrr25(N)* and *hrr25(ΔM)* mutations may lead to reduced endocytosis quantitatively. We didn't pursue the possibility due to the lack of qualitative defects in these two mutants.

Hrr25(NLSm) does not localize to the spindle pole bodies

The N-terminal region of Hrr25 possesses a cluster of positively charged residues (Fig. 2). We hypothesized that those amino acid residues might compose a nuclear localization signal (NLS), which directs Hrr25 to the nucleus. To test this hypothesis, we generated an *hrr25(NLSm)* mutant (Fig. 2), carrying the R4Q, R7E, K8N, R10H, R13Q, and K14R mutations, and performed a fluorescence microscopy analysis of its cellular localization. When grown in dextrose medium, GFP-tagged Hrr25(NLSm) was able to localize to the nucleus correctly (data not shown), suggesting that this cluster of positively charged residues are not necessary for Hrr25's localization to the nucleus.

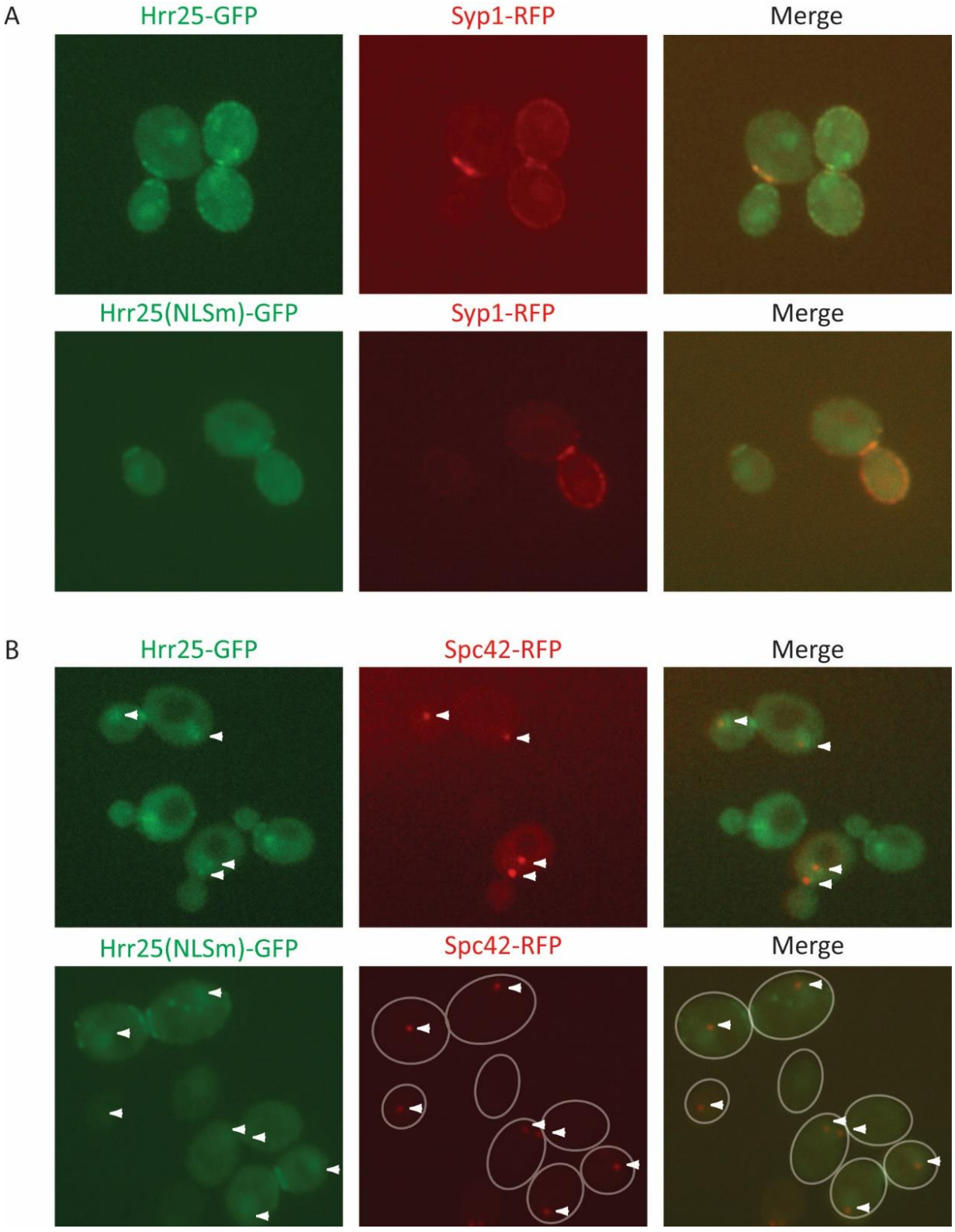

Figure 10. Colocalization analysis of GFP-tagged Hrr25(NLSm) with RFP-fused endocytosis site reporter Syp1 and SPB reporter Spc42.

We next determined whether Hrr25(NLSm) affects the localization of Hrr25 to other cellular compartments. As seen in Figure 10, panel A, Both full-length Hrr25 and Hrr25(NLSm) colocalized with Syp1. Surprisingly, internal dotted foci from cells expressing GFP-tagged Hrr25(NLSm) did not colocalize with the SPB marker Spc42, suggesting that the extreme N-terminal region of Hrr25 is required for its localization to the spindle pole bodies (Fig. 10, panel B).

The middle region of Hrr25 is required for its localization to P-bodies under water stress conditions

Hrr25 is known to localize to P-bodies under a number of stress conditions to participate in mRNA turnover (Zhang et al., 2016). Both the kinase domain and kinase activity of Hrr25 are required for its localization to P-bodies. The middle region has been suggested to be required for P-body localization. However, this possibility has not been directly tested. Accordingly, we analyzed the cellular localization of mCherry-tagged Edc3, a protein localized to P-bodies, in cells co-expressing GFP-tagged Hrr25(ΔM), as well as with full-length Hrr25, Hrr25(ΔM), and Hrr25(N).

Cells were grown overnight in medium with 5% dextrose or 2% raffinose as the sole carbon source and fluorescence microscopy was conducted when culture OD_{600} reached 0.75-0.85. Cells were additionally treated with water for 2 hours to induce P-body formation as described and observed under the microscope.

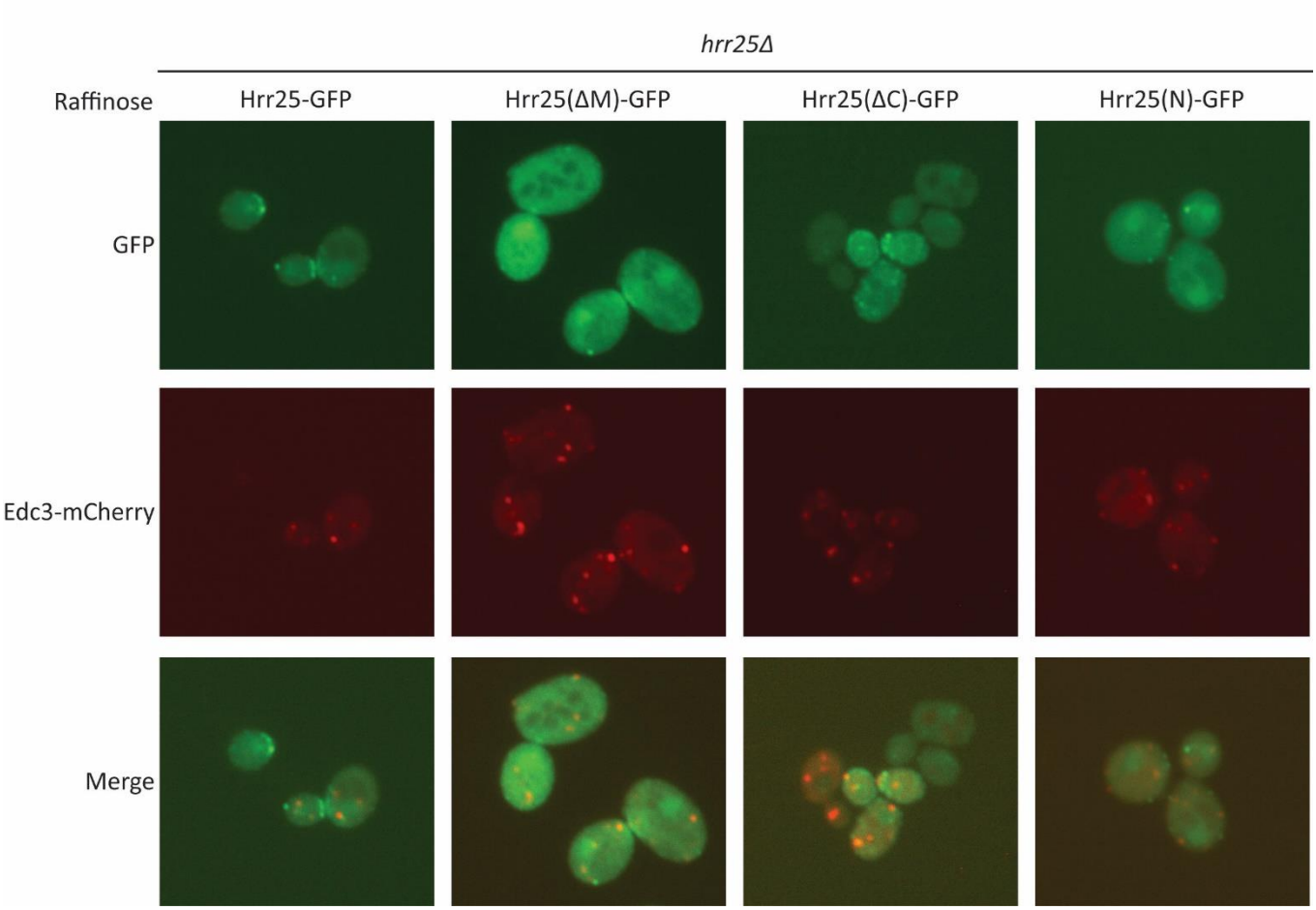

Figure 11. Colocalization analysis of GFP-tagged full-length Hrr25, Hrr25(ΔM), Hrr25(ΔC) and Hrr25(N) with mCherry-tagged P-body marker Edc3 in cells grown raffinose medium.

Little to no P bodies were formed in dextrose-grown cells and the expression of P-body marker Edc3-mCherry was low (data not shown). In contrast, compared to dextrose-grown cells, cells grown in raffinose medium showed increased Edc3 expression and exhibited bright cellular foci, suggesting that raffinose as the sole carbon source constitutes enough stress for cells to form P-bodies. However, all of the four GFP-tagged Hrr25 constructs show little co-localization with Edc3-mCherry (Fig. 11). In cells grown in raffinose medium, followed by water stress, full-length Hrr25 and Hrr25(ΔC), formed dotted foci that colocalized with Edc3 (Fig. 12). In contrast, under the same stress condition, Hrr25(ΔM) and hrr25(N) failed to co-localize with Edc3, indicating that the middle region of Hrr25 is required for its localization of Hrr25 to P-bodies.

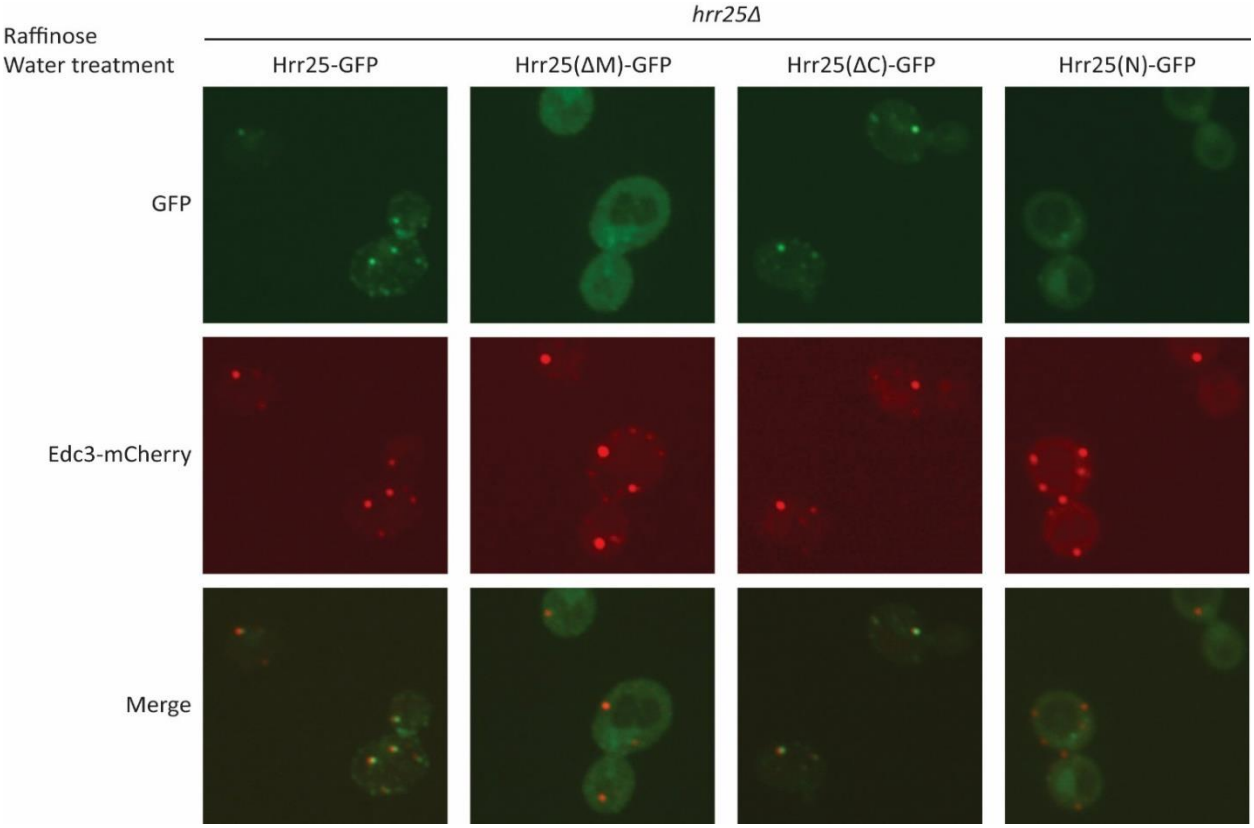

Figure 12. Colocalization analysis of GFP-tagged full-length Hrr25, Hrr25(ΔM), Hrr25(ΔC) and Hrr25(N) with mCherry-tagged P-body marker Edc3 in cells grown raffinose medium followed by water treatment.

Consistent with findings of Zhang *et al.* (Zhang et al., 2016), we found that both Hrr25(K38A) and Hrr25(PLSm), generated in our lab, failed to colocalize with Edc3 (data not shown). We also found that Hrr25(PLSm) colocalized with Spc42 and Syp1 (data not shown).

Discussion

In this study, we have identified the domains of Hrr25 that contribute to its localization to endocytic sites, P-bodies, SPBs, and are important for normal cell morphology and growth. The combined results of chapter II are shown in Table 1.

Feature	Domains of Hrr25					
	The N-terminus			The middle region		The C-terminus
	Full length	Kinase activity	Charged amino acids at the extreme end	Full length	P-body localization signal 1	Full length
Cell growth	+++	+++	No	++/+	-	+/-
Cell morphology	+++	+++	No	No	No	No
Localization to endocytic sites	Yes	Yes	No	Yes	No	No
Localization to P-bodies	Yes	Yes	No	Yes	Yes	No
Localization to spindle pole bodies	Yes	Yes	Yes	Yes	No	No

Table 1. A color-coded diagrammatic representation of Hrr25 domain functions. A Yes/No indicate whether the domain is required for the Hrr25 function. Color key: yellow represents functions discovered in this study, blue represents functions identified by a different group and that were confirmed in this study, pink indicates that the results obtained in this study are different from previous findings.

Here we show that the kinase activity provided by the N-terminus of Hrr25 is required and sufficient to support cell growth and to confer normal cell morphology. Similarly to cells carrying a *HRR25* deletion mutation, cells expressing either Hrr25(K38A) or any of Hrr25 truncations lacking the N-terminus (Hrr25(M), Hrr25(C), Hrr25(M+C)), were extremely slow growing, were elongated and formed unusual grape-like clusters. Conversely, cells expressing Hrr25(N) showed increased fitness compared to *hrr25Δ* and Hrr25(K38A) and had no morphological defects. The Hrr25(N) mutants grew worse than Hrr25(ΔM) or Hrr25(ΔC) mutants, thus displaying a synergistic effect of the C-terminal region and the middle region truncations on cell growth rates.

Hrr25 localizes to cytoplasm, nucleus, bud neck, endocytic sites, P-bodies, and spindle pole bodies (Brockman et al., 1992; Lusk et al., 2007; Peng et al., 2015a; Zhang et al., 2016).

Truncation mutants Hrr25(M), Hrr25(C), Hrr25(M+C) localized diffusely to the cytoplasm, indicating that the middle region and/or the C-terminal region are not sufficient to drive Hrr25 to distinct cellular sites. Consistent with published literature, we showed that kinase activity of Hrr25 is crucial its subcellular localizations. We found that Hrr25(E52D) mutant did not localize to dotted cellular foci, but was able to localize to the nucleus, suggesting that nuclear localization of Hrr25 does not require its kinase activity. In search for the nuclear localization signal (NLS) of Hrr25, we created a Hrr25(NLSm) construct by introducing point mutations to charged amino acid residues at 4-14. Interestingly, we found that Hrr25(NLSm) localized to the nucleus, but no longer localized to SPBs. To our knowledge the N-terminal charged residues of Hrr25 have not been reported to have a function in mitosis. In meiosis however, these residues have been found to play a prominent role in binding Mam1, a monopolin subunit, by forming a hydrogen bond network that largely stabilized Hrr25:Mam1 complex (Corbett and Harrison, 2012). Perhaps the Hrr25 target in the mitotic γTuSC-mediated microtubule nucleation or the Kar9 spindle-positioning pathway binds Hrr25 through a similar mechanism (Peng et al., 2015b).

A recent study identified an amino acid stretch within the middle region of human CK1δ that is required for its centrosomal anchoring (Greer et al., 2014). Another study in *S. cerevisiae* showed that a Hrr25 truncation mutant, that only possessed a kinase domain, failed to localize to SPB (Peng et al., 2015b). Here we show that deletion of the middle region greatly reduced Hrr25 localization to SPBs, but the truncation of the P/Q rich region did not. Thus, Hrr25 requires the presence of the kinase activity, charged amino acids at positions 4-14, and the middle region to successfully localize to SPBs.

We show that Hrr25 localization to stress-induced P-bodies requires the presence of both, the middle region and the kinase domain. These results correlate with published literature, where

the group identified loci within the middle region (PLS1) that were required for Hrr25 localization to P-bodies in water stress conditions (Zhang et al., 2016). The group also reported that Hrr25(PLSm) mutant, where Arg294 and Lys297 were substituted for Ala, was unable to localize to SPBs. The Hrr25(PLSm) mutant generated in our lab did not localize to P-bodies but localized to endocytic sites and SPBs. These differenced could be due to utilization of yeast strains from different backgrounds.

In this thesis we discovered that the middle region of Hrr25 is required for its localization to endocytic sites. Two lines of evidence support that conclusion. First, GFP-tagged Hrr25 truncation mutants lacking the middle region (Hrr25(ΔM) and Hrr25(N)) failed to colocalize with an RFP-tagged endocytic marker Syp1. Second, cells expressing Hrr25(ΔM) and Hrr25(N) were defective in internalization of the fluorescent dye FM4-64. Thus, we have identified a novel function of the middle region of Hrr25.

Chapter III: Hrr25-dependent Pin4 Phosphorylation Is Implicated in Cell Wall Integrity Maintenance

Materials and methods

Growth Media and Growth Conditions

Yeast cells were grown at 30°C in YPD (2% peptone, 1% Bacto-yeast extract, and 2% D-glucose), YNBcasD medium (0.67% yeast nitrogen base, 1% casamino acids, and 2% dextrose), YNBcas5D medium (0.67% yeast nitrogen base, 1% casamino acids, and 2% dextrose) , YNBcasR medium (0.67% yeast nitrogen base, 1% casamino acids, and 2% dextrose), and complete supplement mixture (CSM) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.6 g/liter CSM minus histidine, leucine, and tryptophan). The amino acids L-leucine, L-lysine, L-histidine, L-methionine, L-tryptophan, and/or uracil were added to selective growth media to meet auxotrophic requirements (Amberg, 2005). If indicated, 1M sorbitol was added to YNBcasD liquid medium. For yeast two-hybrid analysis involving the *HIS3* reporter gene under the control of Gal4, 3-amino-1,2,4-triazole was added to the CSM medium. If stated in text, YPD medium with 5mM and 10mM caffeine, YPD medium with 0.002% SDS, and YPD medium with 1M sorbitol were used for serial dilution analysis. Solid media contained 2% agar in addition to the components described above.

Yeast Transformations

Plasmids were transformed into yeast strains using the high efficiency lithium acetate-PEG method as described (Amberg, 2005).

Cell extract preparation and immunoblotting

Total cellular protein extracts were prepared by lysing yeast cells in extraction buffer (1.85N NaOH – 7.5% β-mercaptoethanol) followed by precipitation with trichloroacetic acid

(TCA). TCA was neutralized with unbuffered Tris and total cellular proteins were resuspended in 1X SDS-PAGE loading buffer. Protein samples were boiled for 3 min. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane for immunoblotting.

Myc- and HA- tagged proteins were probed with anti-myc primary antibody and anti-HA primary antibody (9E10 and 3F10, Roche Applied Science), respectively. Primary antibodies were detected by HRP-conjugated goat anti-mouse secondary antibody or HRP-conjugated goat anti-rat secondary antibody. Chemiluminescence was induced by an ECL reagent. Chemiluminescence images of Western blots were captured using the Bio-Rad Chemi-Doc photo documentation system (Bio-Rad Laboratories, Hercules, CA). For Pgk1 (3-phosphoglycerate kinase) loading control images, nitrocellulose membrane blots were deprobed first in stripping buffer (2% SDS, 0.1M β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.7) for 45 mins at 70°C with shaking and then reprobed with anti-Pgk1 antibody. Alternatively, the same protein samples were loaded onto a separate SDS-PAGE gel and immunoblotting was conducted using anti-Pgk1 antibody, followed by HRP-conjugated goat anti-rabbit secondary antibody.

Phosphatase treatment

TCA-precipitated total cellular proteins were neutralized with unbuffered Tris and incubated with 400 U of λ protein phosphatase (PPase) (New England BioLabs, Inc.) in a final volume of 20 µl at 30°C for 90 min. When indicated, phosphatase inhibitors (1 µM sodium orthovanadate, 10 mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 10 mM NaF) were added to inhibit λ PPase. Treated samples were resuspended in 1 × SDS-PAGE loading buffer with 100 mM dithiothreitol (DTT). Protein samples were boiled for 3 min before being separated by SDS-PAGE and subjected to Western Blot.

Cycloheximide Chase Assay

Cells expressing indicated HA-tagged proteins were grown in liquid YNBcasD medium to OD_{600} 0.7 – 0.8. Protein synthesis was inhibited by addition of 50 µg/mL cycloheximide. Every 10 minutes, a 1 mL sample of the cell culture was withdrawn and immediately subjected to cell extract preparation as described. HA-tagged proteins were detected by Western blotting.

Immunoprecipitation

Total cellular extracts were prepared in IP buffer (50 mM Tris-HCl, pH 7.6, 80 mM NaCl, 0.1% Triton X-100, and protease inhibitors). Cell extracts (~5mg of proteins) were incubated at 4 °C for 1 h with 20 µl of monoclonal anti-myc antibody (9E10, Roche Applied Science), after which, 30 µl of a 50% slurry of protein G-Sepharose (Roche Applied Science) was added to each sample and the samples were further incubated at 4 °C for 2 h. Immunoprecipitates bound to the Sepharose beads were released by boiling in 1 × SDS-PAGE loading buffer after being washed three times with 1 ml of IP buffer. Proteins in the immunoprecipitates were analyzed by Western blotting.

Caffeine treatment

Caffeine was dissolved in warm water to obtain a 100mM stock solution. Cell cultures were grown overnight in YNBcas5D medium to reach OD_{600} ~ 0.4, and each cell culture was divided into three equal parts. Caffeine stock solution was added to respective culture tubes to reach final concentration of 5mM and 10mM, and water was added to the control tubes. Cultures were further incubated to reach OD_{600} 0.7 – 0.8. 1ml of culture sample was withdrawn for cellular lysate extraction and Pin-HA in the samples were detected by Western blotting.

Methyl methanesulfonate (MMS) treatment

Wild type and mutant yeast strains expressing HA-tagged Pin4 were grown overnight in YNBcas5D medium and diluted in the morning to OD_{600} ~0.5. Diluted cultures were incubated at 30ºC for 30 minutes. After incubation, aliquots of cell cultures were transferred into fresh tubes and incubated in a 30ºC water bath in the safety hood for MMS treatment. MMS was added to the cultures at the final concentration of 0.1%. Treated cultures were vortexed 4 times for 30 seconds after initial MMS addition, and vortexed for 30 seconds every 15 minutes afterwards. 1ml culture samples were withdrawn after 90 minutes and 120 minutes of MMS treatment and subjected to cellular lysate extraction. Pin4-HA was detected by immunoblotting.

Yeast two-hybrid analysis

The yeast two-hybrid strains AH109 and Y187 (Clontech Laboratories, Inc.) were transformed with bacterial plasmids indicated in text, and transformants were selected on SD medium without leucine and tryptophan. Transformants were streaked on CSM plate supplemented with adenine and histidine for the analysis of an *ADE2* reporter gene. CSM medium without tryptophan and histidine was used for the analysis of a *HIS3* reporter gene under the control of a *GAL1* promoter. 3-Amino-1,2,4-triazole (3-AT), an inhibitor of His3, was added to CSM medium when required.

Mating and Tetrad Dissection

(See Chapter 2)

Fluorescence microscopy analysis

(See Chapter 2)

Bioinformatic analysis of protein interaction networks

Interaction networks of Hrr25 and Mps1 were exported from the BioGrid public database and merged for analysis in CytoScape software (Oughtred et al., 2019; Shannon et al., 2003).

Results

Genes that form genetic and/or physical interactions with Mps1 and Hrr25

MPS1 is an essential gene encoding a dual-specificity kinase. Mps1 has been shown to be involved in SPB duplication and spindle mitotic function, sister-kinetochore bi-orientation in mitosis, among others (Aravamudhan et al., 2016; Luca and Winey, 1998; Straight et al., 2000; Weiss and Winey, 1996; Winey et al., 1991). Like Hrr25 during mitosis, Mps1 localizes to the bud neck (Huh et al., 2003). Given their similar SPB functions and bud neck localization, we hypothesized that Hrr25 and Mps1 might work in the same genetic network. We thus created a fused Mps1–Hrr25 interaction network using the CytoScape software to look for novel Hrr25 targets. There are 114 genes shared by Hrr25 and Mps1 in this network (data not shown). Four genes were chosen for further analysis based on their subcellular localization and functions: *CDC12, MOB1, DSN1, and PIN4*. Cdc12 is a bud neck protein and is a septin ring component (Chang et al., 1997; Huh et al., 2003). Pin4 is a poorly studied protein involved in DNA damage response and cell wall integrity (Pike et al., 2004; Traven et al., 2010). Neither Cdc12 nor Pin4 have been previously reported to interact with Mps1 or Hrr25. Mob1 is a known Mps1 target and localizes to SPBs and cytoplasm (Huh et al., 2003; Luca and Winey, 1998). As a component of the mitotic exit network (MEN), it is required for cytokinesis and cell separation (Luca and Winey, 1998; Meitinger et al., 2011). Dsn1 is essential for both meiotic and mitotic chromosome segregation (Euskirchen, 2002; Ye et al., 2016)and has been reported to be phosphorylated by Hrr25 during meiosis (Ye et al., 2016).

A *hrr25Δ* does not alter the cellular localization of Pin4, Cdc12, Dsn1, and Mob1

To test whether the subcellular localization of GFP-tagged Pin4, Cdc12, Dsn1, and Mob1, was mediated by Hrr25, we sought to perform a fluorescence microscopy assay of these proteins expressed in *hrr25Δ* mutant strain. Since deletion mutant cells are extremely slow growing, we introduced *ltv1Δ* to partially suppress the growth defect of *hrr25Δ* mutant cells. Accordingly, *ltv1Δ* and *hrr25Δ ltv1Δ* mutant cells were transformed with a centromeric plasmid encoding GFP-tagged *PIN4*, *CDC12*, *DSN1*, or *MOB1*. Transformants were grown overnight in YNBcas5D medium and the intracellular localization of GFP tagged proteins was analyzed by fluorescence microscopy.

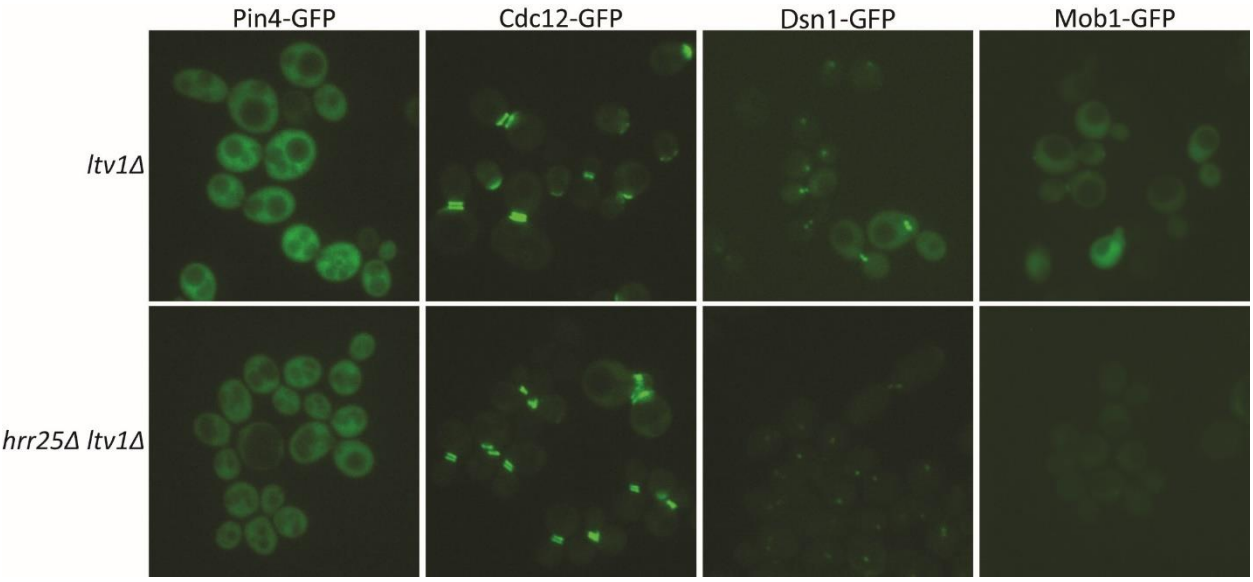

Figure 13 Analysis of intracellular localization of GFP-tagged Pin4, Cdc12, Dsn1, and Mob1 in *ltv1Δ* and *hrr25Δ ltv1Δ* mutant cells using fluorescence microscopy. Cells were grown in YNBcas5D medium to mid-logarithmic phase before cells were concentrated for fluorescence microscopy.

Figure 13 shows that *hrr25Δ* did not affect the intracellular localization of Pin4, a cytoplasmic protein, Cdc12, a bud-neck protein, Dsn1, a kinetochore protein, and Mob1, a protein localized both in the cytoplasm and at the spindle pole bodies. The GFP fluorescence signal was reduced in *hrr25Δ ltv1Δ* mutant cells compared to *ltv1Δ* mutant cells, especially for

GFP-tagged Dsn1 and Mob1. Altogether, our data indicate that *hrr25Δ* does not grossly alter the intracellular localization of Pin4, Cdc12, Dsn1, and Mob1.

Pin4 and Mob1 are potential Hrr25 targets

According to the ISB PhosphoPep database and the *Saccharomyces* Genome Database, Dsn1, Cdc12, Pin4, and Mob1 are phosphoproteins, suggesting that they are subject to post-translational modifications by protein kinases and possibly, phosphatases (Bodenmiller et al., 2008). Despite our failure to detect a change in their intracellular localization due to *hrr25Δ*, we determined whether the phosphorylation of these four proteins might be affected by *hrr25Δ* by using Western blotting. To facilitate the detection of protein mobility shifts on SDS-PAGE, we constructed plasmids expressing C-terminal 3xHA-tagged Dsn1, Cdc12, Pin4, and Mob1 under the control of their respective endogenous promoter and transformed them into *ltv1Δ* and *hrr25Δ ltv1Δ* strains. Transformants were grown overnight in YNBcas5D medium to reach OD_{600} 0.7-0.85. Cell extracts were prepared, proteins were separated by SDS-PAGE, and HA-tagged proteins were detected by Western blotting.

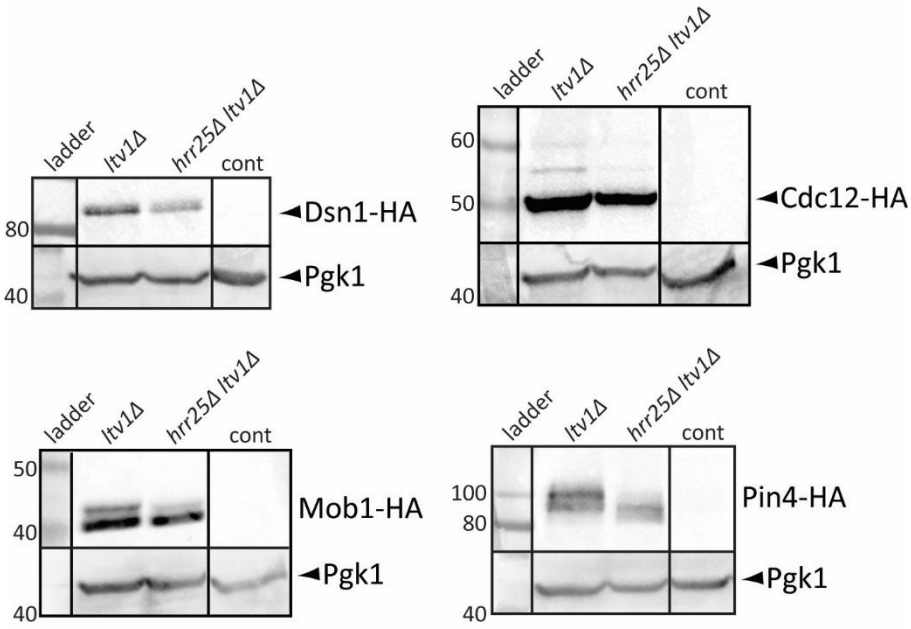

Figure 14. Western Blot analysis of HA-tagged Dsn1, Cdc12, Pin4 and Mob1 in *ltv1Δ* and *hrr25Δ ltv1Δ* cells grown in YNBcas5D medium.

As seen in Figure 14, *hrr25Δ* caused a decrease in the protein amount of all constructs analyzed, consistent with reduced GFP fluorescence from GFP-tagged proteins in *hrr25Δ* cells in Figure 13. Moreover, there was a clear mobility shift for Pin4-HA in *hrr25Δ ltv1Δ* cells compared to the *ltv1Δ* control. A faster mobility on SDS-PAGE is often an indication of protein dephosphorylation, suggesting that *hrr25Δ* results in reduced Pin4 phosphorylation. Figure 14 also shows a slight increase in the mobility of one of the two forms of Mob1-HA on SDS-PAGE from *hrr25Δ ltv1Δ* mutant cells compared to *ltv1Δ* cells. The mobility of Cdc12 and Dsn1 did not seem to be affected by *hrr25Δ*. Together, our data suggest that Pin4, and to a lesser extent, Mob1 may be new targets of Hrr25.

Pin4 and Mob1 are phosphorylated *in vivo*

HA-tagged Pin4, Dsn1, Cdc12, and Mob1 migrated as more than one species on SDS-PAGE (Fig. 14). To confirm whether they were indeed phosphoproteins, we conducted a phosphatase treatment experiment on these proteins expressed in *ltv1Δ* and *hrr25Δ ltv1Δ* cells.

When treated with lambda protein phosphatase, the broad Pin4 band seen in both *ltv1Δ* and *hrr25Δ ltv1Δ* immunoblots migrated faster and a dominant fast-migrating species appeared (Fig. 15, compare lane 3 to lane 2 and lane 6 to lane 5). However, in the presence of phosphatase inhibitors, the effect was largely blocked (Fig. 15, compare lanes 2, 3, and 4, and lanes 5, 6, and 7), indicating that Pin4 is hyperphosphorylated. Importantly, phosphatase treatment of cell extracts from *ltv1Δ* and *hrr25Δ ltv1Δ* cells generated a faster, presumably unphosphorylated, Pin4 species of the same size, suggesting that the change in Pin4 mobility in *hrr25Δ* is due to reduced phosphorylation. A similar phenotype, albeit much weaker, was observed for Mob1 (Fig. 15). Phosphatase treatment did not lead to an obvious change in the mobility of Cdc12 and Dsn1 on SDS-PAGE, suggesting that these two proteins might be unphosphorylated or hypophosphorylated (Fig. 15). Together, our data suggest that we may have discovered a new target for Hrr25, Pin4.

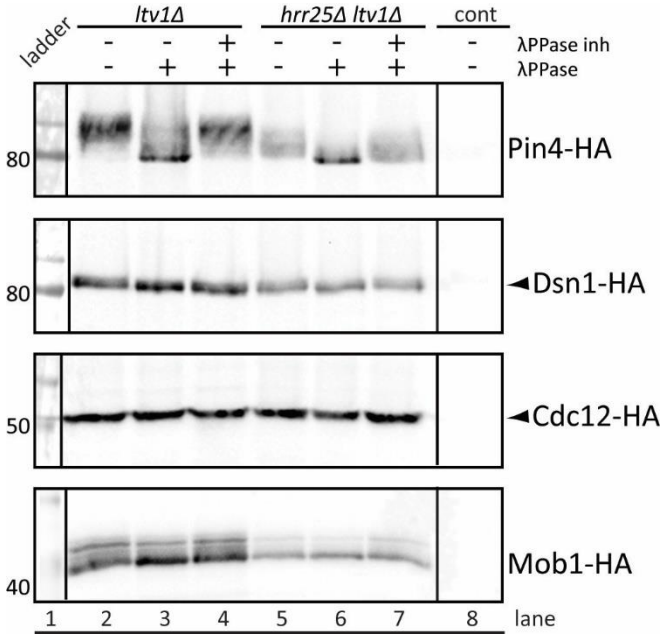

Figure 15. Phosphatase treatment of HA-tagged Pin4, Dsn1, Cdc12, Mob1 in *ltv1Δ* and *hrr25Δ ltv1Δ* cells grown in YNBcas5D. Lambda protein phosphatase treatment was conducted as described in materials and methods. Proteins were separated by SDS page and HA-tagged proteins were detected by Western blotting.

Hrr25 kinase activity is required for Pin4 and Mob1 phosphorylation

The *hrr25Δ* cells are very slow growing even in the presence of *ltv1Δ.* Reduced phosphorylation of Pin4, and to a lesser extent, Mob1, in *hrr25Δ* could be due to an indirect effect associated with slow cell growth. To support our hypothesis that Hrr25 is required for Pin4 and Mob1 phosphorylation, we sought to investigate Pin4 and Mob1 phosphorylation in a yeast strain carrying a partial loss-of-function *hrr25* mutant, *hrr25(E52D),* which causes only a mild growth defect. If Hrr25 were indeed the kinase mediating the phosphorylation of Pin4 and Mob1, we would observe a decrease in their phosphorylation in the *hrr25(E52D)* strain, similar to the effect observed in *hrr25Δ* mutant cells. Accordingly, we transformed a plasmid encoding HA-tagged *PIN4* or *MOB1* into a *hrr25(E52D)* mutant and the corresponding isogenic wild-type strain. Transformants were grown in YNBcas5D, and HA-tagged Pin4 and Mob1 were detected by Western blotting.

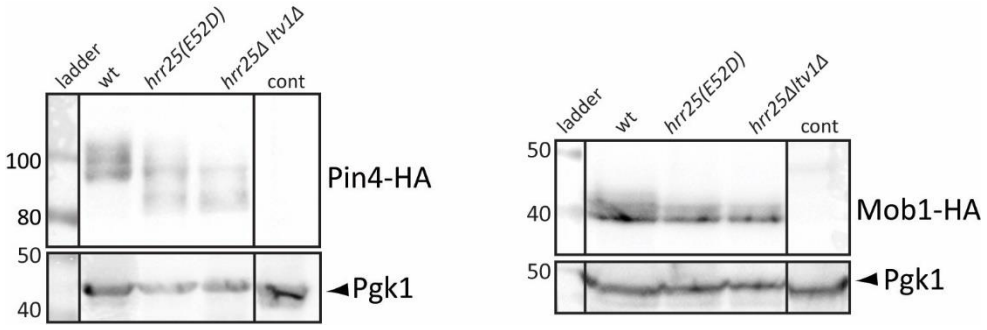

Figure 16. A Western Blot analysis of HA-tagged Pin4 and Mob1 in wild-type BY4741 (wt) and isogenic *hrr25(E52D)* and *hrr25Δ ltv1Δ* mutant cells. Cells were grown in YNBcas5D medium and total cellular proteins were prepared and separated by SDS-PAGE and probed with anti-HA antibody.

Figure 16 shows that the mobility of Pin4 is increased in *hrr25(E52D)* mutant cells compared to wild type. The slight difference in Pin4 migrating pattern on the Western blot between *hrr25(E52D)* and *hrr25Δ ltv1Δ* could be explained by incomplete and total loss of kinase activity in these mutants, respectively. Mob1, on the other hand, exhibited a similar

increase in mobility in *hrr25(E52D)* and *hrr25Δ ltv1Δ* mutant cells. Thus, we conclude that Hrr25 is required for Pin4 and Mob1 phosphorylation.

Pin4 and Mob1 are stable in *HRR25* and in *hrr25Δ*

To test whether Hrr25-dependent phosphorylation of Pin4 and Mob1 affects their stability, we performed a cycloheximide chase analysis of HA-tagged Pin4 and Mob1 in *ltv1Δ* and *hrr25Δ ltv1Δ* strains. Cells were grown in YNBcas5D medium overnight to mid-logarithmic phase. When cell cultures reached OD_{600} 0.7-0.8, cycloheximide was added at 50 μg/mL to inhibit protein synthesis. Samples were collected at 10, 20, and 30 minutes after the start of cycloheximide treatment. Total cellular lysates were prepared, and HA-tagged proteins were detected by Western blotting. The results of the experiment were depicted in Figure 17.

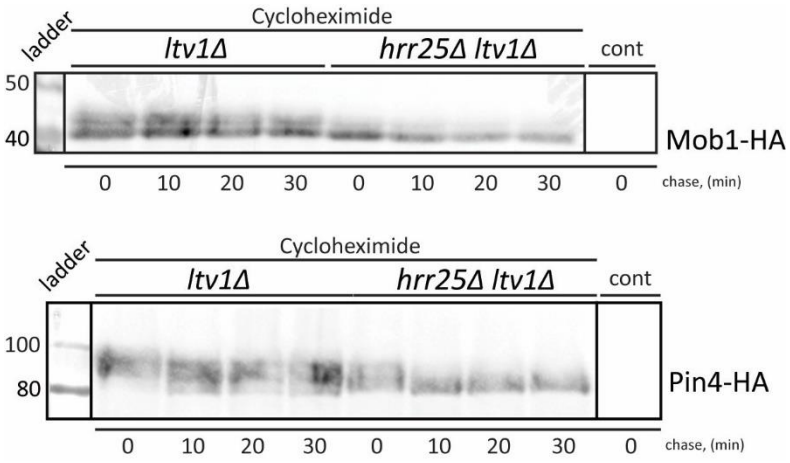

Figure 17. Cycloheximide chase of HA-tagged Pin4 and Mob1 in *ltv1Δ* and *hrr25Δltv1Δ*.

Pin4 phosphorylation was reduced in *hrr25Δ ltv1Δ* cells compared to *ltv1Δ* mutant cells at time point 0 of the cycloheximide chase assay, confirming our earlier findings. Although we failed to detect a change in Pin4 stability in the presence of *hrr25Δ*, a significant decrease in Pin4 phosphorylation was observed in both *ltv1Δ* and *hrr25Δ ltv1Δ* cells at the 10 minute time point. At the 20 and 30 time points after cycloheximide treatment, Pin4 phosphorylation appeared to be stable in both mutants. We also failed to observe a change in Mob1 stability in the presence of

hrr25Δ. In conclusion, Hrr25-dependent phosphorylation of Pin4 and Mob1 does not seem to affect their stability. Unexpectedly, we found that cycloheximide treatment could lead to dephosphorylation of Pin4, suggesting that such a treatment might inhibit another protein kinase(s) and/or activate a phosphoprotein phosphatase(s).

Pin4 physically interacts with Hrr25

A decrease in Pin4 phosphorylation in *hrr25* mutant cells suggests that Hrr25 is required for Pin4 phosphorylation. However, Pin4 regulation by Hrr25 can be direct or indirect. To differentiate these two possibilities, we performed a co-immunoprecipitation analysis of HA-tagged Pin4 and myc-tagged Hrr25 to determine whether they interact directly. Cell extracts from cells expressing Pin4-HA and with or without co-expressing Hrr25-myc were prepared and Hrr25-myc was immunoprecipitated with anti-myc antibody and protein G agarose beads. Protein samples were resolved by SDS-PAGE and Western Blot analyses of HA- and myc-tagged proteins were conducted.

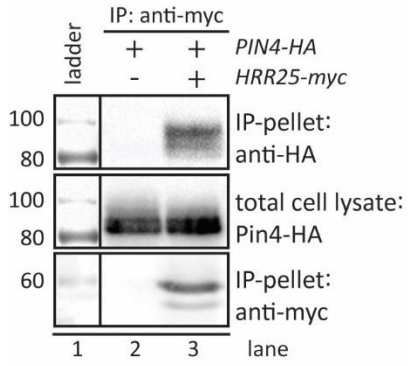

Figure 18. A co-immunoprecipitation analysis of Hrr25-myc with Pin4-HA. A plasmid encoding HA-tagged Pin4 was transformed into a wild type strain and a *hrr25Δ* mutant expressing Hrr25-myc. Transformants were grown in YNBcas5D medium. Cell extracts were prepared and subjected to immunoprecipitation with anti-myc antibody. anti-myc immunoprecipitates were analyzed for HA and myc-tagged proteins using Western blotting. Both the pellets and total cell lysates from the immunoprecipitation experiment were analyzed.

Figure 18 shows that Pin4-HA was recovered in the anti-myc immunoprecipitate in cells coexpressing Hrr25-myc. In cells expressing Pin4-HA but not Hrr25-myc, Pin4-HA was not

recovered. Figure 18 also shows that Hrr25-myc had a preference for hyperphosphorylated Pin4 in comparison to hypophosphorylated Pin4. Together, our data indicate that Pin4 physically interacts with Hrr25 *in vivo*.

We performed a co-immunoprecipitation assay on the interaction between Hrr25-myc and Mob1-HA and failed to detect an interaction between these two proteins. Although the result was negative, we would like to note that it is not uncommon to fail in the detection of an interaction between a protein kinase and its substrate in a co-immunoprecipitation assay (Collins et al., 2017).

The kinase domain and C-terminal region of Hrr25 are required for Hrr25's interaction with Pin4

To demonstrate the physical interaction of Hrr25 and Pin4 using an alternative approach, we conducted a yeast two-hybrid assay to examine their interaction. Since it is sometimes difficult to capture the physical interaction of the active kinase with its substrate, we included the kinase-dead Hrr25(K38A) mutant in the analysis. We thus generated plasmids encoding fusion proteins of the Gal4 DNA binding domain (GBD) and Hrr25 or Hrr25(K38A) and transformed into the yeast two-hybrid strain AH109. We also generated plasmids encoding fusion proteins of the Gal4 transcriptional activation domain (GAD) and Pin4 and introduced them into the yeast two-hybrid strain Y187. AH109 and Y187 transformants were then mated for the detection of an interaction between Hrr25 and Pin4 using the *ADE2* and *HIS3* reporter genes under the control of Gal4-dependent promoters.

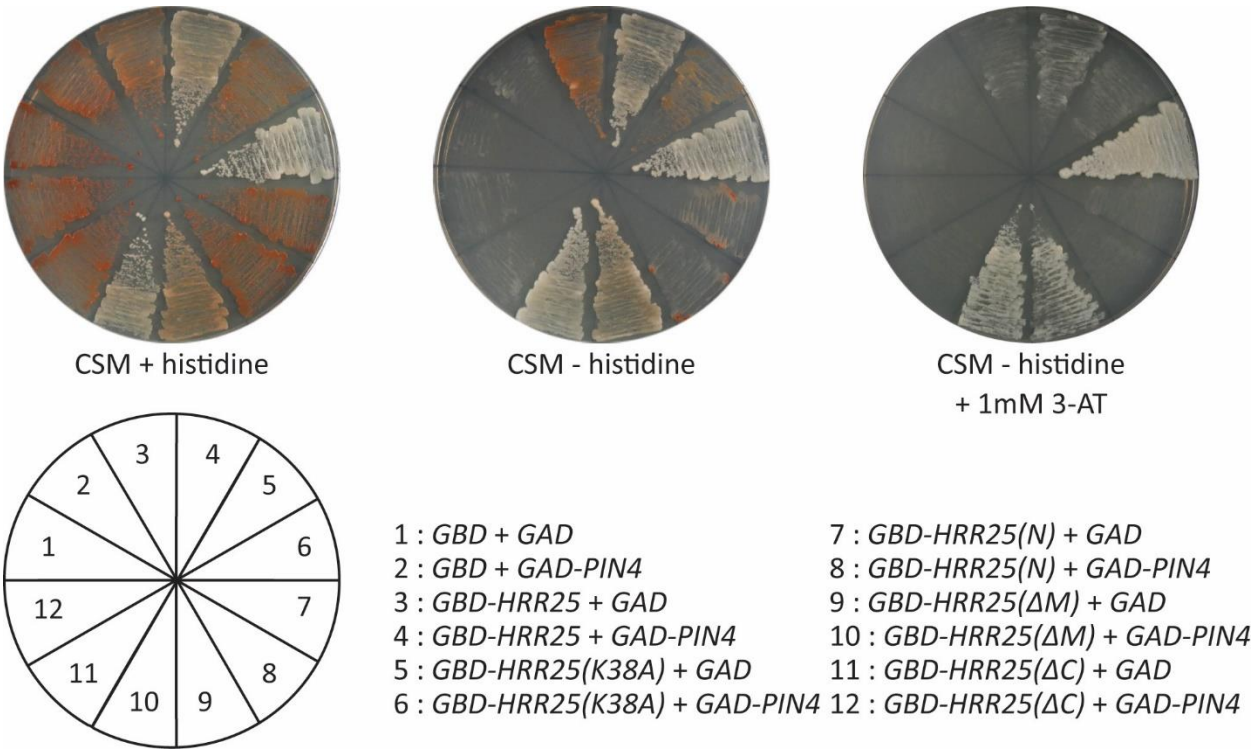

Figure 19. A yeast two-hybrid interaction analysis of Hrr25, Hrr25(K38A), Hrr25(N), Hrr25(ΔM), Hrr25(ΔC) and Pin4. AH109 cells expressing *GBD*, *GBD-HRR25*, *GBD-HRR25(K38A)*, *GBD-HRR25*(N), *GBD-HRR25*(*ΔM*), or *GBD-HRR25*(*ΔC*) were crossed to Y187 cells expressing *GAD* or *GAD-PIN4*. The resulting diploid strains were streaked on solid CSM medium supplemented with adenine and histidine (CSM + histidine), or with adenine only (CSM – histidine), or with adenine and 1 mM 3-amino-1,2,4-triazole (3-AT).

The results from the yeast two-hybrid analysis were presented in Figure 19. Unlike cells coexpressing *GBD* and *GAD* or *GAD-PIN4*, cells coexpressing *GBD-HRR25* or *GBD-HRR25(K38A)* and *GAD-PIN4* appeared whiter on CSM plate supplemented with adenine and histidine. On CSM medium lacking histidine, cells co-expressing *GBD-HRR25* or *GBD-HRR25(K38A)* and *GAD-PIN4* were able to grow, while cells coexpressing *GBD* and *GAD* or *GAD-PIN4* were not. Cells coexpressing *GBD-HRR25* and *GAD* were able to grow on the CSM plate without histidine, indicating that *GBD-HRR25* alone can activate the expression of the *HIS3* reporters. Addition of 3-AT to inhibit His3 eliminated the growth of the control strains, but not the cells coexpressing *GBD-HRR25* or *GBD-HRR25(K38A)* and *GAD-PIN4*. Together, these

data support the result of our co-immunoprecipitation analysis and demonstrating a physical interaction of Hrr25 and Pin4.

To determine the contribution of individual domains of Hrr25 to Pin4 interaction, we sought to perform a yeast two-hybrid analysis of an interaction between different Hrr25 truncation mutants and Pin4. Plasmids encoding a fusion protein of GBD and Hrr25(ΔM), Hrr25(ΔC), Hrr25(N), Hrr25(M), Hrr25(C), or Hrr25(M+C) were constructed and transformed into AH109 cells and transformants were crossed to Y187 cells expressing GAD or GAD-Pin4. On the CSM plate without histidine, cells coexpressing GBD-fused Hrr25(M), Hrr25(C), or Hrr25(M+C) and GAD or GAD-Pin4, did not grow, indicating that the middle and the C-terminal regions are not sufficient to support an Hrr25 interaction with Pin4 (Fig. 20). Compared to cells coexpressing *GBD-HRR25(ΔM)* and *GAD*, cells coexpressing *GBD-HRR25(ΔM)* and *GAD-PIN4* appeared whiter on the CSM plate supplemented with histidine and were able to grow better on the CSM plate without histidine and on the CSM plate with 3-AT. Thus, the middle region of Hrr25 is disposable for its association with Pin4. Conversely, cells coexpressing *GBD-HRR25(ΔC)* or *GBD-HRR25(N)* and *GAD-PIN4* or *GAD* formed red colonies on CSM plate with histidine and unable to grow on the CSM medium without histidine, indicating that Hrr25(ΔC) and Hrr25(N), lacks the C-terminal region of Hrr25, no longer interact with Pin4 (Fig. 19).

Together, our results indicate that both the kinase domain and the C-terminal P/Q rich region of Hrr25 are required for Pin4 interaction. Thus, we have uncovered a novel function for the P/Q rich region of Hrr25.

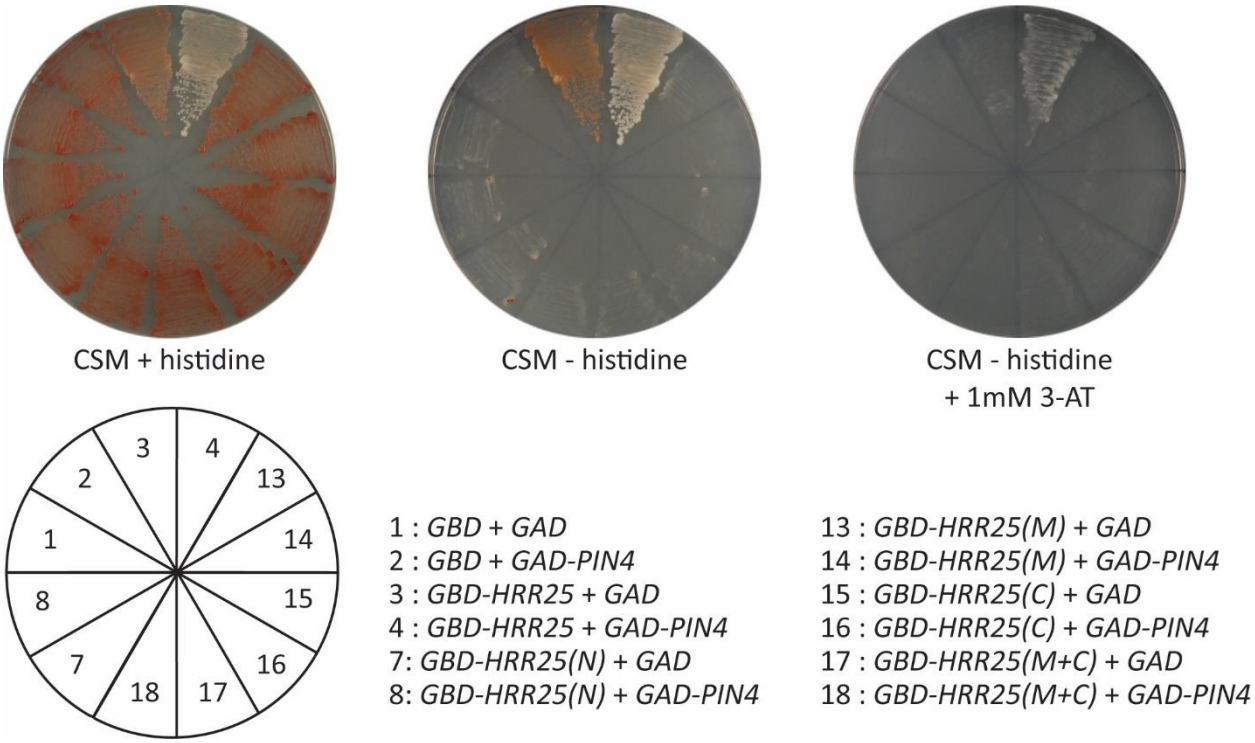

Figure 20. A yeast two-hybrid interaction analysis on the interaction between GBD and GAD fusion proteins as indicated.

Deletion of the C-terminal region of Hrr25 largely abolishes its interaction with Pin4 in a co-immunoprecipitation assay

We carried out a co-immunoprecipitation assay to confirm the yeast two-hybrid result on the requirement of the C-terminal region of Hrr25 for Hrr25's interaction with Pin4. Total cellular lysates were prepared from cells expressing Pin4-HA and untagged Hrr25, Hrr25-myc or Hrr25(ΔC)-myc and subjected to immunoprecipitation with anti-myc antibody and protein G agarose beads. Figure 21 shows that Pin4 is efficiently recovered with Hrr25-myc, but not with Hrr25(ΔC)-myc, indicating that the C-terminal P/Q rich region of Hrr25 is important for Hrr25's interaction with Pin4. To our knowledge, this is the first function that has been solidly attributed to the C-terminal region of Hrr25.

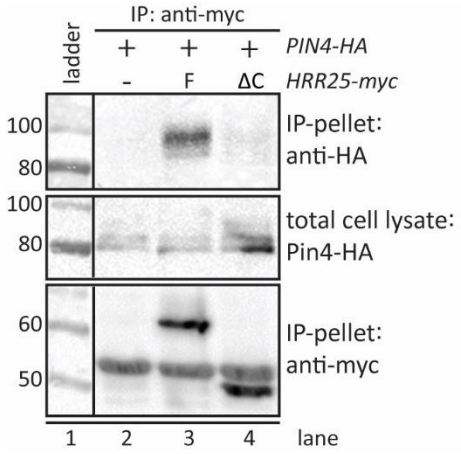

Figure 21. A co-immunoprecipitation analysis of Pin4-HA with Hrr25-myc and Hrr25(ΔC)-myc. A plasmid encoding HA-tagged Pin4 was transformed into a wild type strain, and *hrr25Δ* with Hrr25-myc or Hrr25(ΔC)-myc. Transformants were grown in YNBcas5D medium, cell extracts were subjected to immunoprecipitation with anti-myc antibody and analyzed by Western blotting with either anti-myc or anti-HA antibody. Both the immunopellets and total cell lysates fractions from the immunoprecipitation experiment were analyzed.

HRR25* and *PIN4* show genetic interactions with *BCK1* and *SLT2

Pin4 has been reported to be involved in the cell wall integrity (CWI) pathway. Traven *et al.* have discovered a strong synthetic growth defect of *pin4Δ* with mutations in two genes encoding components of the MAP kinase cascade in the CWI pathway, namely MAPKKK Bck1 and MAPK Slt2 (Traven et al., 2010). However, they did not report an upstream regulator of Pin4. Given the physical and biochemical interactions between Hrr25 with Pin4, we hypothesized that it is Hrr25 that regulates Pin4 in that process. If Hrr25 were the upstream regulator of Pin4 in a pathway, required for the maintenance of cell wall integrity, we would expect to observe synthetic growth defects of *hrr25(E52D)* with *bck1Δ* and *slt2Δ*.

To test that hypothesis, we sought to generate *bck1Δ pin4Δ, bck1Δ hrr25(E52D), slt2Δ pin4Δ, bck1 Δhrr25(E52D)* double mutants and characterize their growth phenotypes. Haploid yeast strains carrying a *bck1Δ* or a *slt2Δ* mutation were crossed with strains carrying a *pin4Δ* mutation or an *hrr25Δ* strain carrying a *hrr25(E52D)* mutant allele on a centromeric plasmid.

The four diploid strains were next induced to undergo meiosis in liquid sporulation medium and tetrad dissections were performed.

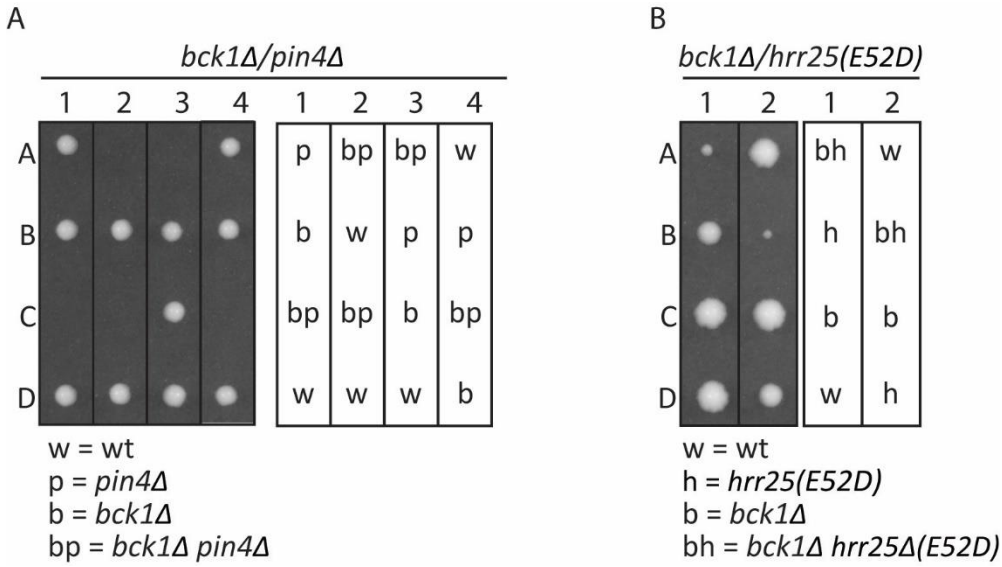

Figure 22. *bck1Δ* forms synthetic growth defects with *pin4Δ* and *hrr25(E52D).* A tetrad analysis of *BCK1/bck1Δ PIN4/pin4Δ* (panel A) and *BCK1*/*bck1Δ HRR25*/*hrr25(E52D)* (panel B) on YPD plates.

Figure 22 shows that the *pin4Δ* and *bck1Δ* single mutants grew at a rate similar to wild type, whereas the *hrr25(E52D)* mutant had a mild growth defect. The *bck1Δ pin4Δ* double mutation caused synthetic lethality (Fig. 22A), consistent with the findings of Traven *et al* using strains of the W303 background (Traven et al., 2010). The *bck1Δ hrr25(E52D)* double mutants had a strong synthetic growth defect (Fig. 22B), which supports our hypothesis of Hrr25 regulating Pin4. Similarly, tetrad analysis of *slt2Δ/pin4Δ* and *slt2Δ*/*hrr25(E52D)* diploids generated *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutants, which grew much slower than respective single mutants (data not shown). Thus, both *pin4Δ* and *hrr25(E52D)* had synthetic growth defects with *bck1Δ* and *slt2Δ*. These results supported our hypothesis that Hrr25 regulates Pin4 in a pathway that is important for cell wall integrity.

pin4Δ and *hrr25(E52D* lead to synthetic growth defects with *slt2Δ*

To quantify synthetic growth defects due to *slt2Δ* and *hrr25(E52D)* or *pin4Δ*, a 5-fold serial dilution assay on the growth of wild-type and mutant strains on YPD plate was performed. Figure 23 shows that neither the *slt2Δ* nor *pin4Δ* single mutant had a growth defect on YPD medium and that the *hrr25(E52D)* mutant had a growth defect. In contrast, *slt2Δ hrr25(E52D)* and *slt2Δ pin4Δ* exhibited severe growth defects, indicating that *slt2Δ* leads to synthetic growth defects with *pin4Δ* and *hrr25(E52D)*

Deletion mutations of *SLT2* or *PIN4* lead to compromised cell growth on growth medium containing cell wall stressors, such as caffeine (Traven et al., 2010; Truman et al., 2009). We tested the growth of *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutants, along with the *slt2Δ, pin4Δ,* and *hrr25(E52D)* single mutants, on YPD plates containing 5mM and 10mM caffeine. Both *slt2Δ* and *pin4Δ* single mutants were sensitive to 5mM and 10mM caffeine. The *hrr25(E52D)* single mutant didn't exhibit sensitivity to caffeine. The *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutants exhibited severe growth defects on YPD plate containing 5mM caffeine and were complete inviable on the 10mM caffeine plate.

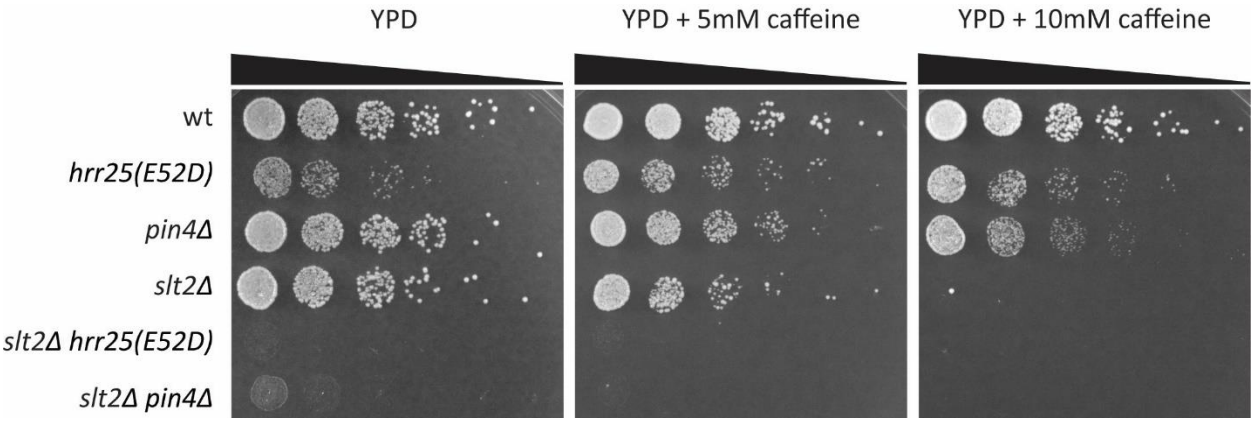

Figure 23. 5-fold serial dilutions of wild type, *hrr25(E52D)*, *slt2Δ*, *pin4Δ*, *slt2Δ pin4Δ*, and *slt2Δ hrr25(E52D)* mutant cells on YPD plates containing 0mM, 5mM, and 10mM caffeine as indicated.

Sorbitol rescues synthetic growth defects of *slt2Δ hrr25(E52D)* and *slt2Δ pin4* mutants

Growth defects caused by *slt2Δ* can be suppressed by an osmotic stabilizer, such as sorbitol (Torres et al., 1991). To test whether sorbitol addition would rescue synthetic growth defects due to *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutations, we performed a serial dilution analysis on YPD plates with and without 1M sorbitol at 30 ºC. The *slt2Δ* mutant phenotype has been reported to be enhanced by high temperature. Thus, a separate set of plates were incubated at 37 ºC.

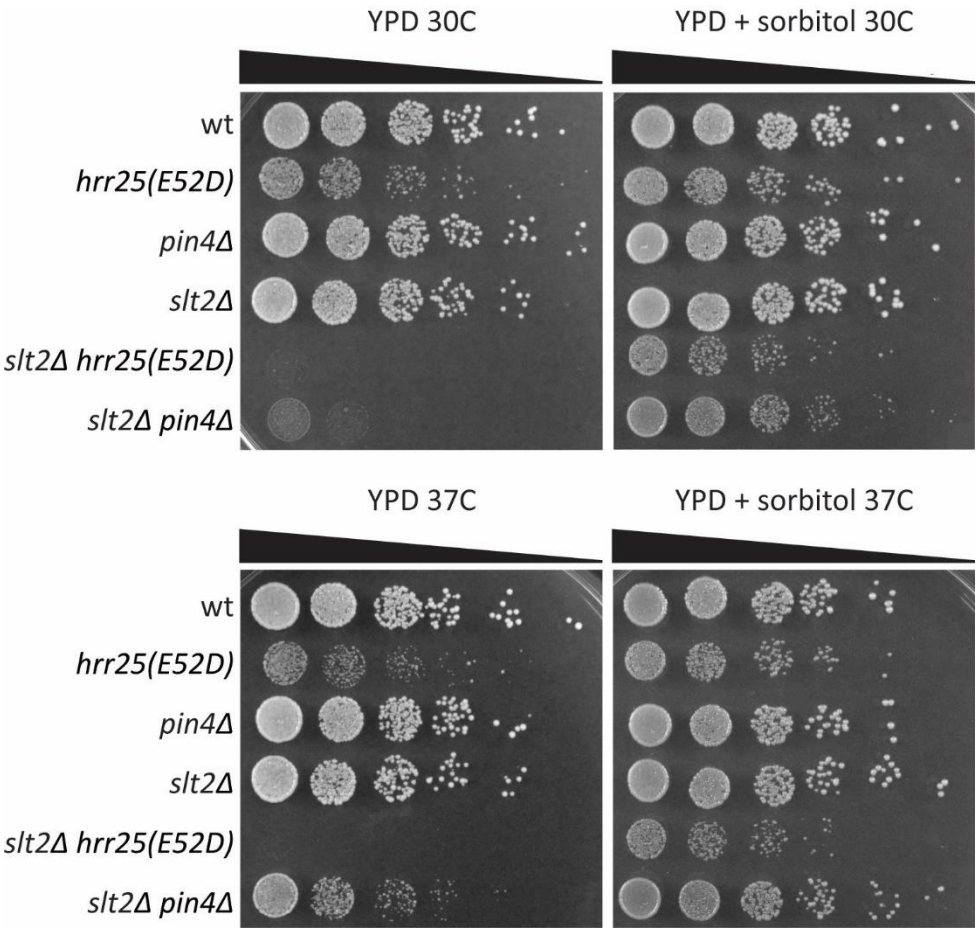

Figure 24. Serial dilution analysis of wild type, *hrr25(E52D)*, *slt2Δ*, *pin4Δ* single mutants, and *slt2Δ pin4Δ*, *slt2Δ hrr25(E52D)* double mutants on YPD plates containing osmotic stabilizer sorbitol at 30ºC and 37ºC.

At 30ºC, the addition of sorbitol to YPD plate improved the growth of *hrr25(E52D)* single, *slt2Δ pin4Δ* double, and *slt2Δ hrr25(E52D)* double mutants. Surprisingly, in contrast to

previous findings, 37° growth failed to exacerbate the growth defect of the *slt2Δ* mutant, which could be explained by the utilization of different background strains. Even more surprisingly, 37° growth partially suppressed the growth defect of the *slt2Δ pin4Δ* double mutant (Fig. 24, lower left panel). At 37°, the addition of 1M sorbitol resulted in a further increase in the cell growth of the *slt2Δ pin4Δ* double mutant. However, the temperature increase did not seem to impact the cell growth of *hrr25(E52D)* single and *slt2Δ hrr25(E52D)* double mutants. Together, our data suggest that Hrr25 and Pin4 are important in the maintenance of cell wall integrity.

PIN4 overexpression does not rescue the growth defect of a *slt2Δ hrr25(E52D)* double mutant

Our data suggest that Pin4 is a target of Hrr25 in a pathway that is important for the maintenance of cell wall integrity. However, the mechanism by which Hrr25 and Pin4 are implicated in cell wall integrity is unknown. How Hrr25 regulates Pin4 is also unclear. We sought to overexpress *PIN4* in a *slt2Δ hrr25(E52D)* double mutant to see whether it can suppress the mutant growth phenotype.

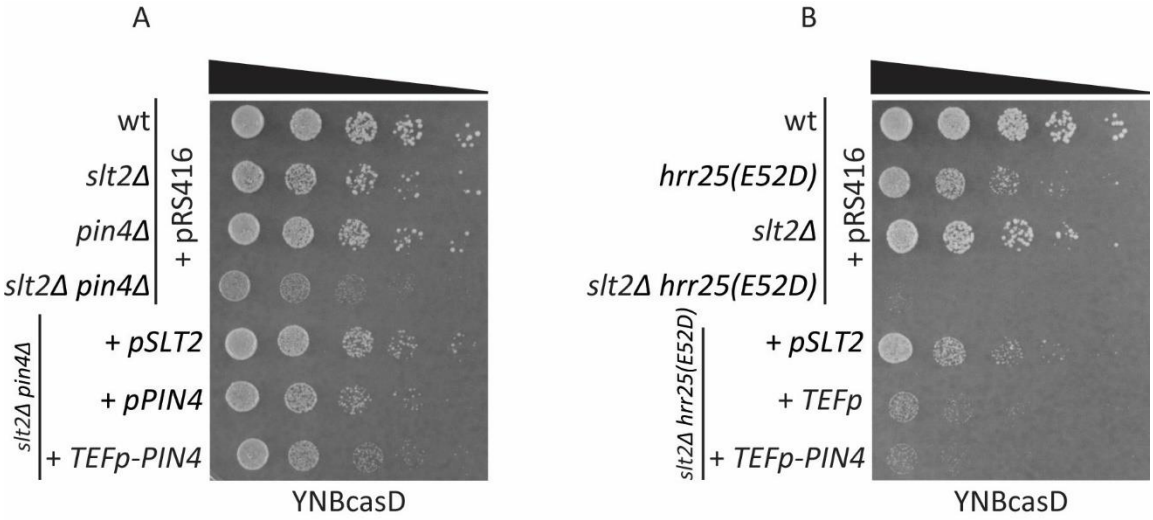

Figure 25. A 5-fold serial dilution analysis of indicated strains carrying various plasmids on YNBcasD plates.

Figure 25 shows that the introduction of a plasmid encoding 3xHA-tagged *SLT2* into *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutant cells largely restored their growth to *pin4Δ* and *hrr25(E52D)* single mutants respectively, indicating that the double mutants used for the analysis were real and that the C-terminal HA-tagged *SLT2* construct is largely functional. The *slt2Δ pin4Δ* strain transformed with a centromeric plasmid encoding HA-tagged *PIN4* under the control of its endogenous promoter or the strong *TEF2* promoter partially suppressed its growth defect. Overexpression of *PIN4* in *slt2Δ hrr25(E52D)* failed to suppress the slow growth phenotype of the double mutant. Therefore, *PIN4* overexpression is unlikely to overcome the effect of its reduced phosphorylation in *hrr25* mutant cells.

Hrr25, but not Bck1, is required for basal and caffeine-induced phosphorylation of Pin4

To further confirm that Hrr25 regulates Pin4 in a parallel pathway to the Bck1/Mkk1/2/Slt2 kinase cascade that impacts on cell wall integrity, we wanted to test the effect of caffeine on Pin4 phosphorylation in the *hrr25(E52D)* and *bck1Δ* single mutants. Accordingly, a plasmid encoding HA-tagged *PIN4* was introduced into the mutant strains and a Western blot analysis was carried out on Pin4-HA in transformants grown in the absence or presence of 10 mM caffeine.

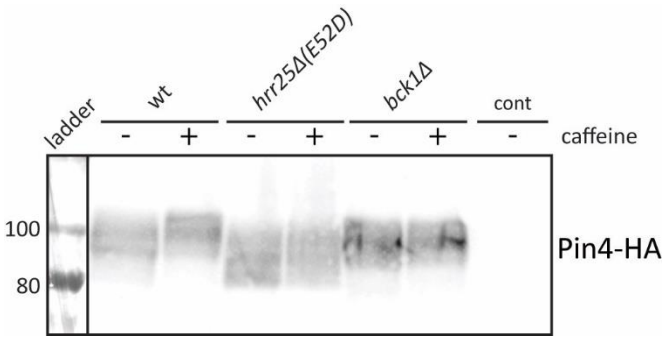

Figure 26. A Western Blot analysis of Pin4-HA in wild type, *hrr25(E52D)*, and *bck1Δ* grown in YNBcasD medium with or without 10mM caffeine.

As selected, caffeine treatment increased Pin4 phosphorylation in wild type cells (Fig. 26), which was indicated by its reduced mobility on SDS-PAGE gels. In *hrr25(E52D)* mutant cells, Pin4 phosphorylation was reduced both in the presence and the absence of caffeine treatment. In contrast, Pin4 phosphorylation was largely not affected by *bck1Δ*. Together, our data suggest that Hrr25 regulates Pin4 in an independent pathway that is important for cell wall integrity, separate from the Bck1/Mkk1/2/Slt2 kinase cascade.

A *pkc1Δ* does not affect the phosphorylation of Pin4

Pkc1 is a protein kinase that regulates the CWI pathway upstream of Bck1. Unlike mutations in the genes encoding the components of the Bck1/Mkk1/2/Slt2 kinase cascade, *pkc1Δ* leads to inviability, which can be suppressed by the addition of sorbitol. These results suggest that Pkc1 regulates another protein that is important for cell wall integrity, independent of the Bck1/Mkk1/2/Slt2 cell wall integrity pathway. In the light of our discovery that mutations in *hrr25* and *pin4* lead to synthetic defects with *bck1* and *slt2* mutations, we wanted to test whether Pkc1 functions upstream of Hrr25.

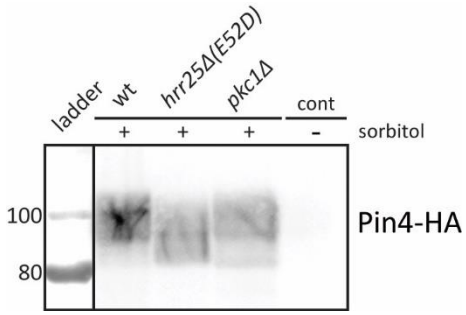

Figure 27. A Western Blot analysis of Pin4-HA in wild type, *hrr25(E52D),* and *pkc1Δ* grown in the presence of 1M sorbitol.

Accordingly, we transformed *pkc1* mutant cells with a plasmid encoding HA-tagged *PIN4* and selected transformants on a plate containing 1M sorbitol. Cells were cultured in liquid YNBcasD medium supplemented with sorbitol and a Western blot analysis was carried out. Figure 27 shows that *pkc1Δ* did not reduce Pin4 phosphorylation as observed in the *hrr25(E52D)* mutant. We conclude that Hrr25 regulation of Pin4 is independent of Pkc1.

The C-terminal P/Q-rich region of Hrr25 is required for efficient cell growth on caffeine

Using two alternative approaches, a co-immunoprecipitation analysis and a yeast two-hybrid assay, we have shown that C-terminal region of Hrr25 is required for Pin4 interaction. We sought to investigate the consequence of an Hrr25 C-terminal region truncation on cell growth on plates containing caffeine. Accordingly, *hrr25Δ* cells carrying plasmids encoding 3xMyc-tagged *HRR25*, *hrr25(E52D),* and *hrr25(ΔC)* were subjected to a serial dilution onto YPD plates supplemented with 0mM, 5mM, and 10mM caffeine.

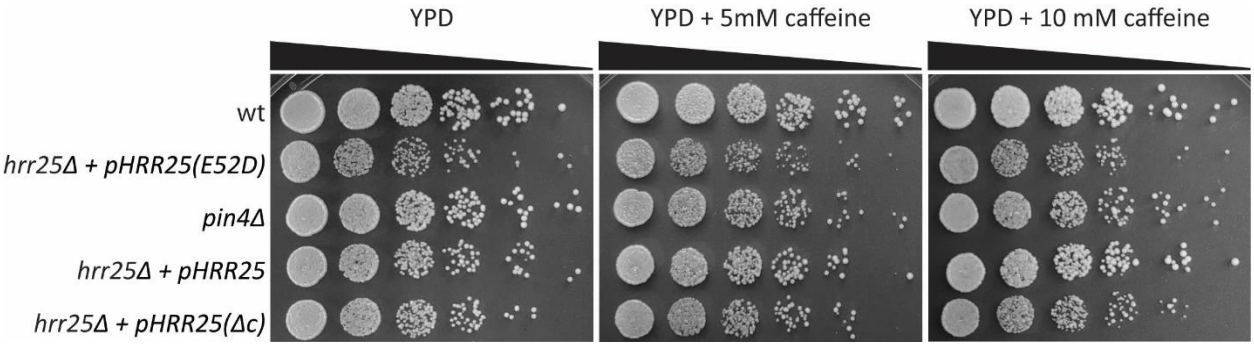

Figure 28. 5-fold serial dilutions of indicated cells on YPD plates supplemented with 0mM, 5mM, and 10mM caffeine.

As expected, a *pin4Δ* single mutant exhibited mild caffeine sensitivity at both concentrations (Fig. 28). Compared to wild type *HRR25,* an *hrr25(ΔC)* mutant was increasingly sensitive to caffeine, showing progressive reduction in growth rate on plates supplemented with 5mM and 10mM caffeine. Together, our data indicate that the interaction between Pin4 and Hrr25 via the C-terminal region of Hrr25 is required for cell growth under cell wall stress conditions.

A *rlm1Δ* mutation has no synthetic growth defects with *pin4Δ* or *hrr25(E52D)*

Rlm1 is a transcription factor and a well-known target of Slt2 in the CWI pathway (Jung et al., 2002). To test whether mutations in *HRR25* and *PIN4* lead to synthetic growth defects with *rlm1Δ,* we sought to examine the growth phenotype of *pin4Δ rlm1Δ* and *rlm1Δ hrr25(E52D)* double mutants on YPD medium with and without caffeine.

We generated *pin4Δ rlm1Δ* and *rlm1Δ hrr25(E52D)* mutants through tetrad analyses and used a serial dilution assay to examine their growth on YPD plates supplemented with 0mM, 5mM and 10mM caffeine.

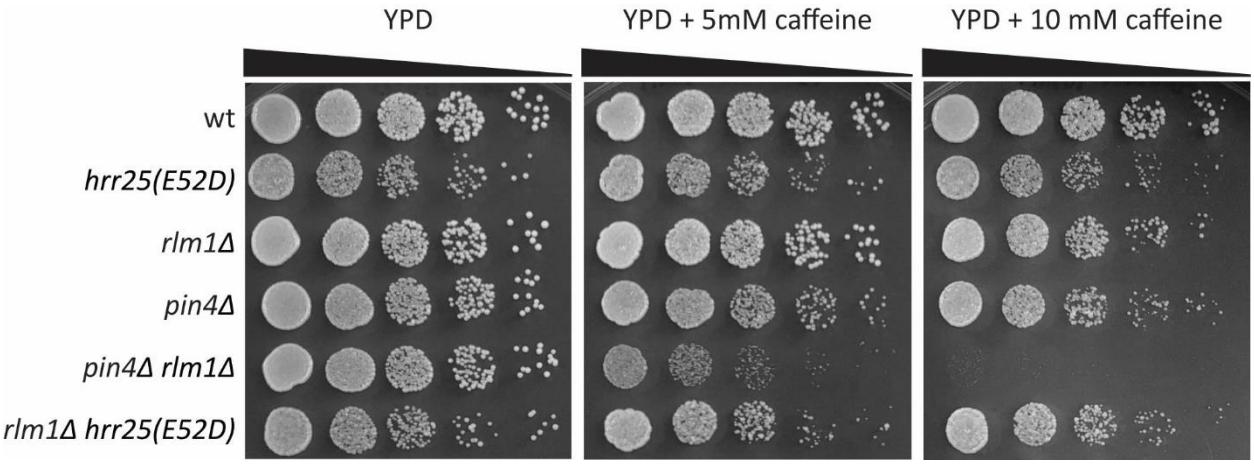

Figure 29. A 5-fold serial dilution analysis on the growth of *pin4Δ rlm1Δ* and *rlm1Δ hrr25(E52D)* on 0mM, 5mM, and 10mM caffeine.

As seen in Figure 29, neither the *pin4Δ rlm1Δ* nor *rlm1Δ hrr25(E52D)* double mutants exhibited synthetic growth defects on YPD plate. *pin4Δ* and *rlm1Δ* led to synthetic growth defects on YPD plates containing 5mM and 10 mM caffeine. In contrast, an *rlm1Δ hrr25(E52D)* double mutant did not show increased sensitivity to caffeine compared to the *rlm1Δ* and *hrr25(E52D)* single mutants.

Plasmid-borne *PIN4* and *RLM1* recover the growth defect of a *pin4Δ rlm1Δ* double mutant grown in the presence of caffeine

To confirm that the *pin4Δ rlm1Δ* double mutant cells used in the serial dilution experiment described above was real, centromeric plasmids encoding HA-tagged *PIN4* and *RLM1* were introduced into the *pin4Δ rlm1Δ* strain and a 5-fold serial dilution analysis was conducted on a YNBcasD plate, and on YPD plates with 0mM, 5mM, and 10mM caffeine added.

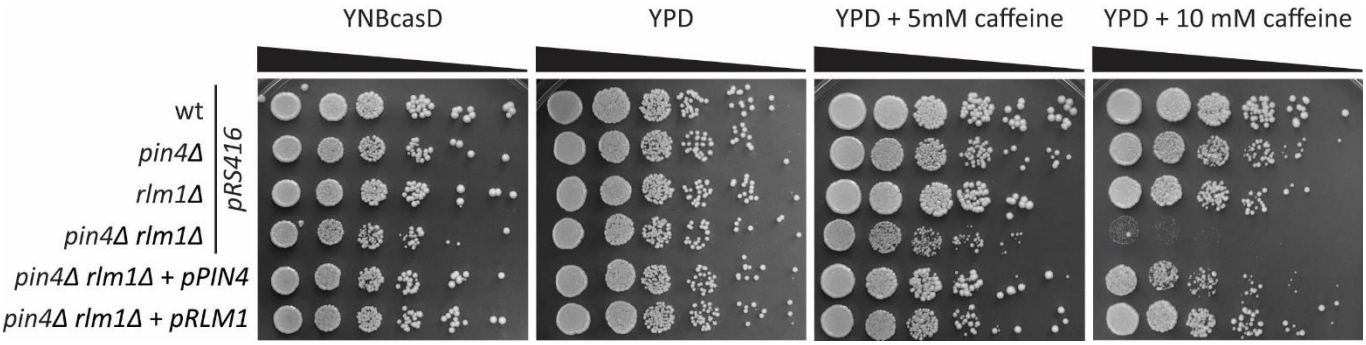

Figure 30. A Serial 5-fold serial dilution analysis of *pin4Δ rlm1Δ* cells carrying a plasmid encoding*PIN4* or *RLM1* as indicated on YNBcasD plate and on YPD plates supplemented with 0mM, 5mM, and 10mM caffeine.

On YNBcasD plate, all strains grew at approximately the same rate, except for the *pin4Δ rlm1Δ* double mutant, which was slower than wild type (Fig. 30). That growth defect was almost undetectable on the YPD plate. On YPD plates supplemented with 5 mM and 10 mM caffeine, *pin4Δ rlm1Δ* double mutant cells carrying a plasmid encoding *PIN4* and *RLM1* grew significantly better than the double mutant carrying an empty vector, indicating that the double mutant is real. Figure 30 also showed that

A Western Blot analysis of Rlm1-HA in slt2, pin4, hrr25(E52D) mutant strains

Increased caffeine sensitivity of *pin4Δ rlm1Δ* double mutant cells, but not *hrr25(E52D) rlm1Δ* mutant cells, suggested the possibility of a complex relationship between Pin4, Hrr25, and Rlm1. To further investigate the relationships among Hrr25, Pin4, and Rlm1, we sought to examine Rlm1-HA expression in *pin4Δ, hrr25(E52D),* and *slt2Δ* mutant cells using the Western blotting technique. Slt2 is known to phosphorylate Rlm1 and thus was included in our experiment.

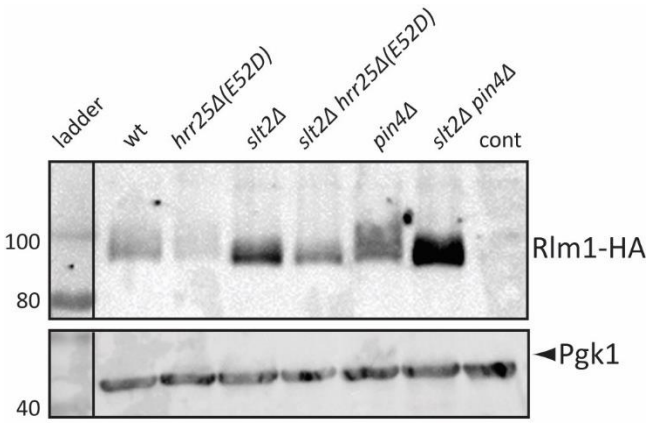

Figure 31. A Western Blot analysis of HA-tagged Rlm1 in wild type and mutant strains as indicated.

Slt2 phosphorylates Rlm1 *in vivo* (Jung et al., 2002). As expected, *slt2Δ* reduced Rlm1 phosphorylation (Fig. 31). In *hrr25(E52D)* mutant strain, Rlm1 phosphorylation remained the same, but the protein level decreased significantly. In the *slt2Δ hrr25(E52D)* double mutant, Rlm1 was dephosphorylated and the protein level of Rlm1 was reduced, indicating that the effects of *slt2Δ* and *hrr25(E52D)* were additive. In the *pin4Δ* mutant cells, Rlm1 protein level was increased compared to the wild type. In *slt2Δ pin4Δ* double mutant cells, Rlm1 was dephosphorylated and the protein level of Rlm1 was increased compared to wild type, indicating that the effect of *pin4Δ* and *slt2Δ* mutations are also additive.

RLM1 overexpression is lethal to *slt2Δ* mutant cells

An analysis of *RLM1* overexpression in *slt2Δ, pin4Δ, slt2Δ hrr25(E52D),* and *slt2Δ pin4Δ* mutants might provide insights into the mechanism by which these genes work together in regulating cell wall integrity. Accordingly, we generated a plasmid encoding *RLM1* under the control of the relatively strong *ADH1* promoter. The plasmid was then transformed into wild type and *slt2Δ, pin4Δ* and *slt2Δ pin4Δ* mutant cells.

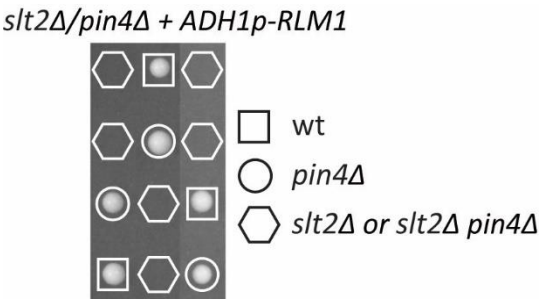

Figure 32. Tetrad analysis of a diploid strain heterozygous for *slt2Δ* and *pin4Δ* carrying a centromeric plasmid encoding *RLM1* under the control of the *ADH1* promoter. Tetrads were dissected on YNBcasD plate.

Multiple attempts were made, but we couldn't get transformants with *slt2Δ, slt2Δ hrr25(E52D), and slt2Δ pin4Δ*, which we found to be strange. We did get transformants with a *pin4Δ* mutant. We then transformed the plasmid into a *SLT2/slt2Δ PIN4/pin4Δ* diploid strain and used tetrad analysis to get the mutant strains carrying the plasmid encoding *ADH1-RLM1*. Figure 32 shows that spores of the *slt2Δ* or *slt2Δ pin4Δ* genotypes didn't grow into visible colonies on YNBcasD plate, indicating that *RLM1* overexpression is lethal in cells carrying a *slt2Δ* mutation. Our data suggest that high levels of unphosphorylated/hypophosphorylated Rlm1 is toxic to yeast cells.

Analysis of potential targets of Pin4

Pin4 is a putative RNA-binding protein (Pike et al., 2004). RNA-binding proteins can bind to mRNA targets and regulate their stability or translation. This type of regulations often leads to changes in target protein levels. In order to determine the mechanism by which Hrr25 and Pin4 regulate cell wall integrity, several proteins were chosen based on their involvement in the maintenance of cell wall integrity and subjected to a Western Blot analysis on their expression in wild type versus *pin4Δ* mutant cells. If the mRNA transcript of a target of Pin4 is bound through its putative RNA-binding domain, we expect to see a change in the protein level of the target.

Six proteins involved in maintenance of cell wall integrity were tested: Ecm8, a protein of unknown function; Srv2, a cyclase-associated protein, Inn1, a contractile actomyosin ring associating protein; Pfy1, profilin; Rad52, a protein involved in the repair of double-strand breaks; Rvs161, a protein regulating the polarization of the actin cytoskeleton. The genes encoding these six proteins were tagged with an N-terminal 9xHA tag and the expression of the fusion constructs was under the control of the *ADH1* promoter. Plasmids encoding these constructs were than transformed into wild type and *pin4Δ* mutant cells and transformants were grown in liquid YNBcas5D medium to OD_{600} 0.7-0.8. Total cellular proteins were collected and separated by SDS-PAGE and HA-tagged proteins were detected using Western blotting.

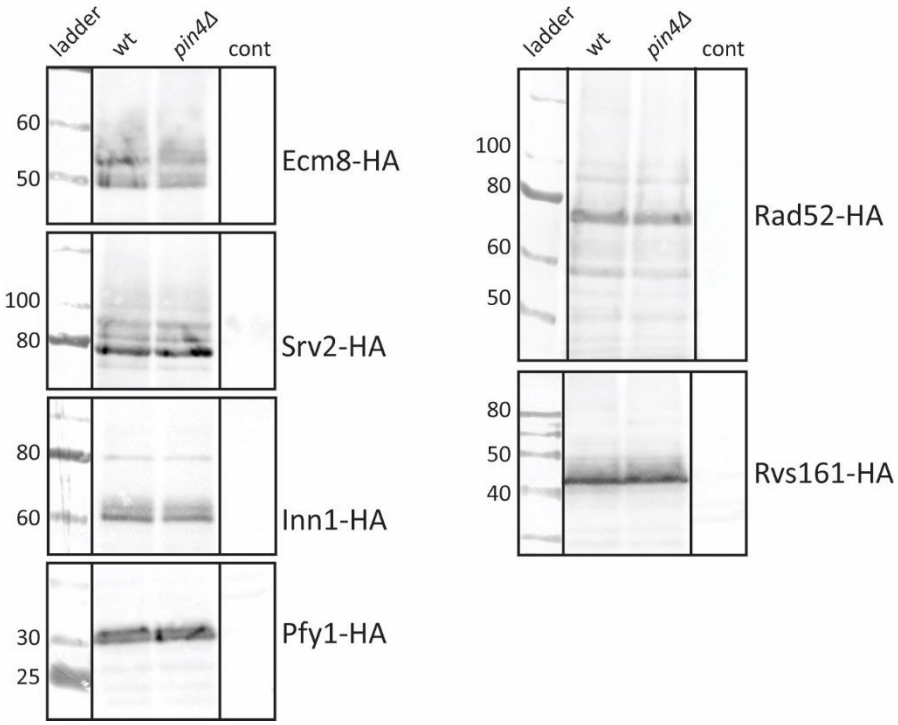

Figure 33. A Western blot analysis of the expression of HA-tagged Ecm8, Srv2, Inn1, Pfy1, Rad52, and Rvs161 in wild type and *pin4Δ* mutant cells.

As seen in Figure 33, a *pin4Δ* did not affect the protein level of any of the constructs tested, suggesting that these six genes are not regulated by Pin4 in the way by which some RNA-

binding proteins, such as Puf3, regulate their target gene expression through effecting changes in the stability or translation of bound mRNAs.

Pin4 Thr305 is required for its function in DNA damage response and CWI

Pin4 consists of 668 amino acid residues, with an N-terminal RNA recognition motif (RRM) domain, followed by a nuclear localization signal, and a C-terminal SQ/TQ motif (amino acids 302-668) (Pike et al., 2004). The N-terminal RRM domain has been shown to be critical for both the DNA damage response and the cell wall stress response (Pike et al., 2004; Traven et al., 2010). The threonine residue 305 within the C-terminal SQ/TQ domain has been identified to be required for Pin4's role in the DNA damage response through its regulation of Pin4 interaction with Rad53. It has been proposed that threonine 305 is phosphorylated by Mec1/Tel1 kinases and its phosphorylation contributes to the DNA damage response (Pike et al., 2004). However, a thorough analysis of the contribution of individual Pin4 domains to the cell wall stress response has not been reported. To that end, we generated a series of Pin4 truncation and mutant constructs shown in Figure 34 and evaluated their functionality by their ability to complement a *pin4Δ* mutation in a *pin4Δ slt2Δ* double mutant.

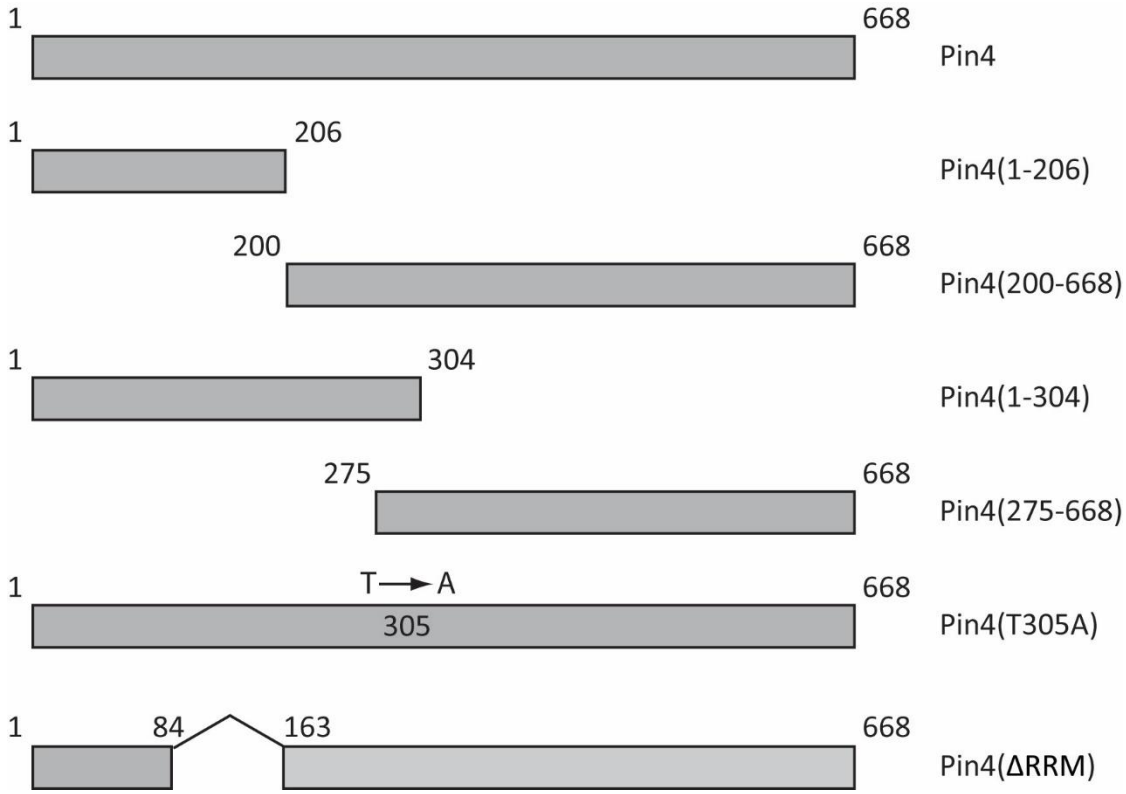

Figure 34. A diagrammatic representation of Pin4 truncation and mutant constructs used for functionality analysis: Pin4(1-206), Pin4(200-668), Pin4(1-304), Pin4(275-668), Pin4(ΔRRM), and Pin4(T305A).

Centromeric plasmids encoding *PIN4* constructs as shown in Figure 34 were constructed and introduced into a *slt2Δ pin4Δ* double mutant. The growth phenotype and caffeine sensitivity of the resulting transformants were assessed using a serial dilution analysis on cell growth on YNBcasD plates and YPD plates supplemented with 5mM caffeine.

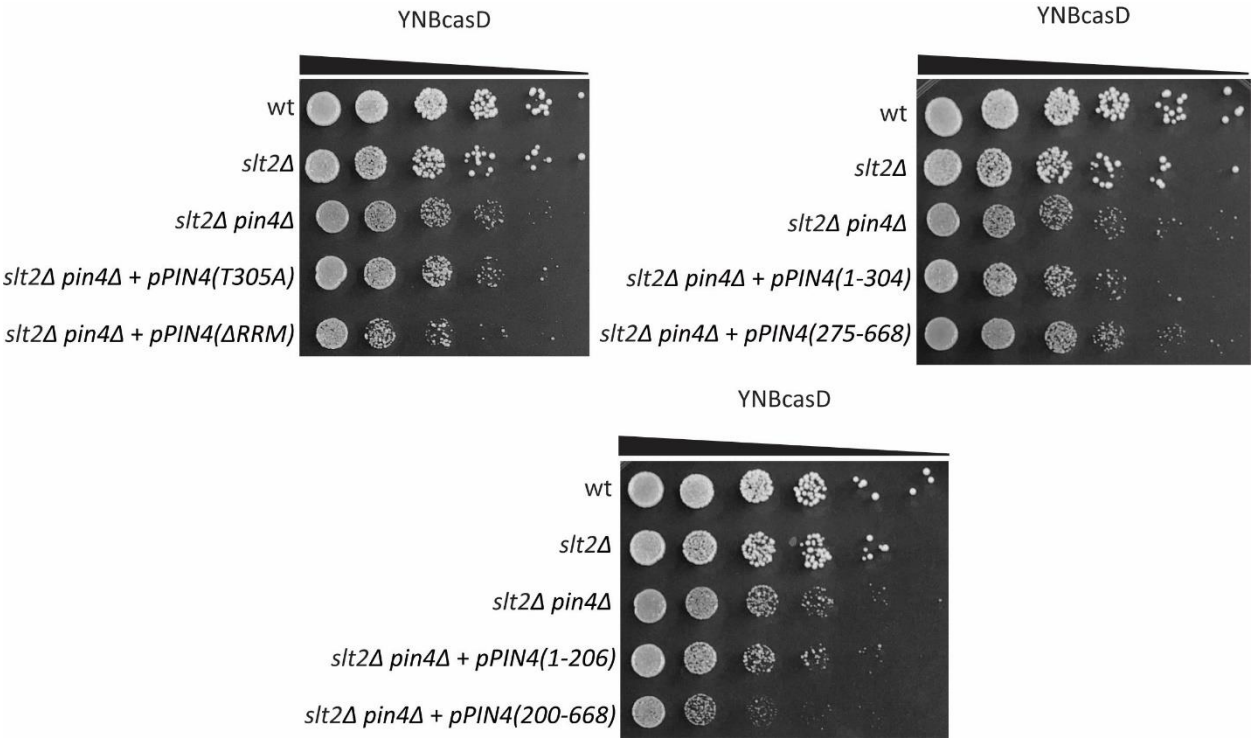

Figure 35. 5-fold serial dilutions of indicated strains on YNBcasD plates.

Figure 35 shows that none of the Pin4 truncation or mutant constructs were able to complement *pin4Δ*. Furthermore, none of the constructs were able to restore cell growth of the double mutant to that of a *slt2Δ* mutant on YPD + 5mM caffeine plate (data not shown). Thus, both the RRM domain and Thr305 are critical for Pin4 functions in the cell wall stress response.

Hrr25 is required for DNA damage-induced phosphorylation of Pin4

Pin4 is known to be activated by hyperphosphorylation in response to DNA damage by Mec1/Tel1 kinases. We have found that Hrr25 is required for basal phosphorylation and caffeine-induced phosphorylation of Pin4. Since Hrr25 is also required for DNA damage response, we wanted to determine whether Hrr25 is also required for Pin4 phosphorylation in response to DNA damage (Hoekstra et al., 1991). Accordingly, we assessed changes in Pin4 phosphorylation in cells treated with the DNA-methylating agent MMS (methyl methanesulfonate). Wild type and *hrr25(E52D)* mutant cells expressing *PIN4-HA* were treated

with or without 0.1% MMS. Cellular lysates were collected directly before MMS addition, 90 minutes, and 120 minutes after MMS addition. The proteins were separated using SDS-PAGE and visualized using Western Blotting

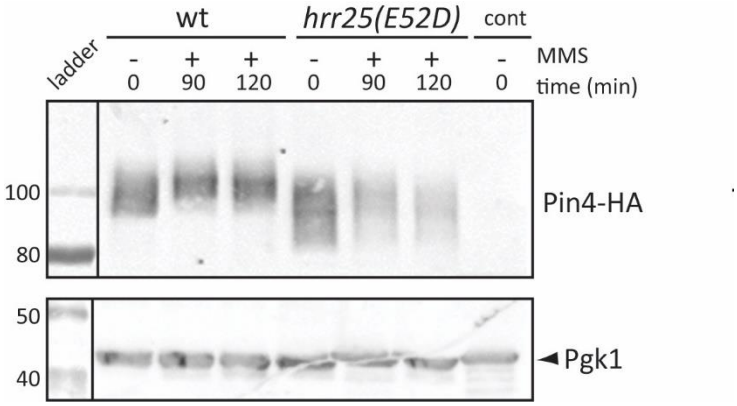

Figure 36. A Western blot analysis of Pin4-HA in wild type and *hrr25(E52D)* cells treated without or with 0.1% MMS.

In wild type cells, increased Pin4 phosphorylation was observed after 90 minutes of MMS treatment (Fig. 36). In *hrr25(E52D),* Pin4 phosphorylation was not affected by MMS, even after 120 minutes of treatment. Thus, Hrr25 is required for Pin4 phosphorylation in response to DNA damage. Mec1/Tel1 are important for cell cycle checkpoint control and Pin4 hyperphosphorylation in response to DNA damage. In light of our discovery that Hrr25 is also required for Pin4 phosphorylation in response to DNA damage, we wanted to determine their relative contribution to Pin4 phosphorylation. Therefore, we examined Pin4 phosphorylation in *mec1Δ tel1Δ* mutant cells.

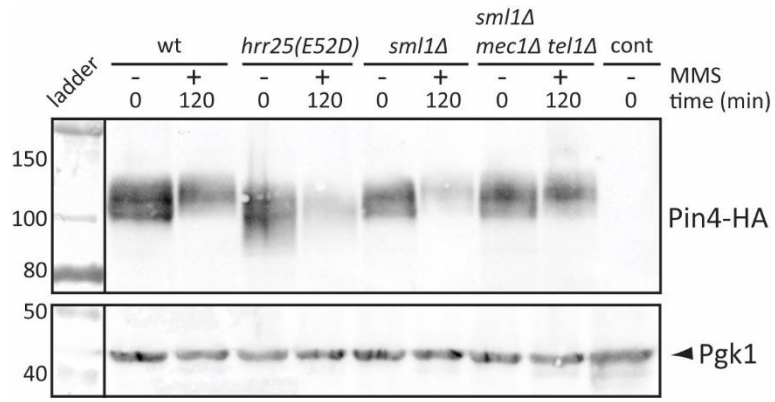

Figure 37. A Western blot analysis of Pin4-HA in the indicated strains treated with or without MMS.

Since *mec1Δ* is lethal, a *sml1Δ* was introduced to maintain the viability of a *mec1Δ tel1Δ* mutant. Figure 37 shows that Pin4 phosphorylation did not seem to be affected by *mec1Δ tel1Δ* in both the absence and presence of MMS treatment. Future research will be directed towards the elucidation of the mechanism by which Hrr25 mediates the DNA damage response.

Discussion

In this study, we analyzed whether any of 4 selected proteins, namely Mob1, Pin4, Cdc12, or Dsn1 were a target of Hrr25. Hrr25 belongs to a highly evolutionarily conserved casein kinase I family, that is known to have diverse functions and a large number of substrates. Thus, when screening for a potential Hrr25 target, we were primarily looking for a noticeable change in the phosphorylation level of the putative substrate when expressed in cells carrying a *hrr25Δ* or Hrr25(E52D) mutations. SDS-PAGE analysis of Cdc12, a septin ring component, and Dsn1, a known Hrr25 target in meiosis, expressed in *hrr25Δ* mutants showed a decrease in protein amount but no detectable phosphorylation change, compared to when expressed in *HRR25*. In addition to that, we showed that *hrr25Δ* does not alter the subcellular localization of Cdc12 and Dsn1, but strongly reduces the GFP fluorescence signal intensity. The decrease in GFP signal output in *hrr25Δ* could be due to reduction of protein amounts observed in SDS-

PAGE experiments. We thus concluded that Cdc12 and Dsn1 were unlikely to be targets of Hrr25. Dsn1 has been reported to be phosphorylated by Hrr25 during meiosis, but not mitosis. In mitosis, however, it is phosphorylated by Yck2, a different CK1 isoform (Robinson et al., 1999). It is possible that Hrr25 and Yck2 differentially phosphorylate or mediate a phosphorylation of Dsn1 depending on the type of division a cell undergoes. An alternative explanation for no detectable phosphorylation change of Dsn1 and Cdc12 in *hrr25Δ* is their initial Hrr25-dependent hypophosphorylation.

In this study we show that Mob1 is a potential Hrr25 target. A *hrr25* null mutation did not abolish Mob1 localization to SPBs and cytoplasm, however the GFP fluorescence signal was greatly reduced. Mob1 was dephosphorylated and the protein levels were reduced in *hrr25Δ* and in Hrr25(E52D) mutant cells, compared to when expressed in cells carrying *HRR25.* However, we found that Hrr25-dependent Mob1 phosphorylation does not affect its stability, and we failed to detect a physical interaction of Mob1 with Hrr25 by co-immunoprecipitation. It has been reported that co-immunoprecipitation assays are often unable to detect a kinase-substrate interaction, thus we cannot conclude that there is no physical interaction between Hrr5 and Mob1 (Collins et al., 2017).

The last protein analyzed, Pin4, exhibited a strong mobility shift when expressed in *hrr25Δ* or in Hrr25(E52D) mutant cells, compared to when expressed in *HRR25*. A phosphatase treatment of cell lysates of cells expressing Pin4 in *HRR25* and in *hrr25Δ* confirmed that the mobility shift observed in *hrr25Δ* was due to Pin4 dephosphorylation. Interestingly, in lambda phosphatase treated cell extracts from both *HRR25* and *hrr25Δ* cells Pin4 travelled as a faster and sharper band of the same size. These results imply that Hrr25 is not the only kinase that mediates basal Pin4 phosphorylation. We showed that Pin4 stability *in vivo* is not dependent on Hrr25-

mediated phosphorylation by performing a cycloheximide chase assay. A significant decrease of Pin4 phosphorylation occurred in both *ltv1Δ* and *hrr25Δ ltv1Δ* cells 10 minutes after cycloheximide addition, which we suppose is due to cellular response to cytotoxicity of cycloheximide, or due to degradation of a short-lived Pin4 regulator that is not Hrr25.

In this study we identify a metabolic pathway that had not been previously associated with Hrr25, the cell wall integrity pathway. Here we find that Hrr25 regulates cell wall integrity by mediating Pin4 phosphorylation in a parallel pathway to the classic CWI MAP kinase cascade. Several lines of evidence support this claim.

First, we showed that *slt2Δ pin4Δ* and *slt2Δ hrr25(E52D)* double mutants have synthetic growth defects that can be exacerbated by cell wall stressors caffeine or 0.002% SDS and recovered by an osmotic stabilizer 1M sorbitol. It has been reported that cells defective in the CWI signaling pathway undergo lysis at 37º C. This phenomenon was observed in cells lacking a functional *BCK1* and *SLT2* (Lee and Levin, 1992; Martin et al., 1993). However, the *slt2Δ* and *bck1Δ* mutants generated in our lab were not sensitive to 37º C and grew better at elevated temperature. These phenotypic differences could be due to utilization of yeast cells of different backgrounds. Interestingly, *slt2Δ* and *pin4Δ* single mutants and *slt2Δ pin4Δ* double mutant grew better at 37º C, than at 30º C, but *hrr25(E52D)* and *slt2Δ hrr25(E52D)* double mutant grew at the same rate at both temperatures.

Second, we show that the genetic interactions of *PIN4* with the components of the CWI pathway are much stronger than those of *HRR25*. A *pin4Δ* was synthetic lethal with *bck1Δ,* had severe relative synthetic growth defects with *slt2Δ,* and the *rlm1Δ pin4Δ* double mutant was sensitive to caffeine. On the contrary, *hrr25(E52D)* was synthetic slow with *bck1Δ,* relative

synthetic growth defects with *slt2Δ* were moderate, and *rlm1Δ hrr25(E52D)* double mutant was not sensitive to caffeine.

Third, we showed that Pin4 gets activated by hyperphosphorylation when grown in medium supplemented with caffeine, and that Hrr25-dependent Pin4 phosphorylation is required for Pin4 activation. Pin4 activation was not affected in *pkc1Δ* or *bck1Δ,* and *PIN4* overexpression does not recover the growth defect of the *slt2Δ hrr25(E52D)* double mutant. All data combined strongly support our hypothesis of the Hrr25 regulating Pin4 independently from Pkc1, Bck1 and Slt2.

By using two alternative approaches, co-immunoprecipitation analysis and yeast two-hybrid screen, we demonstrated that Hrr25 and Pin4 interact physically, and that the P/Q rich region of Hrr25 is required for binding Pin4. The deletion of the C-terminal region of Hrr25 lead to increased sensitivity of the Hrr25(E52D) to caffeine, indicating that the physical interaction between Hrr25 and Pin4 is necessary for the cell growth when exposed to cell wall stress. This discovery was rather unexpected. Our findings described in first chapter of this thesis highlight the importance on the kinase domain and the middle region of Hrr25 for binding its targets, whereas the C-terminal region is often dispensable.

Identifying the downstream target of Pin4 is crucial for establishing the mechanism by which Hrr25 regulates Pin4. In this study we propose that Rlm1 could be the downstream target of Pin4 in the cell wall integrity pathway. A Western Blot analysis of HA-tagged Rlm1 revealed additive effects of *slt2Δ* with *pin4Δ* or *hrr25(E52D)* on Rlm1 protein levels and phosphorylation. Importantly, Rlm1 protein amount was dramatically increased in cells carrying a *pin4Δ* mutation. We also found that *RLM1* overexpression was lethal to cells carrying a *slt2Δ* mutation. This leads us to think that toxic hypophosphorylated Rlm1 accumulation is the reason for poor growth of

slt2Δ pin4Δ and *slt2Δ hrr25(E52D)* double mutants. Perhaps Pin4 negatively regulates the hypophosphorylated Rlm1 species by binding its mRNA transcript through its putative RNA-binding motif (RRM), while Slt2 positively regulates Rlm1 by phosphorylating it.

In search of the Pin4 target, we tested whether any of the selected cell wall integrity proteins Ecm8, Srv2, Inn1, Pfy1, Rad52, and Rvs161 were a Pin4 target. None of the proteins tested showed a decrease in protein levels in *pin4Δ*. Additionally, Gas1, a β-1,3-glucanosyltransferase, was assessed as a potential Pin4 target, however, the results we got were inconclusive (data not shown). We tested whether Kdx1, a Slt2 has a paralog that interacts with Rlm1 to downregulate Slt2 activity, was regulated by Pin4 (Chang et al., 2013a, b). We found that *pin4Δ kdx1Δ* double mutant had no synthetic growth defects and were not sensitive to caffeine (data not shown). As of yet, we have not been able to identify the Pin4 target in CWI pathway. Although the downstream target of Pin4 remains unknown, we show that the threonine 305 residue and the RRM domain of Pin4 are critical for its functions in the cell wall stress response pathway. The Thr305 residue of Pin4 is known to play a major role in binding the DNA damage response kinase Rad53 (Pike et al., 2004). It is thus possible that Rad53 interacts with Pin4 to regulate cell wall integrity.

Both Pin4 and Hrr25 have been shown to be required for DNA damage tolerance. A deletion of *HRR25* caused hypersensitivity of cells to a DNA damaging agent (Hoekstra et al., 1991). Pin4 has been reported to get phosphorylated by Mec1/Tel1 kinases under DNA damage conditions and associate with the Rad53 FHA1 domain through its Thr305 residue (Pike et al., 2004). Here we show that Hrr25, but not Mec1/Tel1, is required for DNA damage-induced hyperphosphorylation of Pin4. We speculate that Hrr25-dependent Pin4 phosphorylation is required for the subsequent interaction of Pin4 with Rad53 to initiate cellular DNA damage

response. We have thus attempted to co-immunoprecipitate Pin4 and Rad53 from cellular lysates of cells carrying a *HRR25* and a *hrr25Δ*, hoping to see an abolishment of physical interaction of Rad53 with Pin4 in the *hrr25Δ*. We failed in our first attempt to do so, because Pin4 and Rad53 showed no physical interaction in cells bearing *HRR25*. The lack of detectable Pin4 and Rad53 interaction could be due to several reasons. First, it is possible that the total protein amounts extracted from the cells were too low to be successfully visualized via Western Blotting. Second, perhaps the total cellular protein amounts were high enough for detection, but Hrr25-dependent Pin4 phosphorylation was too low to bind Rad53. We thus are going to direct our future work towards the elucidation of the mechanism by which Hrr25 mediates DNA damage tolerance and cell wall integrity.

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